Individual & Interactive Impacts of Mercury & Agriculture on Reproduction in a Freshwater Turtle, *Chelydra serpentina*

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GENERAL AUDIENCE ABSTRACT

In aquatic turtles, females select nest sites that have a high degree of solar exposure, and exploit recently tilled agricultural fields for nesting, presumably because of increased solar exposure and/or easier nest excavation, and the importance of incubation temperature on survival and offspring phenotype. These same disturbed sites are often contaminated by pollutants and turtles can incorporate high levels of pollutants into their eggs which negatively impact hatch success. For my M.S. research, I investigated turtle nest site selection in a system dominated by agricultural and industrial land use, the impact of crop growth on the thermal and hydric dynamics of turtle nests, and I used paired field and laboratory experiments to examine the individual and interactive impacts of agricultural land use and Hg contamination on hatch success and offspring phenotype in *Chelydra serpentina*. Of the 150 turtle nests found during this research, 84% were located in human-disturbed soils. Nest site characteristics were similar among nests found in Hg contaminated and reference areas. Agriculture and control nests did not differ in temperature at the time of nesting, but temperatures diverged as crops grew, with temperatures in nests in agricultural fields averaging 2.5 °C lower than control nests over the course of incubation. Similarly, despite no initial difference, nest moisture levels diverged throughout incubation and moisture averaged 107 kPa lower in agricultural than control soils throughout incubation. In my field and laboratory experiments, I found that in comparison to turtles from control incubation conditions (i.e., warmer), turtles incubated under agricultural thermal regimens (i.e., colder) took longer to hatch, hatched at smaller structural body sizes, lost more mass after hatching, had lower post-hatching structural growth rates, and were more likely to be male. Additionally, thermal conditions associated with agricultural land use interacted with high levels of mercury to impact hatching success and offspring sex ratios. My thesis research provides one of the first documentations of negative interactive effects of mercury pollution and habitat quality on early vertebrate development and highlights the importance of examining the combined influence of multiple global changes on biological systems.

ACADEMIC ABSTRACT

Anthropogenic land use has caused population declines in diverse taxa worldwide. Few studies have focused on the combined influence of multiple aspects of global change (e.g., pollution and deforestation) on the behavior and development of animals despite their frequent co-occurrence. In aquatic turtles, females select nest sites based on solar exposure and exploit recently tilled agricultural fields for nesting. Crop growth subsequently shades nests, and agricultural fields are often contaminated by pollutants which can reduce hatch success of turtles. We investigated nest site selection of *Chelydra serpentina* in a system dominated by agricultural and industrial land use, and also investigated crop impacts on turtle nest microclimate. We then used a 2x2 factorial design to examine individual and interactive impacts of agricultural land use and mercury contamination on hatch success and offspring phenotype in *Chelydra serpentina*. We found 150 nests; 84% were located in agricultural fields or commercial nurseries. Nest microclimate did not differ between agricultural and control nests at the time of nesting but diverged throughout incubation; overall, nests in agricultural fields averaged 2.5 °C and 107 kPa lower than control nests. Agricultural incubation temperatures interacted with maternally-transferred mercury to reduce hatching success and, compared to turtles from control incubation conditions, turtles incubated under agricultural regimens; developed slower, hatched smaller, grew slower, and were more likely to be male. We provide novel evidence of negative interactive effects of mercury pollution and habitat quality on early vertebrate development and highlight the importance of examining combined influences of global changes on animals.

Dedication

To the mother who took me hiking, camping, and fishing, and taught me to believe in myself and work hard; I dedicate this thesis to you.

Attribution

Chapter 1 was co-authored with Brittney H. Coe, Justin D. Congdon, Dean F. Stauffer and William A. Hopkins. All co-authors provided input on the design of the study. Dean F. Stauffer provided guidance on statistical analyses. Molly drafted the chapter, editing was primarily completed by Dr. Hopkins but all co-authors provided editorial reviews.

Chapter 2 was co-authored with Brittney H. Coe, Robin M. Andrews, Dane A. Crossley II, Daniel A. Cristol, and William A. Hopkins. Molly conceived the research question, Brittney H. Coe, Robin M. Andrews, and William Hopkins provided input on the design of the study. Dane Crossley provided Snapping Turtle eggs, Daniel Cristol performed the mercury analyses. Molly drafted the chapter, editing was primarily completed by Dr. Hopkins but all co-authors provided editorial reviews.

Chapter 3 was co-authored with Brittney H. Coe, Robin M. Andrews, Dane A. Crossley II, Daniel A. Cristol, Dean F. Stauffer and William A. Hopkins. Molly conceived the research question, Brittney H. Coe, Robin M. Andrews, Dean F. Stauffer and William Hopkins provided input on the design of the study. Dane Crossley provided Snapping Turtle eggs, Daniel Cristol performed the mercury analyses. Molly drafted the chapter, editing was primarily completed by Dr. Hopkins but all co-authors provided editorial reviews.

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Introduction

Over the last 500 years, anthropogenic habitat alteration has caused extinctions in over 600 vertebrate species, signaling the onset of the sixth mass extinction event on earth (Ceballos et al. 2015). Habitat loss and environmental pollution are two global threats of great concern to species conservation (Meiri and Chapple 2016). The threat of anthropogenic habitat alteration to global biodiversity has led to the development of beneficial new conservation strategies (Doak et al. 2015), yet the human population and global middle class continue to grow and agriclutural land use is predicted to expand to meet increasing consumption demands (Kennedy et al. 2016). Additionally, previously emitted pollutants like mercury (Hg) persist in the environment for hundreds-to-thousands of years (Selin 2009). Because pollution and agriculture are among the most widespread forms of habitat modification in the world (Driscoll et al. 2013, Oakenleaf et al 2015), they are likely to co-occur. Yet, little is known about the cumulative effects of multiple global changes. Due to the widespread prevalence of major global threats, and the predicted future increase or persistence of some, understanding the interactive effects of multiple threats on organisms is critical for conservation (Böhm et al. 2016).

To provide additional information on the individual and interactive impacts of habitat alteration and pollution in a understudied and declining vertebrate group, my master's research investigated the individual and interactive impacts of Hg contamination and agricultural land use on turtle reproduction (nest site selection, nest thermal and hydric dynamics, and impacts on early development of turtle offspring). I elected to work on reptiles because they are a data deficient vertebrate group despite their susceptibility to effects of habitat alteration and pollution;

they have specific temperature and moisture requirements for embryonic development and transfer pollutants to their eggs that can alter offspring sex ratios.

Rapid Global Change. Agents of global change generally occur at a significantly faster rate than natural processes and many animals today are facing environmental conditions not experienced in their evolutionary history (Robertson et al. 2013, Hale and Swearer 2016). As a result, evolutionarily useful environmental cues may no longer be present in modified habitats or such cues may become mismatched from the cost/benefit regimens under which the species evolved (Hale and Swearer 2016). These types of mismatches are a growing conservation concern because they can result in evolutionary traps: when human modified habitats resemble habitats that historically indicated an optimal choice based on environmental cues but currently result in negative fitness consequences if used (Robertson et al., 2013). Examples of impacts of rapid global change on animals include the evolution of longer, narrower bills, and altered vocal performance among house finches (*Haemorhous mexicanus*) in response to noise in urban environments (Giraudeau et al. 2014) and stronger reflections of polarized light off of glass and asphalt than off of water cuing insects to oviposit on surfaces where their eggs cannot hatch (Robertson and Hutto 2006).

Habitat Modification & Turtle Nesting Ecology. In aquatic turtles, nesting females often exploit areas where humans have disturbed terrestrial substrate, increasing solar exposure, and presumably facilitating easier digging for nest construction. Examples of these modified habitats include gardens, road-shoulders, and agricultural fields (Bobyn and Brooks 1994, Castellano et al. 2008, Beaudry et al. 2010, Patterson 2013, Mui et al. 2015). One of the best studied

populations of snapping turtles is in Ontario, Canada where females nest almost exclusively on a gravel and sand artificial dam (Obbard and Brooks 1980, 1981). Interestingly, this population also represents the northern limit of the range of snapping turtles, leading some to believe that the damn has been of local benefit by providing nesting habitat with high solar exposure where little natural nesting habitat would otherwise be available (Bobyn and Brooks 1994, Steyermark et al. 2008).

Agricultural Land Use. While some forms of anthropogenic habitat alteration may expand available habitat (for unexpected examples see: Bernath-Plasted and Koper 2016, Poschold et al. 2016, Beatty et al. 2017, Rowland et al. 2017, and Vad et al. 2017), other forms of habitat modification such as agricultural land use, may create attractive, but possibly unsuitable habitat for nesting turtles (Freedburg 2011, Mui 2015). Agriculture and forestry-related activities are the second most common cause of evolutionary traps (Hale and Swearer 2016). Examples of these types of evolutionary traps include high mortality of lizards drawn into agricultural areas by high insect prey availability (Rotem et al. 2013) and high nest depredation among passerine birds drawn to nest near field/forest edges by high vegetation heterogeneity (Gates and Leslie 1978). Although not well studied, rapidly changing thermal conditions of agricultural landscapes due to the seasonal tilling of fields (i.e., sudden elimination of canopy cover followed by rapid crop growth) may have important biological consequences for organisms because oviparous vertebrates primarily select oviposition sites based on thermal cues and the temperatures that embryos experience during development strongly impact offspring phenotype and survival (Deeming 2004). Consequently, because agricultural fields likely create a mismatch between nest site conditions during selection by females (i.e., no vegetation and

relatively warm soil temperatures) and incubation conditions experienced by embryos (i.e., increasingly dense vegetation and relatively cooler nest temperatures; Freedburg et. al. 2011, Mui et. al. 2015). Yet, the thermal effects of agricultural land use on mismatches between environmental cues, and animal behavior, fitness, and early development, are not well studied.

Embryonic Development of Reptiles. In many oviparous vertebrates, embryonic development is dependent upon incubation temperature (Bull 1980, Bobyn and Brooks 1994, Shine et al. 1997, Demuth 2001, Ashmore and Janzen 2003, Deeming 2004a, DuRant et al. 2012). Phenotypic traits in reptiles affected by incubation temperature include sex ratios, size and growth rates, residual yolk stores, and locomotor performance (Janzen and Morjan 2001). In addition to the average temperature at which eggs are incubated, thermal variance during incubation impacts offspring phenotype in reptiles (Shine et al. 1997, Demuth 2001, Ashmore and Janzen 2003, Les et al. 2007, Niehaus et al. 2012). Consequently, habitat modification which results in altered thermal dynamics of nests is likely to strongly affect offspring phenotype (Kolbe and Janzen 2002).

Reptiles exhibit temperature-dependent sex determination (TSD); the temperature at which eggs are incubated is the primary determinant of the sex of embryos produced (Bull 1980, Janzen 1992). Two major patterns of TSD have been described in reptiles (Bowden et al. 2014). Type I TSD reptiles have one pivotal temperature, with one sex produced at low temperatures and the other at high temperatures. Type II TSD reptiles, such as snapping turtles, have two pivotal temperatures, with one sex produced at intermediate temperatures and the other sex produced at both high and low temperatures.

Mercury Contamination. Environmental pollution in the form of mercury (Hg) contamination has received significant attention due to its propensity to biomagnify and bioaccumulate in its methylated form, CH_3Hg or MeHg (Bloom 1992). Mercury has numerous sublethal effects; in vertebrates, Hg can impact cardiovascular, digestive, renal, immune, nervous, endocrine, and reproductive system function (Rice et al. 2014). Point sources of Hg (such as industrial discharges), affect many members of the biological communities living downstream of the source, yet research efforts thus far have primarily focused on invertebrates (Hildebrand et al. 1980, Mason et al. 1994), fishes (Golet and Haines 2001), and fish-eating vertebrates like certain birds and mammals (Scheuhammer et al. 2008). However, more recent work suggests that a variety of non-piscivorous wildlife can also be affected by Hg pollution, including amphibians, songbirds, bats, and turtles (Bishop et al. 1996, Walker et al. 2007, Wada et al. 2010, Bergeron et al. 2011, Hopkins et al. 2013a). Turtles possess unique life history strategies that make them good model organisms for the study and monitoring of contaminants (Golet and Haines 2001, Hopkins et al. 2013a) and ecotoxicology studies on reptiles are needed (Hopkins 2000).

Total Hg concentrations in adult turtles from some locations exceed concentrations that have been shown to cause sublethal and lethal effects in other aquatic species and waterfowl (Hopkins et al. 2013a). In turtles and other oviparous species, females can maternally transfer Hg to their eggs which can result in increased embryonic mortality and infertility (Bergeron et al. 2011, Todd et al. 2012, Hopkins et al. 2013b). In addition to direct sublethal and lethal effects of Hg, it is possible that this widespread pollutant could interact with other widespread anthropogenic activities (e.g. habitat modification) to negatively impact turtles. For example, because hormones are known to influence offspring sex and even reverse TSD predictions in

reptiles (Guillette et al. 2000, Warner et al. 2009, Matsumoto and Crews 2012, Merchant-Larios and Diaz-Hernandez 2012), and Hg alters the production of estrogens (Zhu et al. 2000) and thyroid hormones ((Wada et al. 2009, Wada et al. 2010, Meyer et al. 2014, but see Wada et al. 2011) which enhance steroidal gene expression and contribute to androgen synthesis (Flood et al. 2013), Hg and sex determining hormones may interact. However, these interactions remain untested.

Testudines & Biodiversity. Testudines (i.e., turtles and tortoises) are among the world's most endangered vertebrate groups (Gibbons et al. 2000). A staggering 47% of the world's testudine species are listed as threatened by the IUCN Red List of Threatened Species and many of the remaining half have yet to be evaluated (Bland and Böhm 2016). Habitat modification and loss has been directly linked to declines of numerous freshwater turtle species (Garber and Burger 1995, Buhlmann and Gibbons 2001, Glorioso et al. 2010).

Turtles are conspicuous and charismatic members of biological communities and their persistence can be a critical component of natural ecosystems. For example, some Amazonian river turtles (*Podocnemis expansa*, *unifilis*, and *sextuberculata*) are important food sources for both humans and wildlife, they are integral seed dispersers of fruit trees, and due to their incredibly high biomass and densities they play an important role in nutrient cycling and energy flow in rivers and lakes (Congdon et al. 1986, Moll and Jansen 1995, Buhlman et al. 2009). Consequently, when turtles are lost from the environment, both a loss of biodiversity and biological imbalance may result.

Study Species Range and Conservation Status. The three species of the genus *Chelydra* have ranges that span from Canada to South America, with one species on each of the Americas. The North American species, *Chelydra serpentina* (common snapping turtle), has the widest latitudinal range of any North American reptile. They are found from the Rocky Mountains to the Atlantic Ocean, and from southern Quebec to southern Florida and Texas (Steyermark et al. 2008). Although, as their name suggests, common snapping turtles are abundant throughout much of their range, on the northern periphery they are listed as a species of special concern under Ontario's Endangered Species Act and as a species of special concern by the federal Committee on the Status of Endangered Wildlife in Canada (COSEWIC). Moreover, throughout much of their range, snapping turtles face numerous threats including collection for human consumption (Ceballos and Fitzgerald 2004, Paisley et al. 2009), pollution (Bishop et al. 1996, Hopkins et al. 2013b), increased predation rates resulting from anthropogenic influences on food webs (Wilhoft et al. 1979, Schmidt 2003), habitat modification (Glorioso et al. 2010), and road mortality (Gibbs and Steen 2005). However, we know virtually nothing about how any of these factors interact with one another to affect snapping turtles, even though these threats often co-occur.

Thesis Objectives. The ultimate goals of my research were to inform restoration activities for snapping turtles in the South River, Virginia and to provide novel information about how offspring development is impacted in an agriculturally modified and polluted landscape. In order to properly develop mitigation efforts to enhance the fitness of turtles, a first step is to identify when and where nesting takes place. The first objective of my thesis was to identify and characterize nesting habitat for snapping turtles in an area modified by agricultural and industrial

activity. Fieldwork for my first objective began in 2013 and results from that season showed that turtles nest in agricultural fields in our study area at high rates. This finding sparked my 2014 objectives: to describe how agricultural land use impacts turtle nest microclimate (thermal and hydric dynamics), and to investigate the individual and interactive effects of agricultural land use and maternally derived Hg on turtle embryonic development, hatch success, and offspring phenotype (e.g., sex ratios, body size, growth, etc). Because Hg and agriculture are among the most widespread forms of habitat modification in the world, they are likely to interact with one another to influence organisms yet, to our knowledge, this thesis represents the first effort to understand the combined effects of these two major global threats on any species.

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Chapter 1: Nesting Ecology & Habitat Use of *Chelydra serpentina* in an Area Modified by Agricultural & Industrial Activity

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Abstract

Habitat loss and pollution have been linked to declines of numerous freshwater turtle species, which are among the most endangered vertebrates in the world. We examined characteristics of nest sites of Common Snapping Turtles (*Chelydra serpentina*) located in a system modified by agricultural and industrial land use. We compared characteristics of 150 turtle nests and patterns of nest depredation in mercury (Hg) polluted and reference sites. Of the nests found in this study, 90% were located in human disturbed soils: 79% in agricultural fields and 11% in commercial nurseries. In both Hg and reference sites, we found that 52% of all nests were located in high density nesting areas in agricultural fields bordered by a river on three sides, providing novel evidence that river geomorphology could be useful for identifying important nesting areas. We did not observe predation in the reference sites but 66% of nests were destroyed at the Hg polluted sites. We provide a predictive model demonstrating that the same characteristics influence nest-site selection in this modified system as in more intact systems, and are related to solar exposure at the time of nesting. We provide evidence that Common Snapping Turtles are attracted to agricultural areas for nesting, which could influence the fate of nests and/or development of embryos. We also suggest that research is needed to verify the importance of river geomorphology on nesting. Additionally, the high depredation rate of turtle nests containing eggs with Hg contamination suggests that the impacts of dietary Hg on predators of turtle nests merits investigation.

Keywords: agriculture; habitat modification; mercury; nest-site selection; predation; turtle

Introduction

Habitat loss and pollution have been directly linked to declines of many aquatic turtle species in the United States (Gibbons et al. 2000). For example, anthropogenic land use has been shown to increase mesopredator population densities, including Northern Raccoons (*Procyon lotor*), which are major predators of turtle nests (Congdon et al. 1983, 1987; Ratnaswamy et al. 1997; Garmestani and Percival 2005). In addition to increasing rates of turtle nest depredation, industrial forms of anthropogenic land use can produce environmental pollutants, such as mercury (Hg), which have adverse effects on both adults and offspring in many vertebrate species (Sakamoto et al. 2001; Crump and Trudeau 2009; Tan et al. 2009). In turtles and other oviparous species, females can maternally transfer high concentrations of Hg to their eggs, which results in increased rates of unfertilized eggs and embryonic mortalities among clutches (Bergeron et al. 2011; Todd et al. 2012; Hopkins et al. 2013a, b). Additionally, Hg is a neurotoxin and high Hg levels can impact animal reproductive behavior and fecundity (Wolfe et al. 1998), but the impacts of high Hg levels on nest site selection by turtles is not understood (Hopkins et al. 2013a, b).

Low reproductive success among aquatic turtles in polluted and modified environments can be mitigated, to some extent, by efforts to increase the amount of suitable nesting habitat or decrease depredation of nests. However, a better understanding of how these factors operate to influence nesting success across different spatial contexts is needed to inform mitigation strategies. In this study, we used riparian habitat surveys along a river upstream and downstream of an historic point source of Hg to: (1) investigate the effects of long-term exposure to Hg on the nesting ecology of the Common Snapping Turtle (*Chelydra serpentina*); (2) describe patterns of nest depredation in different habitat types; and (3) develop a predictive model for nest site selection in a system dominated by agricultural and industrial land use. The model can be used

by land managers to identify habitat with a high likelihood of use by Common Snapping Turtles for nesting, and to inform mitigation efforts aimed at expanding suitable nesting habitat for the species in highly modified landscapes.

Methods

Study organism. Common Snapping Turtles are susceptible to Hg accumulation (and maternal transfer of Hg) because they are long-lived, exhibit delayed reproductive maturity, feed in the benthos and/or at high trophic levels, and have small home ranges (Obbard at al. 1981; Congdon et al. 1994). The onset of Common Snapping Turtle nesting typically occurs during the months of May and June; nesting activity primarily occurs diurnally but some nests are constructed at night (Congdon et al. 1987; Iverson et al. 1997). Females dig nest chambers in areas with high solar exposure and normally deposit a range of 26–55 eggs. Predation rates on turtle nests are highly variable but generally range from 30–100% among years (Congdon et al. 1987) and have been linked to nest site characteristics including nest density, the distance from nests to ecological edges (i.e., river banks, forest edges, field edges, etc.), and the timing of nesting (Robinson and Bider 1988; Kolbe and Janzen 2002b; Bowen and Janzen 2005; Leighton et al. 2008; but see Congdon et al. 1983,1987).

***Study sites*.** We conducted our study near Waynesboro, Virginia, USA, where an historical Hg point source and land conversion for agriculture have produced a heavily modified and polluted landscape. In the center of Waynesboro, a manufacturing plant used a mercuric sulfate catalyst to produce acetate fiber from 1929–1950. The plant released high levels of Hg into the South River until 1976 (Carter 1977). Mercury concentrations in the river sediments and in aquatic organisms are still extremely high for more than 32 km (20 mi) downstream from the initial point source (Bergeron et al. 2010; Wada et al. 2010; Hopkins et al. 2013a). At our study

sites, tissue Hg concentrations in adult female Common Snapping Turtles exceed concentrations known to cause sublethal and lethal effects in other aquatic species and waterfowl (Wolfe et al. 1998) and reduce hatching success through increased rates of egg infertility and embryonic mortality (Hopkins et al. 2013b).

In 2013 and 2014, during early May (prior to the reproductive season), we searched the study area, to 200 m on either side of the South River, for suitable nesting habitat. We defined suitable habitat as areas exposed to the sun with relatively loose substrate and sparse vegetation that would facilitate digging (Kolbe and Janzen 2002a; Beaudry et al. 2010; Paterson et al. 2013). We used aerial photography, kayaking surveys, and scouting on foot to locate sites with suitable nesting habitat. The Reference sites were four stretches of the river (ranging in length from 1.1–2.2 km) located from 10–14 km upstream of the manufacturing plant where females and eggs were not exposed to excessive Hg. The Hg sites were three stretches of the river (ranging in length from 1.5–2.5 km) located from 7–16 km downstream of the plant (Fig. 1.1). The Hg sites have been well characterized in relation to Hg accumulation and effects on Common Snapping Turtles and other species (Bergeron et al. 2010; Wada et al. 2010; Bouland et al. 2012; Hopkins et al. 2013b). However, to verify Hg levels at our sites, during the 2014 nesting season, we sub-sampled blood from nesting females and tissue from turtle eggs for Hg analysis. Both the reference and Hg sites along the South River are dominated by farmland: reference sites were 61% farmland, 23% forest, 10% residential, and 5% water (i.e., the South River and its tributaries); Hg sites were 68% farmland, 20% forest, 8% residential, and 4% water. We characterized land cover using the National Land Cover Database (NLCD 2011 dataset; Homer et al. 2015).

***Nesting surveys*.** We visited areas where we identified suitable habitat daily throughout the nesting season. In 2013, we conducted morning and evening surveys. However, because we found only 2.3% of all nests in the evening (although we discovered 12% of nests in morning surveys freshly depredated, making their oviposition time unknown), we conducted surveys exclusively in the morning in the latter part of the 2013 nesting season and during the entire 2014 season. To find nests, we searched for signs of recent nesting activity and for migrating or nesting females. Common Snapping Turtles create a mark where nests have been made that can be easily recognized by trained surveyors (Steyermark et al. 2008). When females were spotted nesting, we left them undisturbed until the nests were completed.

We assigned a unique identification number to each nest and recorded the geospatial coordinates of the nest site. To mark the precise locations of nests, we also placed a ground stake 1 m away from each nest, which was flush with the ground and unlikely to attract predators (Strickland et al. 2010). We used ArcGIS (Esri, Redlands, California, USA) to determine the distance to the South River or to the nearest water. Additionally, we categorized the soil type in which we located nests using the Soil Survey database of the U.S. Department of Agriculture (Soil Survey Geographic Data Base 2010). When appropriate, we used range finders to ascertain the distance from nest sites to forest and/or agricultural field edges. Lastly, we recorded nest site characteristics of the area around the nest using a 1 m^2 sampling plot. Specifically, we measured soil water content and estimated the percentage composition of canopy cover, bare ground, forb, grass, crop, coarse woody debris, leafy detritus, other detritus, tree, shrub, and rock (Table 1.1), at the nest site using Daubenmire classes (Daubenmire 1959). We estimated soil water content from soil cores taken to a depth of 20 cm soil, collected at two random points within the 1 m^2 sampling plot surrounding the nest. We combined and homogenized the cores to yeild one soil

sample per nest and determined the water content gravimetrically through weighing soil samples and then drying them to a constant mass in an Isotemp drying oven (Fisher Scientific, Ottawa, Ontario, Canada) using the following equation:

$$\text{Water Content (\%)} = \left(\frac{\text{Sample Mass}_{\text{Wet}} - \text{Sample Mass}_{\text{Dry}}}{\text{Sample Mass}_{\text{Dry}}} \right) \text{x } 100$$

In 2013, we re-visited nests (n = 87) daily during the nesting season and bi-weekly once the nesting season ended. We noted threats to hatching success such as predation, flooding, or tilling of agricultural fields that occurred during the first month of incubation. We considered nests depredated on day zero if the nest was never witnessed intact, because we conducted surveys every 24 h during the nesting season. In 2014, we did not use nests to evaluate predation because we collected nests to be used in subsequent experimental studies (Molly Thompson et al., unpubl. data).

Habitat Availability Surveys. To compare characteristics of used nest sites to available habitat, we collected habitat data associated with nests (Table 1.1) and at randomly selected points. We randomly selected 15 sets of coordinates that were both within 100 m (in 2013) or 200 m (in 2014) of either side of the South River and along portions of the river that were within 200 m of areas used for nesting the same season (Fig. 1.2). We increased our sampling area in 2014 because turtles were found nesting greater than 100 m from the South River in 2013.

We also conducted paired random habitat surveys to assess nest site characteristics described above but at a finer scale. Nests encountered were paired with a random point located between 5 and 30 m from the actual nest site on the same day that the nest was discovered (30 m

was the average distance female Common Snapping Turtles traveled from the South River to nest sites in 2013). We used a list of random azimuths and distances to locate the paired random point for each nest (Fig. 1.2).

Statistical methods. We performed all statistical tests in JMP Pro version 11.2.0 (SAS Institute Inc., Cary, North Carolina, USA) or Microsoft Excel 2013 with significance assessed at $\alpha = 0.05$. In all analyses involving eggs or habitat characteristics, we use nests (or random points) as the sampling unit. We used logistic regression to examine patterns of predation in relationship to key habitat characteristics (Marchand and Litvaitis 2004a; Fisher and Wiebe 2006). Nest fate was the dichotomous dependent variable and the following continuous predictor variables were used to model probability of nest success: (1) percentage canopy cover over the nest site; (2) distance to the nearest water body; (3) shortest distance to the South River; and (4) the distance to field and forest edges (Wilhoft et al. 1979; Marchand and Litvaitis 2004a). In 2014, we collected eggs from nests and in 2013 we did not find nests after being destroyed by predators in reference sites. Because of this, we restricted our logistic regression tests on predation patterns to Hg sites during the 2013 nesting season (Appendix A).

To accomplish our objective of developing a predictive model for nest site selection in a system dominated by anthropogenic land use, we evaluated relationships between characteristics of nest sites and random points using univariate logistic regression analyses for each habitat variable measured (Keating and Cherry 2004; Hughes and Brooks 2006) except for soil type, which was tested using a Fisher's exact test. Initially, we compared nest sites to random points separately within reference and Hg sites (using univariate logistic regressions), and directly compared characteristics of nest sites in reference and Hg sites (using *t*-tests for each variable),

but found little variation between sites in both analyses. Because trends between habitat characteristics at nest sites and random points were highly similar between reference and Hg sites for most variables tested (n = 10/14, Appendix B), and only a few characteristics differed in availability (percentage grass and crops) or use (percentage bare ground and distance to the South River), we elected to present pooled data between sites and discuss trends in habitat use compared to random habitat availability along the South River both up and downstream of the Hg source.

At a finer resolution, we compared characteristics of nest sites to paired random points using paired *t*-tests and Wilcoxon signed-rank tests. We tested differences in soil types between nest sites and paired points using Fisher's exact tests. Initially, we investigated differences between nest site characteristics and paired points separately within reference and Hg sites and between sites (Appendix C), as we did for analyses at the larger spatial scale. Because we found little variation in habitat use and availability between reference and Hg sites, we elected to present paired analyses with data pooled between sites.

We chose to use the habitat characteristics of Common Snapping Turtle nest-sites and random points to develop our predictive model, rather than paired habitat data, because differences in selection were very minor at the paired resolution. We used a correlation matrix to identify correlated features (from all listed in Table 1.1) and we retained one feature from each highly correlated pair ($|r| \geq 0.7$) to include in multivariate regression models (Hosmer and Lemeshow 2004; Marchand and Litvaitis 2004b). We also removed habitat characteristics that had very low frequencies of occurrence at both random points and nest sites (i.e., rocks, coarse woody debris, etc.). Using the resulting characteristics, we fit several logistic models based on knowledge of the species and indications of importance from univariate analyses ($P < 0.200$).

Models were then fitted and reduced using Wald tests and Likelihood Ratio Statistics (Hosmer and Lemeshow 2004). We present four final models that we rank using deviance and receiver operating curve (ROC) indices (Pearce and Ferrier 2000). Results of egg contamination with Hg are given as egg total Hg (THg) in parts per million (ppm) dry weight (dwt). Values are means ± one standard error (SE).

Results

In the two seasons (spring-summer, 2013–2014) of this study, we located 150 Common Snapping Turtle nests in the reference (n = 62) and Hg sites (n = 88). The average date of nesting was 2 June ± 1.3 d (SE), and among turtles that nested in the morning (the time of day most surveys were conducted), the average time that we observed turtles finish nesting was 0840 ± 0.2 h (SE). The average egg THg in clutches collected downstream of the Hg source was nearly 10 × higher than concentrations in clutches collected upstream (Hg sites = 2.6 ± 0.2 ppm SE, n = 10; reference sites = 0.3 ± 0.3 ppm SE, n = 8). We found no elevated concentrations of THg (above 0.5 ppm) among samples from the reference sites, supporting our longer term mark recapture efforts that indicate Common Snapping Turtles do not move between our reference and contaminated sites.

We primarily found nests in: (1) agricultural fields (n = 119, 79%); (2) commercial nursery properties with open patches of bare ground or grassy areas (n = 17, 11%); and (3) within 5 m of rivers or tributaries that offered banks with exposed soils (i.e., sparsely vegetated soil; n = 7, 5%). We found no depredated nests in the reference sites in 2013 and we did not evaluate depredation patterns in 2014. Of the 45 nests we found in 2013 at the Hg sites, depredation was high among nests in agricultural fields (n = 23/32, 72%), and in nursery properties (n = 7/9, 78%), but did not occur among nests found located near rivers or tributaries (n = 0/4, 0%).

Of the 150 total nests found, 52% were found in just two of the seven total nesting areas that we identified (Fig. 1.3). The two nesting areas used at high rates were in agricultural fields bordered by the South River on three sides (horseshoe bend nesting areas depicted in Fig. 1.2 and shown in Fig. 1.3). A *post-hoc* Fisher's exact test, with nest density as the binary response, showed that soil types differed between low and high density areas ($P < 0.001$, Fig. 1.5). The two high density nesting areas in our study represented two extremes of predation probability in 2013; the reference site experienced 0% predation (n = 0/20 nests); whereas, the Hg site experienced 83% predation (n = 24/29 nests).

During the 2013 nesting season, we found that 66% of nests were depredated at the Hg sites; whereas, none were depredated at the reference sites (Table 1.2). Logistic regression relating predation to habitat characteristics (in 2013 at the Hg site) showed that increasing distances of nests to forest edges decreased the likelihood of predation ($\chi^2 = -2.00$, df = 32, $P = 0.046$), but that the distance of nests to the nearest water source, the South River, and field edges were not useful predictors of predation (in all cases $P > 0.160$; Table 1.2). Other potential sources of nest destruction that we identified include the tilling of agricultural fields and flooding. We found one nest destroyed by tilling in July; however, it is unknown if more nests were destroyed during crop harvesting in the fall. We found one nest partially washed away by flooding and nine nests to be under a pool of water for over one week (all in Hg sites).

Of the 14 habitat characteristics we examined, 11 were useful in distinguishing between nest sites and random points; percentage canopy cover, forbs, grass, crops, and leafy detritus, other detritus, bare ground, and distance to forest and field edges (in all cases $P \leq 0.009$; Table 1.3). We found that soil composition differed between reference and Hg sites but that nests at both sites were associated with less variation in soil type than random points (Fisher's exact: $P <$

0.001). Nest sites were in loamy soils with sand more than random points and in loamy soils with cobble, clay, and silt less than random points (Fig. 1.4).

At the paired, fine scale resolution, only three of the 11 variables that we examined differed between nest sites and paired random points. Nest sites had lower canopy cover and forbs, and higher levels of bare ground than paired random points (in all cases $P \leq 0.033$; Table 1.4). Compared to nests in the reference sites, turtle nests in the Hg sites had less bare ground (20.1% ± 5.7 less bare ground, mean ± SE) than in reference sites, despite no difference in bare ground availability between the reference and Hg sites ($x^2 = -1.92$, df = 45, $P = 0.129$, Appendix B). We also found that nests in the Hg sites had wetter soil (20.4% ± 0.5 higher soil water content, mean ± SE) despite no difference in water availability compared to the reference sites ($t_{1, 45} = -1.10$, $P = 0.164$, Appendix C).

Of the four competing models discriminating between turtle nest sites and randomly selected points, we recommend the model that had one of the highest AUC values (0.904), the highest number of true positives (Accuracy = 0.708), and the largest percentage decrease in deviance compared to the deviance of the null model (48.3% compared to the next highest at 42.02%; Table 1.5). The selected model predicts the probability of habitat use by Common Snapping Turtles for nesting using the negative influence of canopy cover, the negative influence of ground cover by forbs, the positive influence of bare ground, and the positive influence of detritus (Table 1.6). The probability of a site being suitable for nesting can be estimated as:

$$P=\frac{\mathrm{EXP}(-2.152+(0.042*BG)+(-0.045*FORBS)+(-0.045*CANOPY)+(0.028*DETRITUS))}{1+\mathrm{EXP}\left(-2.152+(0.042*BG)+(-0.045*FORBS)+(-0.045*CANOPY)+(0.028*DETRITUS)\right)}$$

Discussion

We found strong similarities in the nesting ecology of Common Snapping Turtles in Hg contaminated and reference sites; in both areas turtles favored disturbed areas for nesting and

seemed to choose sites primarily based on high solar exposure. However, turtle nests in the Hg sites were located in sites with wetter soil and less bare ground than in reference sites, despite no difference in water content or bare ground availability between the reference and Hg sites. Moreover, 66% of nests in the contaminated sites were depredated compared to 0% in the reference sites.

The differences in soil water content that we observed between sites may be biologically significant; turtles selected nest sites with higher soil water content in the Hg sites than in the reference sites and nest flooding was only observed in the Hg sites (n = 10 nests). Consequently, Hg may impact turtle reproductive success through both maternal behavior (e.g., increased selection of nest sites in floodplains) and maternal transfer of Hg to eggs that causes increased rates of unfertilized eggs and embryonic mortalities (Hopkins et al. 2013a, b). While Hg has been shown to alter neural function and behavior in vertebrates (Wolfe et al. 1998), ours is one of the first studies to suggest that Hg may impact nest site selection in turtles. Yet, the impact of Hg on nest site selection was restricted to soil water content and bare ground; the other 12 nest site characteristics examined showed similar trends in both areas.

The striking difference in depredation rates between our reference and Hg sites may have been related to predator community structure, which is not necessarily related to Hg pollution. The property owner of two of our four reference sites actively hunted mesopredators on his land while tenants at the Hg sites reported that Ground Hogs (*Marmota monax*) and White-tailed Deer (*Odocoileus virginianus*) are the only animals hunted on their property (pers comm.). However, we cannot rule out the possibility that Hg, which readily biomagnifies in predators, directly influences predator populations, community structure, or predator feeding ecology.

In both reference and Hg sites, female Common Snapping Turtles nested largely in agricultural fields and in disturbed soils at commercial nursery properties, which may prove maladaptive. Other studies have observed that Common Snapping Turtles, and other turtle species, often nest in human-disturbed soils including earthen dams, gardens, road-shoulders, and agricultural fields (Bobyn and Brooks 1994; Castellano et al. 2008; Beaudry et al. 2010). Not surprisingly, a recent study of nest site selection by Blanding's Turtles (*Emydoidea blandingii*) in agricultural landscapes found that vegetation cover increased significantly over turtle nests in the agricultural sites but not in reference sites (Mui et al. 2015). Lower incubation temperatures in Common Snapping Turtle nests in agricultural fields and the production of male biased offspring sex ratios have been observed in Minnesota, USA (Freedberg et al. 2011). Additionally, crop canopies impair the ability of hatchlings to use environmental cues for orientation that guide them towards water (Pappas et al. 2013; Congdon et al. 2015). Consequently, agricultural land may provide attractive but unsuitable nesting habitat for aquatic turtles.

Nest density at both reference and Hg sites was related to river geomorphology and soil type. Overall, turtle nest density was highest in agricultural fields that were bordered by the South River on three sides. To our knowledge, the effect of this river geomorphology (i.e., horseshoe bends in rivers) on turtle nest site selection has not been investigated. However, recent work suggests that nesting in these areas could be advantageous because hatchlings emerging from nests in agricultural fields have impaired orientation and horseshoe bends provide three directions for the hatchlings to find water (Pappas et al. 2013; Congdon 2015). In addition, the river deposits substrate in the floodplain along its inner bank, and creates deep pools with woody debris on the outside of bends (Harrison et al. 2011), which likely provide high quality in-

stream habitat for females to use before and after nesting (Braudrick and Grant 2001; Garcia et al. 2012) and may represent high quality hatchling habitat. High density nest areas were found more in fluvaquents, and less in loamy fine sand, compared to low density nest areas. The tendency to avoid sandy areas for nesting has been documented in Painted Turtles (*Chrysemys picta*; Christens and Bider 1987; Ratterman and Ackerman 1989) and may be due to the low water holding capacity of sand because turtle embryos are sensitive to the hydric conditions of nest substrates (Packard et al. 1987; Deeming 2004).

Common Snapping Turtles and other aquatic turtles are known to nest both solitarily and in high densities, and their nest sites and high density nesting areas are often well defined and consistently used (Burke et al. 1998; Robinson and Bider 1988). In some cases, high density nesting has been shown to decrease predation rates, possibly by satiating nest predators with a few nests, leaving the other nests in the area less likely to be depredated (Robinson and Bider 1988; Eckrich and Owens 1995). However, evidence to support this hypothesis is lacking (Burke et al. 1998; Doody et al. 2003; Marchand and Litvaitis 2004a). The two high density nesting areas in our study represented two extremes of predation probability in 2013; the reference site experienced no predation; whereas, the Hg site experienced 83% predation. Although we did not find a relationship between nest density and nest predation, we found that increased distance of nests from forest edges was associated with a decreased likelihood of predation. This may be the result of higher mesopredator density and/or activity near forests than in open fields. Our observations are thus consistent with other studies that found that nesting farther from ecological edges decreases the probability of predation of turtle nests (Kolbe and Janzen 2002b; Leighton et al. 2008; but see Congdon et al. 1983, 1987).

The key habitat characteristics incorporated in our predictive model for Common Snapping Turtle nest site selection were primarily related to solar exposure, which is consistent with the findings of many other studies of nest site selection by aquatic turtles (e.g., Janzen and Morjan 2001; Valenzuela 2001; Paterson et al. 2013). Our model predicts the likelihood that a particular location would be used for nesting by Common Snapping Turtles based on the positive influence of high levels of bare ground and detritus, and the negative influence of high levels of forbs and canopy cover. For example, our model would predict the likelihood of nesting use to be 0.047 for a site with the average characteristics of the randomly selected sites used in this study. The optimal probability cut point for this model is 0.658 (Fig. 1.6). When using our model for management decisions, if the characteristics of a site (i.e., percentage bare ground, forb, detritus, and canopy cover) equate to a probability of use higher than 0.658, then the logistic regression predicts that the site will be suitable for nesting. This model could be used to select sites to survey for nesting turtles or areas that might be prioritized for conservation actions, or to inform restoration activities that aim to increase suitable turtle nesting habitat (e.g., sites may be altered to increase the percentage bare ground and detritus while decreasing the percentage of forbs and canopy cover).

Results from our study can be used to mitigate low reproductive success among aquatic turtles due to anthropogenic activities. Because the 200 m area along both sides of the South River is dominated by agricultural land use in our reference (61%) and Hg sites (68%), measures to promote best management practices for turtles and turtle nests are needed. For example, avoiding monoculture and planting a variety of crops at varying times in areas with high turtle nesting rates may help diversify thermal effects of crops on nests and thus help to produce mixed hatchling sex ratios. Because hatchlings of some turtle species overwinter in their nests and

others (such as Common Snapping Turtles) emerge in the fall when corn and soy are harvested, we support prior recommendations that the cutting height of disc mowers (a traditional harvesting tool) be set to at least 100 mm above the soil surface to help to reduce nest destruction and hatchling injury or mortality likely associated with fall crop harvests (as per Saumure and Bider 1998 and Saumure et al. 2007). Of course, identifying high density nesting areas and protecting them from destructive agricultural activities and excessive predation may be the most beneficial management action in many scenarios.

In addition to highlighting the importance of best management practices for crop planting and harvesting, our work identifies key future research needs. First, because growing crops increasingly shade nests throughout incubation, experimental research is needed to understand how agriculture practices may impact turtle nest success and hatchling characteristics (Mui 2015). Additionally, our work suggests that horseshoe bends in rivers may create high quality nesting habitat, and future work should verify whether this is a general pattern in freshwater turtles that could allow land managers to prioritize protection of habitat of high conservation importance. Finally, Hg studies traditionally focus on fish and fish eating wildlife (Crump and Trudeau 2009) because Hg is methylated and becomes bioavailable in aquatic systems. The predation of Common Snapping Turtle eggs that contain high levels of Hg in eggs (which is well documented; Hopkins et al. 2013a), suggest that turtles provide dietary subsidies of Hg to terrestrial mesopredators and highlight the need for studies on the effects of excessive Hg on nest predators.

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Table 1.1 Habitat characteristics sampled at Common Snapping Turtle (*Chelydra serpentina*) nest sites, random points, and paired random points along the South River, Virginia, USA, from 2013–2014. Habitat variables were used to investigate predation and nest site characteristics and are expressed as the percent of each variable in the 1 m^2 area around the nest site or random point.

Variable	Description
CANOPY	percentage of canopy closure, estimated as the percent of ground obscured by vegetation
BG	percentage of bare ground/dirt
FORB	percentage of broad leafed (non-crop) vegetation
GRASS	percentage of grasses
CROP	percentage of crops (corn or soy)
WOOD	percentage of coarse woody debris, downed trees, or woody tree roots
LEAF	percentage of leafy detritus
DETRITUS	percentage of detritus other than leafy or woody (such as compost in agricultural fields)
TREE	percentage of tree
SHRUB	percentage of shrubs
ROCK	percentage of rocks, including pebbles to roughly 3 cm
RIVER	shortest distance to the South River
WATER	shortest distance (in m) to the nearest water source (river, tributary, or pond)
FOREST	shortest distance (in m) to forest bordering open habitat in the direction of the South River
FIELD	shortest distance (in m) to the edge of an open area in the direction of the South River
SOIL	category of soil classified using SSURGO database
WATER	Percent of water in soil cores taken from within 1 m of nest sites (to 20 cm in depth)

Table 1.2 Comparison of Common Snapping Turtle (*Chelydra serpentina*) nest predation rates in reference (REF) and Hg sites along the South River, Virginia, USA, and relationships between predation rates (during the first month of incubation) and habitat characteristics (linear distance in m between nests and habitat characteristics) during the 2013 season. The number of nests is shown as n, rate is the percent of nests depredated at each site. For each habitat characteristics (mean ± SE), P-values represent results from univariate logistic regression analysis.

	REF Sites		HG Sites						
	n	%	n	%	CANOPY	FIELD	FOREST	WATER	RIVER
Depredated	0	–	31	65.6	0	12.4 ± 1.5	12.6 ± 1.4	31.4 ± 2.7	31.4 ± 2.7
Not depredated	39	100	17	35.4	6.7 ± 6.7	18.4 ± 4.7	25.3 ± 5.9	32.9 ± 6.3	38.4 ± 5.5
B	–	–	–	–	–	-0.0640	-0.1056	-0.0049	-0.0298
X^2	–	–	–	–	–	$X^2_{1,33}$ = -1.41	$X^2_{1,32}$ = -2.00	$X^2_{1,42}$ = 1.2	$X^2_{1,37}$ = -1.2
P-value						0.160	**0.046**	0.281	0.227

Table 1.3 Average habitat characteristics (mean ± SE) of Common Snapping Turtle (*Chelydra serpentine*) sites and randomly selected points and results of univariate logistic regression analyses investigating the extent to which each habitat characteristic could be used to distinguish between turtle nest sites and random points along the South River, Virginia, USA. Data from reference and Hg sites are pooled; the number of each type of characteristic measured is shown as n; regression coefficients (β), one standard error (SE), and) nest Wald chi-square statistic (Wald x^2) from Wald tests for each univariate model against the intercept-only model.

Variable	n	Nest	n	Random	β	SE	Wald x^2	df
CANOPY	150	0.9 ± 0.5	161	26.5 ± 3.2	-0.0510	0.0125	16.63	1
BG	150	71.3 ± 2.2	161	22.9 ± 2.5	0.0426	0.0043	96.05	1
FORB	150	4.9 ± 0.9	161	19.0 ± 1.8	-0.0596	0.0108	30.42	1
GRASS	150	5.2 ± 1.3	161	31.0 ± 2.9	-0.0378	0.0063	36.05	1
CROP	150	5.8 ± 0.7	161	3.2 ± 1.5	0.1385	0.0413	11.23	1
WOOD	150	< 0.01 ± 0.01	161	1.1 ± 0.3	-1.5751	0.9780	2.58	1
LEAF	150	0.6 ± 0.2	161	5.9 ± 1.3	-0.1144	0.0436	6.90	1
DETRITUS	150	14.7 ± 1.5	161	8.7 ± 1.4	0.0193	0.0070	7.63	1
TREE	150	0.1 ± 0.07	161	1.6 ± 0.6	-0.1784	0.1087	2.69	1
SHRUB	150	< 0.01 ± 0.01	161	5.2 ± 1.5	-0.7464	0.5246	2.02	1
ROCK	150	0.7 ± 0.2	161	0.4 ± 0.3	0.0326	0.0415	0.62	1
RIVER	132	58.8 ± 7.9	161	60.5 ± 4.5	–	–	–	–
WATER	138	36.2 ± 2.1	161	48.1 ± 2.9	-0.0119	0.0037	10.01	1
FOREST	126	23.2 ± 2.6	68	54.4 ± 4.6	-0.0282	0.0057	24.40	1
FIELD	124	22.4 ± 2.8	93	55.0 ± 6.0	-0.0240	0.0056	18.33	1

Table 1.4 Habitat characteristics of Common Snapping Turtle (*Chelydra serpentina*) nest sites and paired random points (reference and Hg sites pooled) along the South River, Virginia, USA, in 2014. The number of nests measured is shown as n. Nest averages (mean ± SE) are presented and Paired Mean Difference (Paired Mean Diff.) is the percent difference between nest sites and paired random points, relative to the nest site (i.e., on average, nest sites had 12.8% less canopy cover and 13.2% more bare ground than paired random points). Differences between nest and paired random points were tested with a paired t-test except for FOREST, which was tested with a Wilcoxon signed-rank test.

Variable	n	Nest	n	Paired Mean Diff.	Test Statistic	*P* -value
CANOPY	60	0.33 ± 0.3	60	-12.8	t = 2.31	**0.031**
BG	60	55.8 ± 3.9	60	13.2	t = -2.58	**0.020**
FORB	60	4.0 ± 1.4	60	-7.9	t = 1.85	**0.033**
GRASS	60	9.3 ± 2.6	60	-2.4	t = 0.97	0.336
CROP	60	5.6 ± 0.7	60	-0.2	t = 0.94	0.353
WOOD	60	0	60	-0.8	t = 1.03	0.307
LEAF	60	1.0 ± 0.4	60	-0.1	t = -0.35	0.725
DETRITUS	60	21.9 ± 3.1	60	1.5	t = 0.27	0.791
TREE	60	0.18 ± 0.17	60	-0.3	t = 1.00	0.323
SHRUB	60	0.02 ± 0.02	60	-2.9	t = 0.96	0.342
ROCK	60	0.9 ± 0.4	60	0.2	t = -0.73	0.467
WATER	44	44.1 ± 3.8	44	4.0	t = -1.46	0.151
FOREST	36	17.8 ± 2.3	36	-5.8	z = 96.00	0.113
FIELD	34	16.9 ± 2.8	34	-1.1	t = 0.26	0.799

Table 1.5 Comparison of ROC evaluations and deviance indices of predictive habitat models developed using logistic regression analysis of habitat data collected at Common Snapping Turtle (*Chelydra serpentina*) nest sites and random points along the South River, Virginia, USA, during 2013 and 2014. Predictive models were tested against 20% of each dataset that was not used for model fitting.

		ROC				DEVIANCE		
Rank	MODEL STRUCTURE	AUC	Accuracy	Null	df	Residual	df	Decrease
1	BG + FORB + CANOPY + DETRITUS	0.904	0.708	309.8	225	160.1	221	48.32
2	BG + FORB + CANOPY + GRASS + FOREST + WATER	0.904	0.688	216.9	156	126.5	150	41.07
3	BG + CANOPY + WATER	0.890	0.670	309.8	222	179.6	225	42.02
4	GRASS + FORB + FOREST	0.895	0.665	232.8	169	148.1	166	36.46

Table 1.6 Estimated Coefficients, standard errors, Z-scores, two-tailed P-values and 95% confidence intervals for the model that best predicted Common Snapping Turtle (*Chelydra serpentina*) nesting habitat use within 100–200 m of the South River, Virginia, USA. For each term in the model the estimated regression coefficient (β) and 1 SE are shown; statistical significance (Sig.) of each regression coefficient was tested using the Wald chi-square statistic (Wald x^2).

VARIABLE	β	SE	Wald x^2	*P* -value
BG	0.0419	0.0072	5.86	< 0.001
FORB	-0.0458	0.0217	-2.11	< 0.001
CANOPY	-0.0452	0.0216	-2.09	0.035
DETRITUS	0.0282	0.0101	2.78	0.037
INTERCEPT	-2.1518	0.6216	-3.462	<0.001

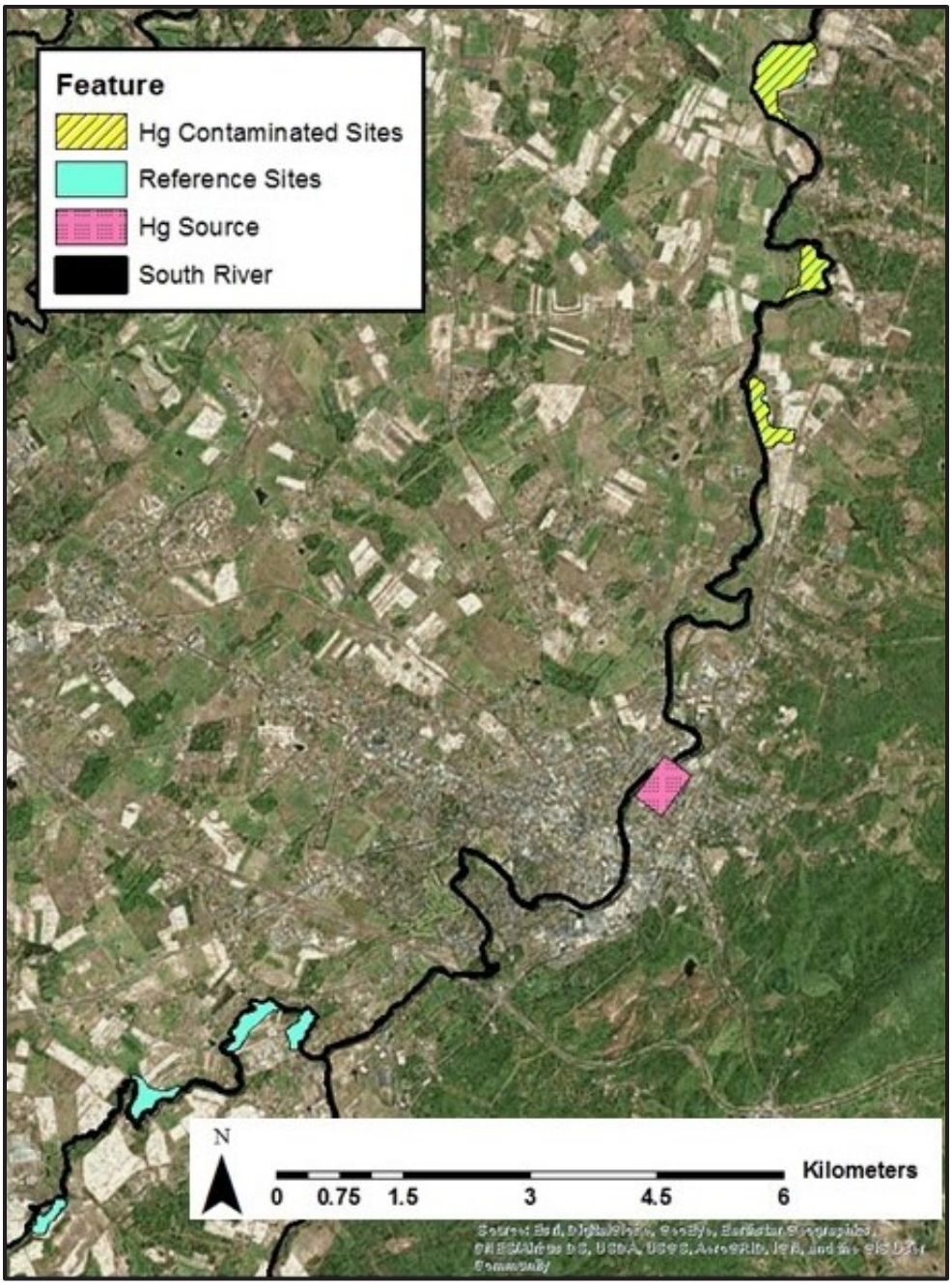

Fig. 1.1 Visual of the study areas along the South River, Virginia, USA, monitored for Common Snapping Turtles (*Chelydra serpentina*) nests during 2013 and 2014. The historical point source of Hg is shown in pink with dots, the Hg contaminated nesting sites in yellow with stripes, and the references sites are shown with blue.

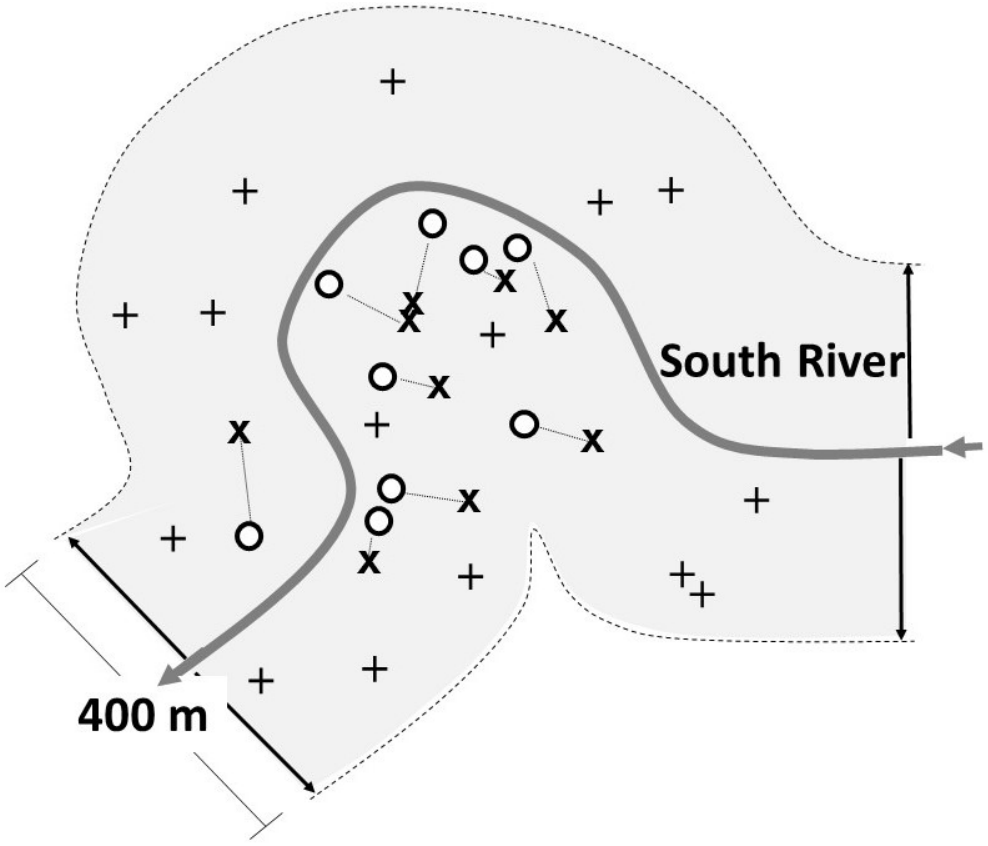

Fig. 1.2 Sampling design used in 2014 for Common Snapping Turtle (*Chelydra serpentina*) nests, random points, and paired random point habitat surveys along the South River, Virginia, USA. Turtle nests (O), paired random points (X), and random habitat surveys (+) were conducted in the areas where nesting was detected (the reference site had four nesting areas, Hg site had three nesting areas), within 100–200 m on either side of the South River throughout the nesting season.

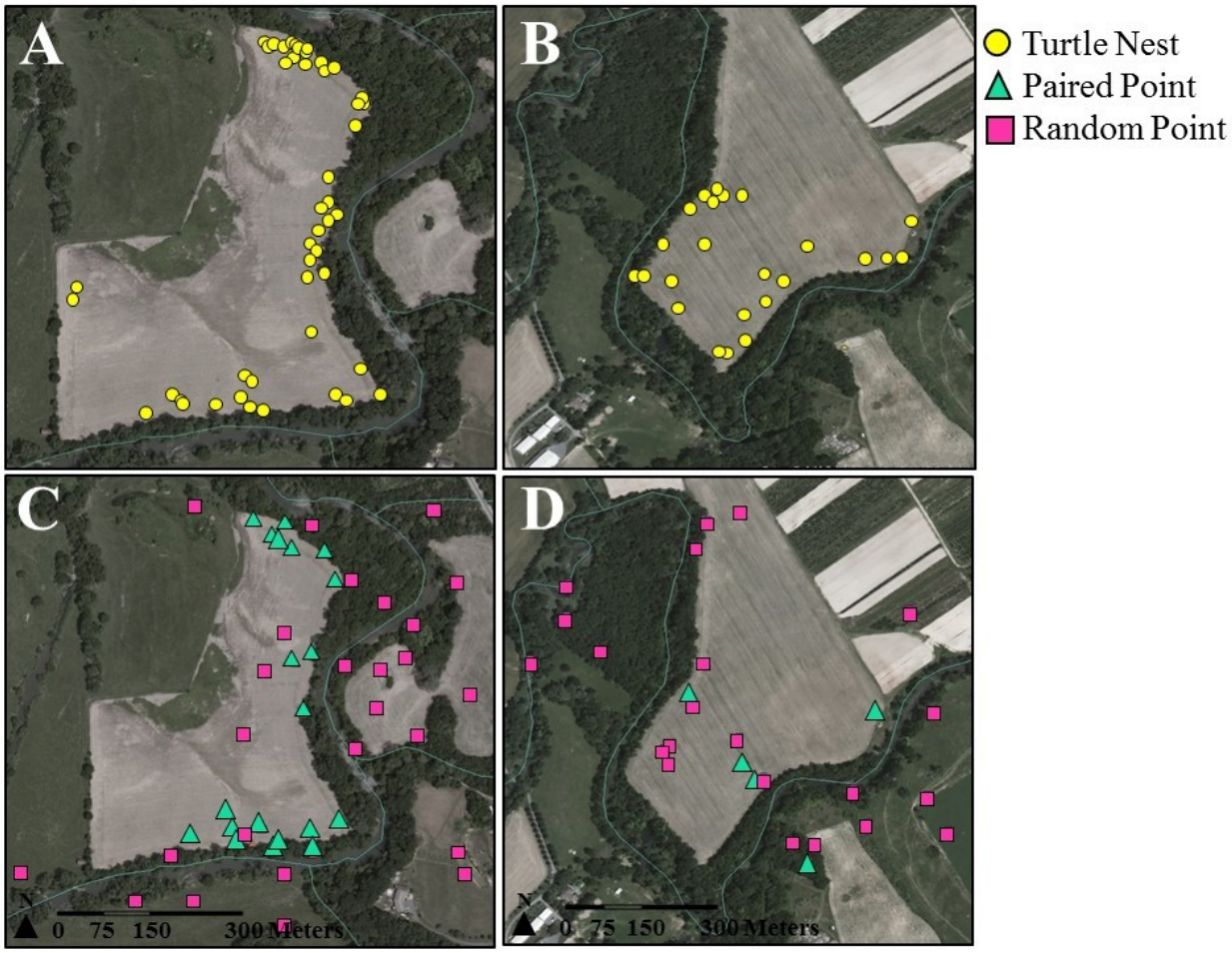

Fig. 1.3 Two high density Common Snapping Turtle (*Chelydra serpentina*) nesting areas (turtle nest sites are shown with yellow circles), one in a Hg site (A) and one in a reference site (B) along the South River, Virginia, used by Common Snapping Turtles during 2013 and 2014. The paired habitat points corresponding to turtle nests are shown with blue triangles, and locations where random habitat points were sampled are shown with pink squares for the Hg site (C) and the reference site (D).

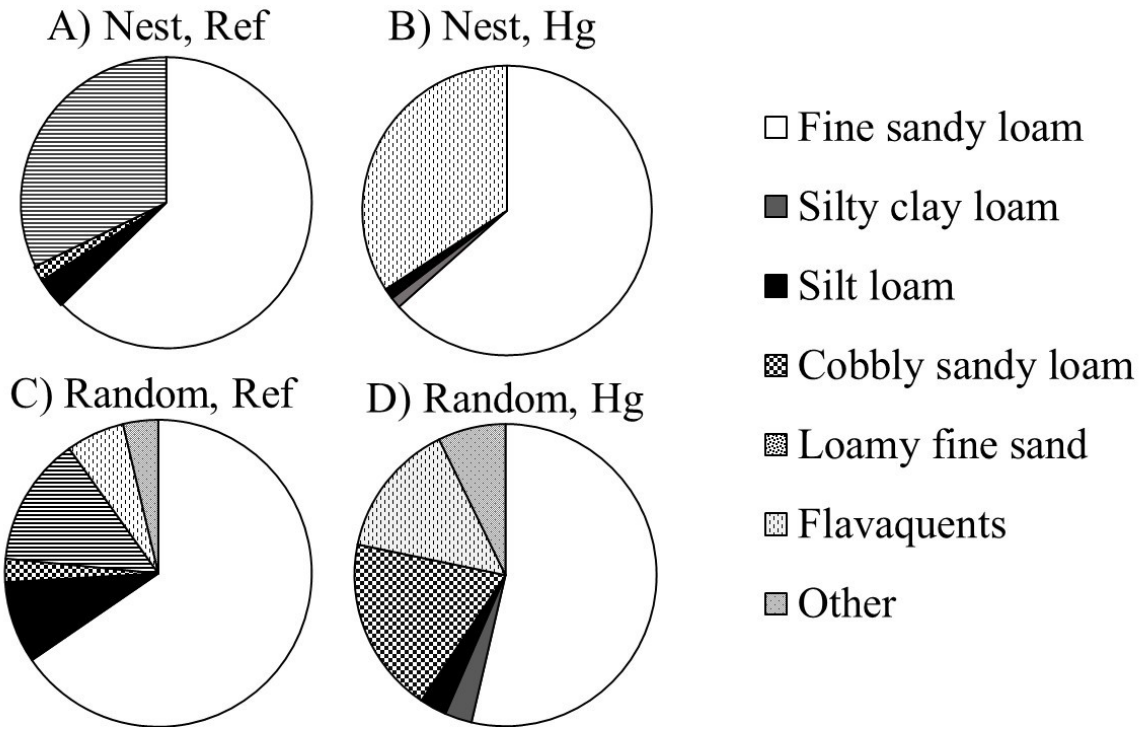

Fig. 1.4 The percent composition of soils (SSURGO categories) at Common Snapping Turtle (*Chelydra serpentina*) nest sites (A & B) and random points (C & D) sampled along the South River near Waynesboro, Virginia, USA, 2013–2014.

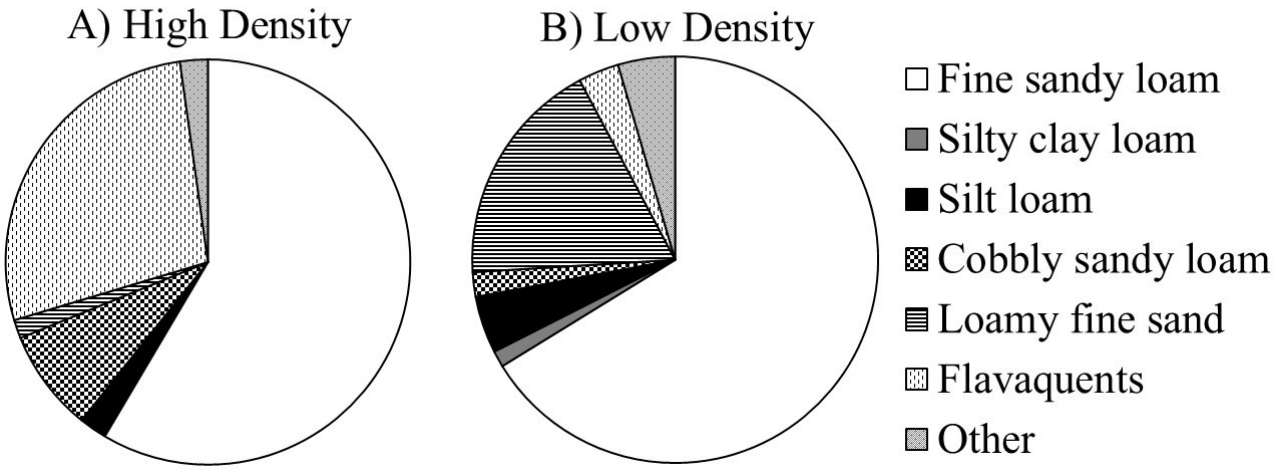

Fig. 1.5 Percent of SSURGO soil categories at high density Common Snapping Turtle (*Chelydra serpentina*) nesting areas (A) and low density nesting areas (B) along the South River, Virginia, USA, 2013–2014.

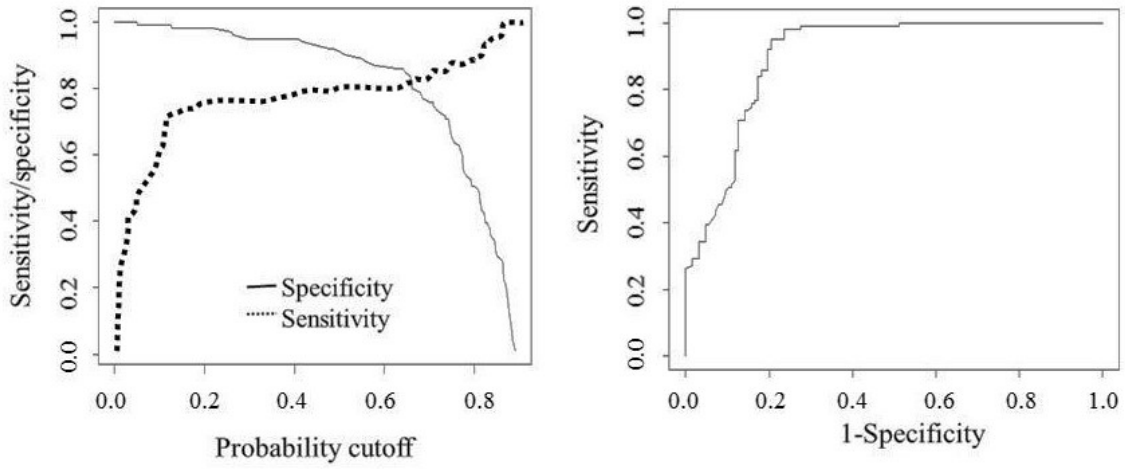

Fig. 1.6 Performance of our highest ranked predictive model developed to identify nesting habitats of Common Snapping Turtle (*Chelydra serpentina*) along the South River, Virginia, USA, 2013–2014. Model specificity versus possible probability cutpoints and sensitivity (Left) showed that the optimal cut point for classification for our models is 0.658; the sensitivity versus 1-specificity (the ROC Curve) of our highest ranked model showed an AUC of 0.904 (Right).

Chapter 2: Agricultural Land Use & Mercury Pollution Interact to Impact Offspring Phenotype of a Freshwater Turtle

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Abstract

Environmental change caused by anthropogenic land use has caused population declines in diverse taxa worldwide. The rate of habitat conversion for anthropogenic use far exceeds the rate of natural ecological and evolutionary processes. Consequently, environmental cues that were evolutionarily useful for animals sometimes fail to confer the same benefits in human-modified habitats and maladaptive behaviors are increasingly common. Relatively few studies have focused on the combined influence of more than one aspect of global change (e.g., pollution and habitat fragmentation) on animals' behavioral decisions and development despite the fact that multiple factors often co-occur spatially and temporally. For example, in numerous species of aquatic turtles, nest site selection is primarily based on environmental cues related to high solar exposure. Consequently, females often select nest sites where humans have disturbed terrestrial substrates and vegetation (e.g., recently tilled agricultural fields) and these same disturbed sites are often contaminated by pollutants. We used Snapping Turtles (*Chelydra serpentina*) as a model to investigate the individual and interactive effects of crop agriculture and mercury pollution on hatch success and offspring phenotype using a factorial design in paired laboratory and field experiments. We hypothesized that following nesting, rapid crop growth would shade and cool nests in agricultural fields and subsequently impact embryonic development. Agriculture and control nests did not differ in temperature at the time of oviposition, but temperatures diverged as crops grew, with temperatures in agriculture nests averaging 2.5 °C lower than control nests over the course of incubation. In both experiments we found that in

comparison to turtles from control incubation conditions (i.e., warmer), turtles incubated under agricultural thermal regimens (i.e., colder) took longer to hatch, hatched at smaller structural body sizes, lost more mass after hatching, and had lower post-hatching structural growth rates. Additionally, thermal conditions associated with agricultural land use interacted with high levels of mercury to decrease hatching success. To our knowledge, this is the first documentation of negative interactive effects of mercury pollution and habitat quality on early vertebrate development and highlights the importance of examining the combined influence of multiple global changes on biological systems.

Keywords: global change; pollution; agriculture; hatch success; offspring phenotype; development; reptile; turtle; anthropogenic; disturbance; interactive

Introduction

Human-induced rapid environmental changes such as deforestation, urbanization, climate change, and pollution have caused global population declines in a wide variety of taxa including mammals, birds, reptiles, amphibians, fishes, and insects (Robertson et al. 2013). Agents of global change, such as anthropogenic habitat alteration and pollution, generally occur at a significantly faster rate than natural processes. As a result, evolutionarily useful environmental cues may no longer be present in modified habitats or such cues may become mismatched from the cost/benefit regimens under which the species evolved, resulting in reduced fitness of organisms relying on these cues (Hale and Swearer 2016). For example, reflections of polarized light off of glass and asphalt are stronger than the light reflections off of water and cue insects to oviposit on surfaces where their eggs cannot hatch (Robertson and Hutto 2006). Mismatches

between current environmental cues and evolved behavioral responses are a growing conservation concern because they can result in evolutionary traps: when human modified habitats resemble habitats that historically indicated an optimal choice based on environmental cues but currently result in negative fitness consequences if used (Robertson et al. 2013, Hale and Swearer 2016).

Agriculture and forestry-related activities are rated as the second most common cause of evolutionary traps (Hale et al. 2016). Agricultural land use can cause evolutionary traps by altering prey abundance and predation patterns. Examples of these types of evolutionary traps include high mortality of lizards attracted to agricultural areas by high insect prey availability (Rotem et al. 2013) and high nest depredation among passerine birds drawn to nest near field/forest edges (Gates and Gysel 1978). Although not well studied, rapidly changing thermal conditions of agricultural landscapes due to the seasonal tilling of fields (i.e., sudden elimination of canopy cover followed by rapid crop growth) may have important biological consequences for organisms. Oviparous vertebrates often select oviposition sites based on thermal cues and the temperatures that embryos experience during development strongly impact offspring phenotype and survival (Deeming 2004). For example, in numerous species of aquatic turtles, females select natural nest sites that have a high degree of solar exposure (such as sandy or rocky patches adjacent to rivers and streams) because of the importance of incubation temperature on embryonic survival and offspring phenotype. However, they also exploit recently tilled agricultural fields for nesting, presumably because of increased solar exposure and/or easier nest excavation, (Freedberg et al. 2011, Mui et al. 2015). Yet, the developmental consequences arising from animals being attracted to nest in agricultural fields are not well studied.

To make matters more complex, pollution and anthropogenic land use are often spatially correlated (Vorosmarty et al. 2010). However, relatively few studies have focused on interactions among more than one global change (e.g. climate change, pollution, or habitat fragmentation) on animal behavior and development despite their common co-occurrence in space and time (Robertson et al. 2013, Hale and Swearer 2016). Importantly, temperature interacts with chemical pollutants to affect organisms (Noyes et al. 2009, Hooper et al. 2013, Moe et al. 2013) and crop agriculture alters the thermal dynamics of landscapes (Freedberg et al. 2011). As an important example, mercury (Hg) has recently been found to exert temperature-mediated negative reproductive effects in wildlife (Hallinger and Cristol 2011). Mercury is a ubiquitous pollutant across the globe; approximately 5,000-8,000 metric tons of Hg are emitted globally each year (UNEP 2013) and Hg persists in the environment for hundreds-to-thousands of years (Selin 2009). Females with elevated Hg tissue burdens transfer Hg to their offspring which causes increased rates of embryonic mortality and reduced fertility in several vertebrate groups including marine and freshwater turtles (Perrault et al. 2011, Hopkins et al. 2013b), amphibians (Bergeron et al. 2011, Todd et al. 2012), fishes (Crump and Trudeau 2009) and humans (Sakamoto et al. 2001). Because Hg pollution and crop agriculture are among the most widespread forms of habitat modification in the world (Driscoll et al. 2013, Oakenleaf et al. 2015), they are likely to frequently interact with one another to influence early vertebrate development yet, the interactive effects of these two forms of global change have not been investigated.

In this study, we used Snapping Turtles (*Chelydra serpentina*) as a model to investigate the individual and interactive effects of crop agriculture and Hg pollution on hatch success and offspring phenotype using a factorial experimental design in paired laboratory and field

experiments. Freshwater turtles are of conservation concern because they are among the worlds most endangered vertebrate groups (Buhlmann et al. 2009) and are often important members of biological communities due to their roles as predators, prey, seed dispersers, and nutrient cyclers (Congdon et al. 1986, Moll and Jansen 1995).

We hypothesized that in the weeks following nesting in agricultural fields, rapid crop growth would shade and cool turtle nests and subsequently impact embryonic development. To our knowledge, only one prior study directly examined the effects of agricultural conditions on turtle nest temperature and offspring phenotype (Freedberg et al. 2011). They found that nests in crop fields experienced significantly higher predation rates and produced male-biased offspring sex ratios, compared to nests located in natural prairie fields (Freedberg et al. 2011). Our study builds on these observations, and on prior knowledge of thermal effects on reptile embryonic development and hatchling phenotype, in that we (1) document high resolution (hourly) differences in thermal profiles of replicated nests in agricultural (shaded) and non-shaded conditions controlling for maternal effects, (2) mimic these complex thermal profiles in the laboratory to evaluate the role of temperature in isolation of other variables (such as water availability) in determining hatching success and phenotype, and (3) investigate the interactive effects of agriculture and Hg contamination (Freedburg 2011, Bowden et al. 2014, Warner 2014). We predicted that a cooling effect due to crop shading would impact offspring phenotype by prolonging incubation, decreasing hatching body size and post-hatch growth rate, and decreasing performance. Additionally, we hypothesized that the adverse effects of high egg concentrations of Hg on hatch success would be intensified by cooler agricultural incubation conditions.

Methods

Study species. The common Snapping Turtle is ideal for investigating the effects of Hg and habitat modification on embryonic development and offspring phenotype. Due to their long life span (+70 years), high trophic-level (Punzo 1975), and sedentary nature (Obbard and Brooks 1981), tissue concentrations of Hg in Snapping Turtles can reach high levels and reflect Hg contamination of a very small area compared to more widely-ranging vertebrate predators (Hopkins et al. 2013a). Snapping Turtles maternally transfer Hg to their eggs, which reduces reproductive success by increasing the numbers of unfertilized eggs and embryonic mortality (Hopkins et al. 2013b). Like many aquatic turtles, Snapping Turtles often select human modified habitats for nesting (Kolbe and Janzen 2002, Paterson et al. 2013), including agricultural fields (Freedberg et al. 2011). The developmental rate of Snapping Turtle embryos is affected by temperature, and embryos cannot complete development if they are incubated above 31˚C or below 17˚C (Yntema 1978).

Study area. Our study area was along the South River, centered in Waynesboro, Virginia, where a historical point source of Hg and extensive land conversion for agricultural use has produced a heavily modified and Hg-polluted landscape. A manufacturing plant in Waynesboro used a mercuric sulfate catalyst to produce acetate fiber from 1929-1950. From this period until 1976, the plant released high levels of Hg directly into the South River (Carter 1977). Considerable amounts of Hg remain in the river and the floodplain; tissue concentrations of Hg in fish, birds, amphibians, snakes, and turtles are high and have not decreased over time (Eggleston 2009, Bergeron et al. 2010, Bouland et al. 2012, Drewett et al. 2013, Hopkins et al. 2013a).

Experimental design. This research was part of a larger study on turtle nesting ecology across a range of natural and human-modified habitat types (Thompson et al. 2017 *in press,* Thompson et al. *in prep*). Here, we report the independent and interactive effects of agriculture and Hg contamination on hatching success, incubation period, and hatchling phenotype. To test our hypotheses, we used a 2x2 factorial design to conduct two simultaneous incubation experiments that we refer to as the 'field' or 'laboratory' experiments. Relationships between crop growth in agricultural fields and turtle nest temperature, moisture, and offspring sex ratios will be presented in Chapter 3.

Mercury level was the first factor of the 2x2 design. The two treatment groups were: eggs that contained high concentrations of maternally derived Hg, hereafter referred to as 'Hg' (eggs collected from within 16 km downstream of the industrial Hg source), and eggs that contained only background concentrations of Hg, hereafter referred to as 'Ref' (eggs collected 6-14 km upstream from the industrial Hg source). To verify embryonic Hg exposure levels, we quantified Hg for each clutch either from maternal blood (if we were able to collect the female after nesting) or from one randomly selected egg in each clutch (when we were unable to obtain maternal blood). Because turtles produce all eggs within a clutch synchronously, eggs of the same clutch have similar maternally-derived trace element concentrations (Van Dyke et al. 2013). In cases where we could capture the female and obtain blood, we estimated egg concentrations using regression equations describing the strong positive relationship between egg and maternal blood concentrations in turtles from these field sites (Hopkins et al. 2013a). We froze egg and maternal blood samples at -20 ℃ until Hg analysis (Hopkins et al. 2013b, Van Dyke et al. 2013). We lyophilized and homogenized the subsampled eggs and report their total Hg (THg) concentrations on a dry mass (dwt) basis. The percent moisture of eggs was 75.5 ±

0.7% SE (n = 44). We used blood total Hg (THg) concentrations (on a wet mass, "wwt", basis). Samples were analyzed at the College of William & Mary using combustion-amalgamation-cold vapor atomic absorption spectrophotometry (Direct Mercury Analyzer 80, Milestone, Shelton, CT, USA). For quality assurance, we analyzed standard reference materials alongside experimental samples. Mean percent recoveries of THg for the standard reference materials (fish protein and tuna) were 104.3 ± 1.0 % SE (n = 14) and 100.3 ± 0.3 % SE (n = 14), respectively. All blood and egg samples were run in duplicate and the relative percent difference from duplicate samples averaged 6.3% ± 1.2 SE.

Agriculture (the amount of crop-related shade over nests) was the second factor of the 2x2 design. The two treatment groups were: a control, hereafter referred to as 'Open' (all crops removed and non-crop vegetation was limited to 10% canopy cover), and the agricultural treatment hereafter referred to as 'Shade' (crops were allowed to grow unchecked). Treatment plots were constructed in a field that was planted with corn in early May 2014. This field had high density nesting (> 20 nests in < 6.0 Hectares) the prior year (Thompson et al. 2017 *in press*). A random subsample (n = 10) of the 2013 nest sites was used to select the locations of our ten experimental plots. All plots (n = 5 per treatment) were circles six meters in diameter. In Open treatment plots, non-crop vegetation was limited to 10% cover to maintain the average characteristics that Snapping Turtles select for nesting (Janzen 1994, Kolbe and Janzen 2002). We estimated the percent of each plot covered by non-crop vegetation every eight days.

On collection, clutches were randomly assigned to either the field experiment (n =18 clutches total; Hg =10, Ref =8) or the laboratory experiment (n = 25 clutches total; Hg =13, Ref =12). Ideally, each clutch would have been split four ways to distribute similar clutch effects across lab and field groups, however, the number of eggs in each group would have been too

small for statistically robust comparisons had we done this. We therefore assigned whole clutches to either the field or laboratory experiment, and then randomly split each clutch between Open and Shade treatments. Clutches assigned to the field experiment were randomly assigned to locations within treatment plots and were incubated in the field. Clutches assigned to the laboratory experiment were incubated using incubation regimens that were generated from temperature data collected from our field experimental plots.

Egg collection. We collected 43 entire clutches of eggs from nests along the South River, predominantly from agricultural fields located both upstream and downstream from the initial Hg point source (Virginia DGIF Permit No. 048080, VT IACUC No. 14-110). The fields in which we found the nests had been planted with either corn (61%, n = 26/43) or soy (28%, n = 12/43) during the month of May 2014. The remaining nests collected were found in eroded stream banks (5%, n = 2/43) and in commercial nursery properties (in human disturbed soils: 5%, n = 2/43 and in a manicured lawn: 2%, n = 1/43). The majority of clutches were collected immediately after we observed females complete oviposition (67%, n = 29/43). When we did not see the female oviposit (33%, n = 14/43), only fresh nests were used and clutches were immediately candled on collection and staged using Yntema's 1968 descriptions to verify recent oviposition. The average developmental stage of embryos from all nests was 4.1 ± 1.1 SE (n = 43). We used developmental stage at collection and the average temperature in artificial nests to calculate approximate lay dates for nests that we did not see females oviposit using developmental rates outlined by Yntema (1968, 1978). On average, embryos spent 94 ± 1.2% SE of the incubation period in their assigned experimental incubation treatment.

On collection, we marked each egg with a unique ID, weighed all eggs to the nearest 0.01g, and recorded egg length to the nearest 0.01 mm.

Field experiment. Clutches assigned to the field experiment were transplanted into reconstructed nests in the experimental plots and left undisturbed until retrieval just prior to their expected hatch dates. We transplanted up to four nests in each of the ten treatment plots. The top and bottom of each transplanted nest were placed at the average depths of Snapping Turtle nests, and were 105 mm and 150 mm below the soil surface, respectively (Congdon et al. 1987). We placed iButton temperature data loggers (DS1923, Embedded Data Systems, KY, USA) to record thermal profiles at 1-h intervals at the top and bottom of nests. Vertical temperature stratification can produce phenotypic variation within nests (Wilson 1998, Refsnider et al. 2013a,b). Because 3 of our 72 iButtons malfunctioned (4%), 69 iButtons were used to calculate thermal profiles (Top; $n = 34$, Bottom; $n = 35$). Turtles often release bladder water while digging their nests; we therefore sprayed water onto the dirt as we sealed nest chambers (Marchand and Litvaitis 2004). We placed wire mesh predator guards over each nest that successfully prevented predation (Riley and Litzgus 2013). Prior to burying eggs, we standardized clutch size to 34 in all transplanted nests by adding artificial eggs (i.e., the sum of the real eggs and artificial eggs was always 34). Clutch size was controlled to **a)** to avoid bias in thermal gradients within nests through standardization of nest dimensions and **b)** to control for the impact of clutch size on the thermal masses/inertias of nests. We used a clutch size of 34 eggs based on the average clutch size and mass at our study site (Thompson et al. 2017 *in press*, Hopkins et al. 2013b). Because all clutches were split between treatments we never had extra eggs after standardizing the clutch size. Artificial eggs were hollow polypropylene plastic balls (CIC Ball Company, PN) roughly

the same size as real Snapping Turtles eggs (diameter = 25 mm; Steyermark et al. 2008)) filled with a wire pulling lubricant (ClearGlide^{TM}) which closely mimics the thermal properties of amniotic eggs (Ardia et al. 2010, Coe et al. 2015).

Eggs were retrieved from field nests between August 18 and September 27 (retrieval date based on oviposition date and treatment group) and brought back to the laboratory to complete incubation under constant temperatures corresponding to the average nest temperature recorded in the field during the previous three weeks (23.3℃ for Open, and 20.5℃ for Shade treatments). During egg retrieval, we numbered the eggs in the order they were removed as we dug down into each nest chamber. We classified the first half of eggs removed as the top layer and the second half as the bottom layer. When eggs were retrieved, four of the 18 Open treatment clutches had begun hatching (but none had begun to exit the nest); no eggs in any of the Shade treatment clutches had pipped. We used the retrieval date as the last day of incubation for the four Open nests that began to hatch in the field. All embryos spent > 90 % of their total incubation in the field.

Laboratory experiment. To obtain ecologically relevant temperature profiles that could be used to determine laboratory incubation regimens, we constructed one artificial nest in the center of each field treatment plot (n = 5/treatment). Artificial nests were constructed with the same dimensions, and in the same manner, as transplanted nests but contained 34 artificial eggs. Rather than iButtons, we placed two Hobo-Temp data logger probes attached to cables (Onset Computer Corporation, Pocasset, Massachusetts) at the top and bottom of eggs in each artificial nest, set to record temperature at 1-h intervals. This configuration allowed us to upload

temperature data regularly throughout the duration of the study without disturbing the artificial nests since the Hobo-Temp logger itself sits above the soil surface.

Incubation regimens for the laboratory experiment were calculated from temperatures recorded by the Hobo-Temp loggers in the artificial nests. First, we averaged temperatures from the top and bottom of each artificial nest and then we averaged over the five replicate artificial nests per treatment for each hour of incubation (Fig. 2.1). Because the laboratory experiment was running simultaneously as we generated our thermal profiles from artificial nests in the field, temperatures used in the laboratory experiment had to be slightly staggered behind temperatures recorded in the field. After the initial four days of temperature data were recorded from artificial nests (May 21-25), they were used to program the next four days of incubation in the lab (May 25-29). We continued to upload temperature data from the field every four days and used it to program the next four days of incubation in the lab throughout the duration of incubation (Fig. 2.1).

Clutches assigned to the laboratory experiment were split between Open and Shade incubation treatments in Memmert IPP 55 Plus incubators (n = 4 incubators; 2 per treatment, Model IPP 400, Memmert GmbH+Co.KG, Schwabach, Germany). Incubators were calibrated using iButton data loggers prior to the study and monitored throughout incubation using built-in probes and two iButton loggers placed in the center of each incubator. Eggs were incubated in plastic containers (grouped by clutch) with vermiculite (1:1, water: vermiculite) and capped with a perforated lid. We rehydrated and rotated egg containers among shelves every four days (Packard et al. 1987).

Hatching. We recorded hatching and processed hatchlings the same way in both experiments. We checked incubators twice daily for pipping or hatching. Once eggs approached hatching, they were covered by a small perforated plastic cup to ensure the proper identification of hatchlings. Incubation period was characterized as the time between oviposition and pipping (Gutzke et al. 1984). Pipped eggs and hatchlings were placed in individual Tupperware^{TM} containers and kept at 23℃ in an environmental chamber. We added 2 cm of distilled water to containers once hatchlings fully emerged from their shells. Hatchlings subsisted on yolk reserves for the remainder of the study.

Hatchling Attributes. Upon full emergence from their shells, we weighed hatchlings to the nearest ± 0.01 g and measured their carapace and plastron length with electronic calipers to the nearest ± 0.01 mm. Size measurements were taken for all hatchlings again at day 21-25 post hatch. Relative growth rates for length measurements and mass loss during the 20-day period were calculated as:

$$\% \text{ Change} = \frac{(\text{Final} - \text{Initial})}{\text{Initial}} * 100 \qquad (1)$$

where 'Initial' was the measurement (either mass or length) taken from the hatchling upon full emergence and 'Final' was the second measurement taken 21-25 days post-hatch.

We quantified hatchling performance using two measures, locomotor velocity and righting response. Locomotor performance trials were conducted on days 20-24 post hatch and righting response trials were conducted the following day. All performance trials were carried out in an environmental chamber at 23 ± 0.1℃ SE to ensure consistent body temperatures among hatchlings. Locomotor performance was quantified by measuring hatchlings' speed on a 1m linear track lined with photocells projecting infrared beams interfaced with a laptop computer

(Columbus Instruments, Columbus, OH). If a turtle stayed stationary for three seconds during the trial, we gently prodded it once every three seconds until it started moving again. If hatchlings did not travel the 1m distance within 10 minutes, the trial was terminated. We conducted two trials and gave hatchlings a 45-minute rest between trials. Speed from the fastest trial for each individual was used in statistical analyses. Righting trials were carried out in a square plywood box divided into nine individual arenas of the same size (9 x 9 cm) with 1 cm of sand on the bottom (Delmas et al. 2007). We gave turtles two minutes to acclimate to their individual arena before placing them on their carapace and stepping behind a curtain to be out of sight of the turtles during the trials. All trials were videotaped and the latency time (i.e., how long turtles took to initiate righting effort) and mechanical righting time (i.e., how long it took turtles to reposition themselves) were recorded for each hatchling from the videos (Paitz et al. 2010). Turtles were given no more than 30 minutes to right themselves and were given a 10-minute rest between their two trials. We used the speed from the faster of the two trials (for both endpoints) for statistical analyses.

Data analysis. Hatch success was analyzed using generalized linear mixed models (GLMM) using R software and the lme4 package (R Foundation for Statistical Computing, Vienna, Austria). All other analyses were performed in JMP Pro 11 (SAS Institute Inc., Cary, NC, USA) or Microsoft Excel 2013. For analyses with random effects, we used linear mixed models (LMM). Variance components were estimated for the LMMs using the restricted maximum likelihood (REML) algorithm, rather than ANOVA, to account for unbalanced data (Patterson and Thompson 1971, Corbeil and Searle 1976). In all cases significance was assessed at $\alpha = 0.05$.

For the field experiment, mean temperature responses were examined for the entire incubation period (hereafter referred to as 'overall') and the thermosensitive period (TSP). The TSP was calculated as the middle third of incubation and is the period during which hatchling sex is determined (Yntema 1979, Yntema and Mrosovsky 1982, Bull 1985). We also examined average daily amplitude (half of the average daily range in °C) and average daily constant temperature equivalents (CTE) during the TSP. Briefly, CTE models calculate the temperature above and below which half of development occurs. CTEs differ from mean temperatures because they account for the influence of temperature on developmental rate of embryos (Georges 1989). Our CTE models were parameterized using $T_0 = 16.01$, the lowest temperature at which development occurs in Snapping Turtles (Yntema 1978, Freedberg et al. 2011). We examined the effect of agriculture treatment (Open v. Shade) on incubation temperature in the field experiment using LMM (REML). The LMM for the field experiment included agriculture treatment and nest layer as fixed effects, lay date as a random effect, nest layer as a fixed effect, and two-way interactions. We compared the actual incubation temperatures experienced by eggs in the laboratory experiment to the average temperature (of the top and bottom probes) in field nests by generating standard deviations between field and laboratory temperatures.

In both experiments, we analyzed hatch success using generalized linear mixed models (GLMM). Our GLMM models used a binomial distribution and logit link-function, with hatched versus not hatched as the dichotomous response variable. Standard errors were estimated using the Delta method. In models for both experiments, we included agriculture treatment, mercury treatment, and their interaction as fixed effects, and we included clutch as a random effect to account for lack of independence between hatchlings from the same mother (Wilson et al. 2002). We modeled our agriculture treatment as a categorical factor ($t = 2$) and the mercury treatment as

a continuous factor using egg concentration (dwt) of THg for each clutch. Mercury concentration was modeled as a continuous variable, rather than using a more simple categorical approach, to account for a wide range in Hg contamination levels among eggs from different clutches in the Hg treatments (Hg range field = 0.32 – 4.69 ppm, Hg range laboratory = 0.33- 4.86 ppm, dwt, Fig. 2.2). To further probe the nature of the significant interactive effects, we used pairwise least-squares means contrasts to conduct *post hoc* analyses on the lsmeans and multcomp packages (R Foundation for Statistical Computing, Vienna, Austria). In this *post hoc* case, mercury treatment was included as a categorical predictor to simply explore the interaction further. In the laboratory experiment, mold infected 186 of the 738 eggs early in development and resulted in a significant source of mortality (n = 160; Ref = 85, Hg = 75). Consequently, all eggs infected with mold were removed from the study (regardless of whether they hatched) and we present analyses on the 552 eggs that were not infected.

All other tests of incubation period and offspring phenotype used LMMs (REML) with clutch (the experimental unit) included in the model as a random effect. Egg mass was included as a covariate in LMMs used for analyses of incubation period, size at hatching, and growth. Hatchling mass was used as a covariate for locomotor performance analyses. Assumptions of parametric statistics were verified for all tests using residual analysis and Shapiro-Wilks tests. We used the same fixed and random treatment effects for all hatchling size and performance measures as those described for regression models of hatch success. To account for a lack of independence among the hatchling size and performance measures we corrected the statistical significance level used for each response using a sequential Bonferroni adjustment (Rice 1989, Appendix D). We chose to use a Bonferroni adjustment, rather than a multivariate approach such as principal component analysis (PCA), because different covariates (egg mass and hatchling

mass) are more appropriate for the hatching size and performance measures, and because the use of biological endpoints (rather than PCA scores) allows more intuitive comparisons between our two experiments which was an explicit *a priori* goal of our study.

Results

Field experiment. Average temperature and average daily constant temperature equivalent (CTE) at the top and bottom of nests in the field, both overall and during the thermosensitive period (TSP), were affected by agricultural treatment (in all cases $P < 0.001$, Table 2.1). Overall mean temperatures were 2.5℃ lower in Shade treatment plots than Open plots (Fig. 2.3), and as expected, mean temperatures were lower at the bottom of nests than at the top of nests (0.2℃ lower, Tables 2.1 & 2.2). When mean temperatures were examined for the TSP alone, the effect of agriculture treatment remained significant (difference of ~3.6 ℃ between Shade and Open treatment groups) but nest layer did not (Tables 2.1 & 2.2). Average daily CTEs during the TSP in the Shade treatment averaged 4.5 ℃ lower than those in the Open treatment (Tables 2.1 & 2.2). During the TSP, CTEs and average daily amplitude varied with nest layer but the magnitude of this effect was smaller among nests in the Shade treatment plots ($n = 18$) than those in the Open plots ($n = 18$, Tables 2.1 & 2.2).

The average egg THg in clutches collected downstream of the mercury source was about 10x higher than clutches collected upstream (Egg THg ppm, dwt: Hg = 2.6 ± 0.2 SE, $n = 10$; Ref = 0.3 ± 0.3 SE, $n = 8$; Fig. 2.2). While the difference in egg THg between our Ref and Hg groups was high, we found no main effects of the mercury treatment on turtle development in this experiment (in all cases $P \geq 0.128$, Table 2.3).

In contrast to the lack of effect of THg, our agriculture treatment affected most endpoints tested. Hatch success was high in all treatment groups (85-92 % success, $n = 18$) and the likelihood of embryos surviving to hatching did not differ among treatment groups (GLMM: z =

1.9, $P = 0.054$, Table 2.3) although more eggs hatched in the Open treatment group (90.4 %) than in the Shade treatment group (84.8 %). Of the embryos that survived to hatch, incubation period was strongly impacted by agriculture treatment with incubation periods lasting an average of 18.1 d longer in the Shade treatment group than in the Open treatment group (Table 2.3). In addition to taking longer to hatch, the Shade treatment group produced hatchlings with smaller structural sizes (carapaces were 5.5% shorter) and post-hatching growth rates (34.9% less carapace growth) than hatchlings from the Open treatment group (Table 2.3). Hatchling mass did not differ between hatchlings from the different treatment groups (Table 2.3). Hatchlings from all treatment groups lost mass during the 20-25 day post-hatching period, however, there was a trend of hatchlings from the Open treatment group losing less mass (23.2% less) than those from the Shade treatment, although this difference was not statistically significant (Table 2.3, Appendix D). Neither agriculture nor mercury treatment impacted terrestrial velocity or latency time to righting, but mechanical righting time was affected by the agriculture treatment; turtles from the Open treatment group righted faster than those from the Shade group (Table 2.3).

Laboratory experiment. The laboratory experiment successfully replicated field thermal regimens (Fig. 2.1). Actual and target (field) average daily incubation temperatures differed by 0.30 ± 0.03℃ SE and the average hourly standard deviation (for the entire incubation period) between actual and target temperatures was 0.86 ± 0.02 SE over the course of the laboratory experiment. Egg THg concentrations were over 14x higher in Hg than Reference clutches (Egg THg ppm, dwt: Hg = 3.0 ± 0.2 SE, $n = 13$; Ref = 0.2 ± 0.2 SE, $n = 12$; Fig. 2.2). Egg THg concentrations did not differ between the laboratory and the field experiments (REML: $F_{1, 80} = 0.9$, $P = 0.35$) and consistent with the field experiment, mercury treatment had little impact on

offspring phenotype compared to the impact of the agriculture treatment (Table 2.4). Hatch success was high (range = 83-84%) among all treatment groups except the Shade-Hg treatment group, which had an average hatch success of 71 ± 6 % SE (Table 2.4). The negative effect on hatch success disappeared when egg mercury was low, and/or when eggs were incubated under Open thermal regimens, and only appeared when both Hg and Shade treatments were combined (GLMM: $z = 5.1$, $P < 0.001$, Table 2.4). *Post hoc* contrasts supported this finding: the combined Hg and Shade treatment group differed from all other groups (*in all cases* $P \leq 0.001$) but the Ref - Shade, Ref- Open, and the Hg-Open groups did not differ from one another (*in all cases* $P \geq 0.76$).

Similar to the field experiment, hatchlings from the Open treatment group compared to the Shade treatment group had shorter incubation periods (by 18.5 d), hatched with 7.2% longer carapaces and lost 53.2% less mass during the 20-day period following hatching (Table 2.4, Fig. 2.4). Similarly, hatchlings from the Open treatment group tended to be slightly heavier (3.9% heavier) at the time of hatching than those in the Shade treatment but this difference was not statistically significant (Table 2.4, Appendix D). Hatchlings from the Shade treatment group initiated righting about 2.5x faster than hatchlings from the Open treatment (Table 2.4) but neither terrestrial velocity nor mechanical righting time differed among hatchlings from the different treatment groups (Table 2.4, Appendix D).

Discussion

The use of recently planted agricultural fields for nesting by Snapping Turtles had substantial impacts on embryonic development and offspring phenotype. In comparison to turtles from control (Open) incubation regimens, turtles incubated under agricultural (Shade) incubation regimens generally (1) took longer to hatch, (2) had smaller structural sizes at hatching, (3) lost body mass faster after hatching, and (4) had lower growth rates during the 20-25 days post

hatching (Fig. 2.4). Because results were largely consistent between the field and laboratory experiments, our study provides strong evidence that the primary proximate mechanism driving agriculturally-induced changes in embryonic development and offspring phenotype was lowered average incubation temperatures and daily thermal fluctuations due to shading of nests by crops relative to open canopy conditions. Because the majority of previous studies on development in reptiles have used either constant temperature incubations or perfectly sinusoidal temperature fluctuations, our experimental manipulation of the thermal dynamics within nests in the field, and replication of these dynamic conditions in the laboratory, provides a much needed closer approximation to natural conditions (Bowden et al. 2014, Warner 2014).

To our knowledge, this study is the first to directly investigate how agriculturally-induced changes in nest microclimate impact phenotypic traits in hatchling turtles. While Saumure and Bider (1998) found significantly lower growth rates of box turtles (*Clemmys insculpta*) inhabiting agricultural areas, relative to those from reference areas, they did not identify a mechanism behind the phenomenon. Our results are consistent with other studies that demonstrate that vegetation, fences, and residential buildings over or near turtle nests can produce a cooling effect strong enough to impact offspring phenotype and hatching success (Janzen 1994, Mrosovsky et al. 1995, Kolbe and Janzen 2002, Kamel and Mrosovsky 2006). Moreover, our results are consistent with studies that show that turtles incubated at low temperatures have smaller body sizes and lower post-hatch growth rates than those incubated at intermediate or higher temperatures (Brooks et al. 1991, McKnight and Gutzke 1993, Bobyn and Brooks 1994, Rhen and Lang 1995). Similarly, the differences we found between the Open and Shade treatments complement findings that reptiles incubated with higher thermal fluctuations

experience higher survival, growth, and immune response relative to those incubated with no or relatively lower thermal fluctuations (Demuth 2001, Les et al. 2009).

Hatchlings that experience extended incubation periods, reduced body size at hatching, increased post hatch mass loss (e.g., those from the Shade treatment), and decreased growth rates may have lower fitness than those that hatch sooner, at a larger size, and maintain more mass in the month following hatching (e.g., those from the Open treatment). As incubation period increases, yolk stores generally decrease, resulting in hatchlings with reduced yolk reserves (Deeming 2004). Residual yolk is an important source of energy for newly hatched turtles and has been linked to survivorship in some species of aquatic turtles, including Snapping Turtles (Wilhoft 1986, Congdon 1989, Bobyn and Brooks 1994) but not in others (Lee et al. 2007, Van Dyke 2011). Evidence that larger hatchling body size increases the likelihood of post-hatch survivorship has been found in several studies (Janzen 1993, Bobyn and Brooks 1994, Janzen et al. 2000, Paterson et al. 2014) and supports the "bigger is better hypothesis" which postulates that larger hatchling body size confers a survival advantage over smaller body sizes. However, some studies of natural selection on body size of hatchling turtles have found evidence of stabilizing or no selection pressure (Brooks et al. 1991, Congdon et al. 1999, Delmas et al. 2007). While the influence of hatchling body size on survival remains an important topic for future research, the growth rate of juvenile turtles has important impacts on lifetime reproduction; relative to fast growing juveniles, juveniles with slower growth rates experience reduced lifetime reproductive output because they mature later and reach maturity at a smaller body size (Congdon et al. unpublished data). Thus, the reductions in size and early growth of hatchlings observed in our study likely have negative ramifications on offspring fitness.

In both of our experiments, Shade generally caused slight reductions in hatch success and importantly, our laboratory experiment documented an interactive effect between mercury and agriculture on hatch success. In the laboratory experiment, hatch success was stable in the Ref and Shade group (relative to both Open groups) but low in the Hg and Shade combination (% reduction relative to all other groups = 15.1 ± 0.5 SE). Albeit not statistically significant, our field experiment showed 6.3% lower (at $\alpha = 0.054$) hatch success in the Shade than in the Open treatment groups (Table 2.3). Notably, in our study system, 68% of the land within 200m of the South River downstream of the Hg point source is used for agriculture (Homer 2015). Consequently, interactive effects of Hg and the thermal effects of agricultural land use on embryonic development and post-hatch survivorship may be common in this region. To our knowledge, this one of the first documentations of negative interactive effects of Hg pollution and temperature on early vertebrate development.

Although the majority of our results were consistent between the lab and field experiments, locomotor performance responses were inconsistent between the two experiments. Other measures, such as hatchling orientation and dispersal, may be more meaningful performance metrics for investigating the impact of agriculture and mercury on turtle locomotor performance (Davy et al. 2014, Carter et al. 2016, Mitchell et al. 2016). Research on orientation in hatchling snapping and Blanding's turtles (*Emydoidea blandingii*) revealed that, for both species, crop canopies (corn and soy) in agricultural fields impair hatchlings' ability to use environmental cues for successful dispersal from nests (Pappas et al. 2013). The damaging effects of Hg on magnetic orientation in hatchling Snapping Turtles (Landler 2015, Landler et al. 2015) highlights the potential for Hg and agriculture to have stronger deleterious effects in

combination than in isolation. Taken together, our work and others' suggest that the impacts of agriculture on performance in hatchling turtles merits further examination.

Current evidence suggests that many reptile populations worldwide are in rapid decline (Gibbons et al. 2000, Whitfield et al. 2007, Brown et al. 2008, Smith et al. 2012), and land use and pollution are suspected contributors to many declines (Sarre 1998, Dorrough and Ash 1999, Díaz et al. 2000, Brown et al. 2008). Our work adds to a small body of literature on adverse effects of agricultural land use on turtle embryos and hatchlings (Saumure and Bider 1998, Pappas et al. 2013), and raises concern that the selection of agricultural fields for nesting by turtles (Beaudry 2010, Freedberg et al. 2011, Mui et al. 2015), particularly in Hg polluted areas, is maladaptive behavior that may create evolutionary traps that are ultimately damaging to turtle populations (Schlaepfer et al. 2002, Battin 2004). Given that factors such as climate change, land use, introduction of non-native plants, and pollution rarely occur in isolation of one another, future research on how these factors interact to impact animals will be critical to wildlife conservation.

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Table 2.1 Individual and interactive effects of agricultural treatment and nest depth on temperature in turtle nests in experimental field plots. Results of REML analysis of variance on the effects of agricultural treatment (Agriculture) and nest depth (Nest Layer) on incubation temperature in reconstructed turtle nests in experimental field plots in Waynesboro, VA. Mercury treatment groups are pooled. Interactions between effects are denoted with an asterisk (*), significant effects at $\alpha < 0.05$ are shown in bold.

	Nest Layer	Agriculture	Nest Layer*Agriculture
Overall Avg. Temp	$F_{1,63} = 12.8$ ***P* = 0.001**	$F_{1,63} = 948.5$ ***P* < 0.001**	$F_{1,63} = 0.9$ $P = 0.359$
TSP Avg. Temp.	$F_{1,64} = 3.1$ $P = 0.084$	$F_{1,64} = 975.7$ ***P* < 0.001**	$F_{1,64} = 0.7$ $P = 0.419$
Middle 3rd CTE	$F_{1,34} = 26.6$ ***P* < 0.001**	$F_{1,34} = 768.5$ ***P* < 0.001**	$F_{1,34} = 8.7$ ***P* = 0.006**

Table 2.2 Average daily thermal conditions observed in the agriculture treatment groups at the top and bottom of turtle nests in experimental field plots. Summaries of average nest temperature (during each third of embryonic development), average daily amplitude and CTEs (during the middle third of development) among nests in the field experiment are grouped by agriculture treatment (Open or Shade) and nest layer (Top or Bottom); thermal parameters were calculated using daily averages (LS Mean ± 1 SE) and are shown in ℃ and sample sizes range from 16-18 clutches per group.

Thermal Parameter	*Development Period*	OPEN		SHADE	
		Top	*Bottom*	*Top*	*Bottom*
Average Temperature	Overall	24.8 ± 0.2	24.4 ± 0.1	22.2 ± 0.2	22.0 ± 0.2
	First 3rd	25.5 ± 0.2	24.9 ± 0.2	23.9 ± 0.2	23.5 ± 0.2
	Middle 3rd	25.3 ± 0.1	25.0 ± 0.1	21.7 ± 0.2	21.5 ± 0.2
	Final 3rd	23.6 ± 0.1	23.4 ± 0.1	20.8 ± 0.2	20.8 ± 0.2
Amplitude	Middle 3rd	5.1 ± 1.1	3.0 ± 0.8	1.7 ± 0.1	1.0 ± 0.1
Variance	Middle 3rd	8.5 ± 0.8	2.5 ± 0.2	1.6 ± 0.2	0.6 ± 0.1
CTE	Middle 3rd	27.2 ± 0.2	25.7 ± 0.2	22.3 ± 0.2	21.7 ± 0.2

Table 2.3 Individual and interactive effects of mercury and agriculture treatment on hatch success, incubation period, and offspring phenotype in the field experiment. Average values shown are LS Mean ± 1 SE. Results of generalized (GLMM, hatch success) and general linear (REML, all other endpoints) mixed model analysis on the effect of mercury (Ref and Hg) and agriculture treatment (Open and Shade) on hatch success, incubation period, and offspring phenotype. Interactions between effects are denoted with an asterisk (*), significant effects are shown in bold, significance was determined by $\alpha = 0.05$ for hatching success and using a sequential Bonferroni adjustment procedure for all other endpoints.

	TREATMENT GROUP				TREATMENT EFFECT		
Response	*Ref/Open*	*Hg/Open*	*Ref/Shade*	*Hg/Shade*	*Agriculture*	*Mercury*	*Ag*Mercury*
Hatching success (%)	91.1 ± 6	89.7 ± 10	84.8 ± 8	84.7 ± 6	z = 1.9 *P* = 0.054	z= -1.5 *P* = 0.128	z= -1.0 *P* = 0.326
Incubation period (d)	82.4 ± 1.3	82.7 ± 1.5	100.0 ± 1.4	101.3 ± 1.4	$F_{1,32}$ = 112.3 ***P* < 0.001**	$F_{1,32}$ = 0.5 *P* = 0.484	$F_{1,32}$ = 1.3 *P* = 0.275
Hatchling CL (mm)	29.6 ± 0.5	30.0 ± 0.5	28.0 ± 0.5	28.3 ± 0.4	$F_{1,24}$ = 162.9 ***P* < 0.001**	$F_{1,24}$ = 0.2 *P* = 0.688	$F_{1,23}$= 1.1 *P* = 0.319
Hatchling mass (g)	9.4 ± 0.4	9.8 ± 0.3	9.1 ± 0.4	9.5 ± 0.3	$F_{1,26}$ = 3.7 *P* = 0.073	$F_{1,26}$ = 0.7 *P* = 0.428	$F_{1,26}$ = 1.8 *P* = 0.201
Growth CL (%)	3.2 ± 1.1	3.4 ± 1.1	2.5 ± 0.9	1.8 ± 0.6	$F_{1,29}$ = 15.4 ***P* = 0.001**	$F_{1,29}$ = 0.9 *P* = 0.361	$F_{1,29}$ = 0.6 *P* = 0.438
Mass loss (%)	6.7 ± 1.9	5.2 ± 1.7	8.5 ± 1.9	7.0 ± 1.7	$F_{1,26}$ = 5.8 *P* = 0.030	$F_{1,26}$ = 0.3 *P* = 0.598	$F_{1,26}$ = 0.9 *P* = 0.366
Terrestrial velocity (cm s^{-1})	1.9 ± 0.2	1.8 ± 0.2	1.8 ± 0.2	1.6 ± 0.2	$F_{1,32}$ = 1.8 *P* = 0.201	$F_{1,32}$ < 0.1 *P* = 0.886	$F_{1,32}$ = 1.2 *P* = 0.296
Latency time (s)	77.4 ± 28	102.9 ± 31	52.5 ± 28	74.2 ± 31	$F_{1,32}$ = 0.2 *P* = 0.651	$F_{1,32}$ < 0.1 *P* = 0.953	$F_{1,32}$ < 0.1 *P* = 0.889
Mechanical righting (s)	1.0 ± 0.2	1.1 ± 0.2	1.2 ± 0.2	1.3 ± 0.2	$F_{1,26}$ = 5.1 *P* = 0.041	$F_{1,26}$ = 0.1 *P* = 0.712	$F_{1,26}$ < 0.1 *P* = 0.955

Table 2.4 Individual and interactive effects of mercury and agriculture treatment on hatch success, incubation period, and offspring phenotype in the laboratory experiment. Average values shown are LS Mean ± 1 SE. Results of generalized (GLMM, hatch success) and general linear (REML, all other endpoints) mixed model analysis on the effect of mercury (Ref and Hg) and agriculture treatment (Open and Shade) on hatch success, incubation period, and offspring phenotype. Interactions between effects are denoted with an asterisk (*), significant effects are shown in bold, significance was determined by $\alpha = 0.05$ for hatching success and using a sequential Bonferroni adjustment procedure for all other endpoints.

	TREATMENT GROUP				TREATMENT EFFECT		
Response	*Ref/Open*	*Hg/Open*	*Ref/Shade*	*Hg/Shade*	*Agriculture*	*Mercury*	*Ag*Mercury*
Hatching success (%)	82.6 ± 6	83.0 ± 11	84.1 ± 8	70.7 ± 6	z = - 0.3 *P* = 0.734	z = -4.3 ***P* = 0.003**	z = 5.1 ***P* < 0.001**
Incubation period (d)	79.7 ± 1.2	83.8 ± 1.7	99.4 ± 1.2	101.0 ± 1.8	$F_{1,36}$ = 128.6 ***P* < 0.001**	$F_{1,36}$ = 3.1 *P* = 0.092	$F_{1,36}$ = 4.1 *P* = 0.060
Hatchling CL (mm)	30.0 ± 0.4	30.1 ± 0.4	28.3 ± 0.4	28.3 ± 0.4	$F_{1,29}$ = 40.2 ***P* < 0.001**	$F_{1,29}$ = 0.5 *P* = 0.508	$F_{1,29}$ = 1.7 *P* = 0.203
Hatchling mass (g)	10.3 ± 0.2	10.4 ± 0.2	9.9 ± 0.2	10.0 ± 0.2	$F_{1,34}$= 5.7 *P* = 0.029	$F_{1,34}$ = 0.1 *P* = 0.775	$F_{1,34}$ < 0.1 *P* = 0.991
Growth CL (%)	4.5 ± 0.8	4.7 ± 0.8	1.6 ± 1.0	1.7 ± 1.0	$F_{1,33}$ = 17.3 ***P* < 0.001**	$F_{1,33}$ =0.1 *P* = 0.722	$F_{1,33}$ = 0.8 *P* = 0.386
Mass loss (%)	5.8 ± 1.5	4.9 ± 0.6	10.3 ± 1.7	9.8 ± 1.8	$F_{1,34}$= 14.3 ***P* < 0.001**	$F_{1,34}$ = 0.7 *P* = 0.421	$F_{1,34}$ = 0.3 *P* = 0.616
Terrestrial velocity (cm s^1)	1.6 ± 0.2	1.7 ± 0.15	1.7 ± 0.19	1.6 ± 0.26	$F_{1,27}$ < 0.1 *P* = 0.903	$F_{1,27}$ = 0.2 *P* = 0.645	$F_{1,27}$ < 0.1 *P* = 0.929
Latency time (s)	155.5 ± 33	148.2 ± 34	66.5 ± 36	53.1 ± 37	$F_{1,34}$= 14.6 ***P* = 0.002**	$F_{1,34}$ = 0.4 *P* = 0.544	$F_{1,34}$ = 0.3 *P* = 0.569
Mechanical righting (s)	1.3 ± 0.2	1.3 ± 0.2	1.3 ± 0.2	1.3 ± 0.2	$F_{1,29}$ = 0.4 *P* = 0.525	$F_{1,29}$ = 0.4 *P* = 0.519	$F_{1,29}$ <0.1 *P* = 0.963

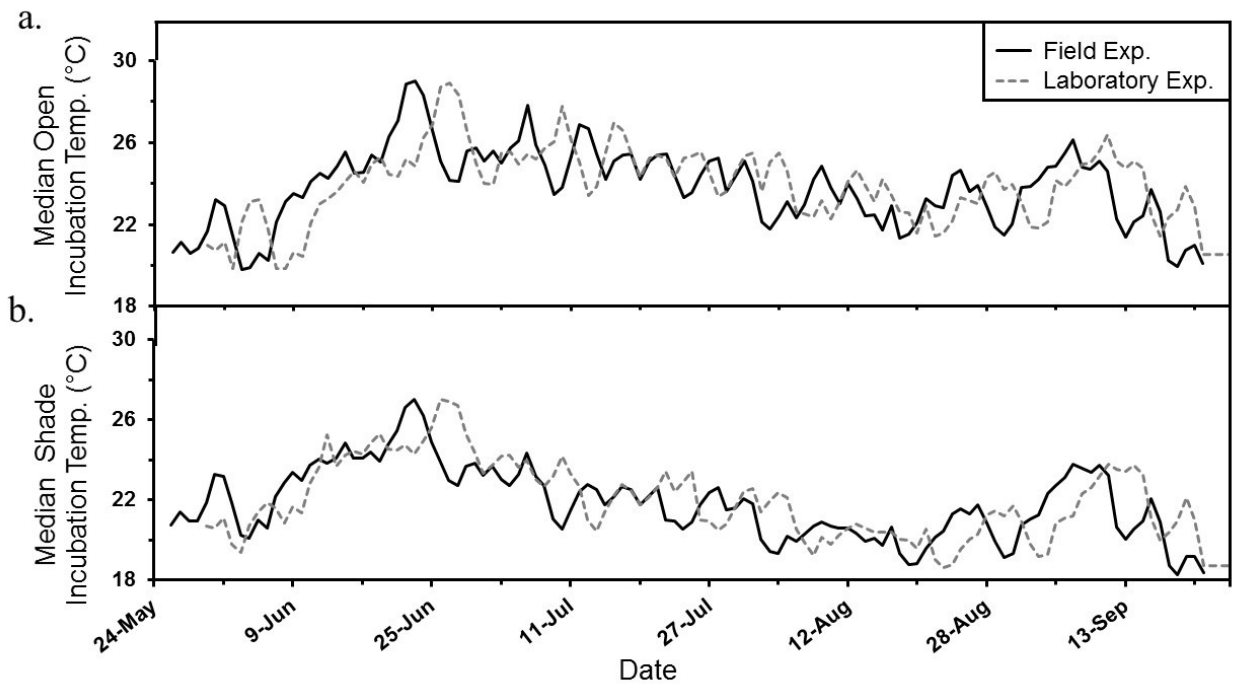

Fig. 2.1 Average daily median temperatures measured from the top and bottom of field nests in the Open (A) and Shade (B) plots in the field experiment (solid line) and daily median temperatures measured from incubators in the laboratory experiment (dotted line). Temperatures simulated in the laboratory are offset by four days from field temperatures (see methods).

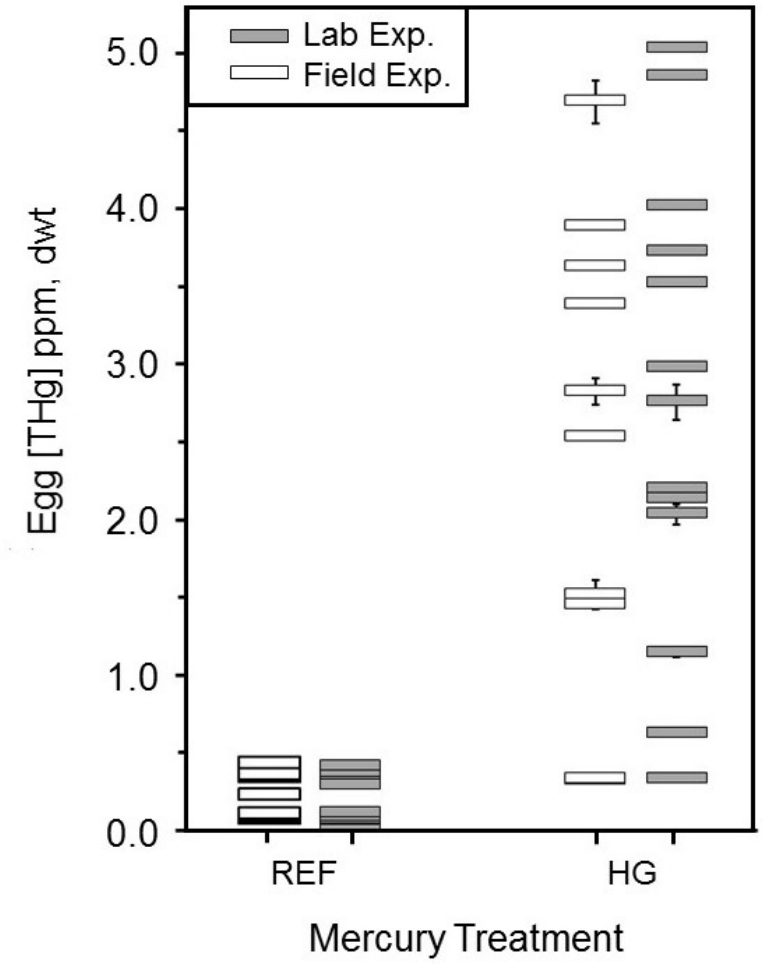

Fig. 2.2 Egg (dry mass) total mercury (THg) concentrations found in *Chelydra serpentina* clutches used in field (white) and laboratory (grey) factorial experiments collected from along the South River, VA, USA. Each bar represents the average THg of the two subsamples taken from one egg of each clutch or the predicted egg THg value from one maternal blood sample.

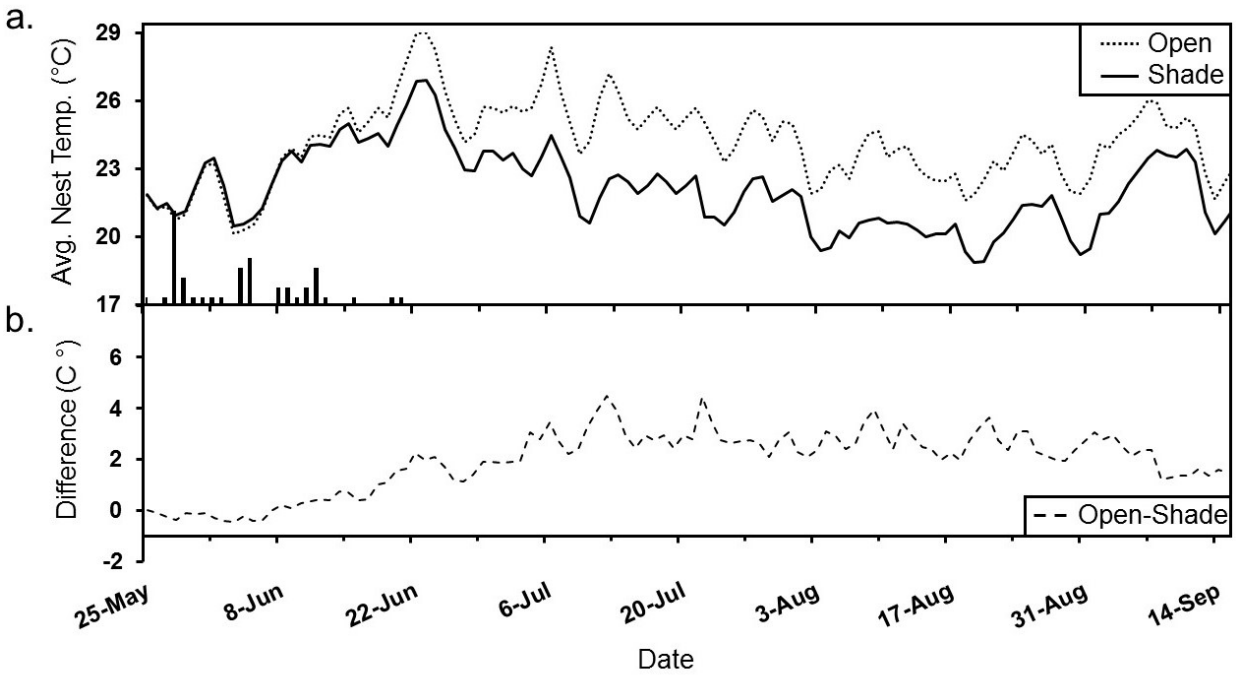

Fig. 2.3 Daily average temperatures (A) for Shade (solid line) and Open (dotted line) treatment plots, and temperature difference (B) between Open and Shade treatment groups(dashed line), from artificial nests. Black vertical bars along the x-axis (A) show the number of nests found along the South River, Virginia, during the 2014 nesting season.

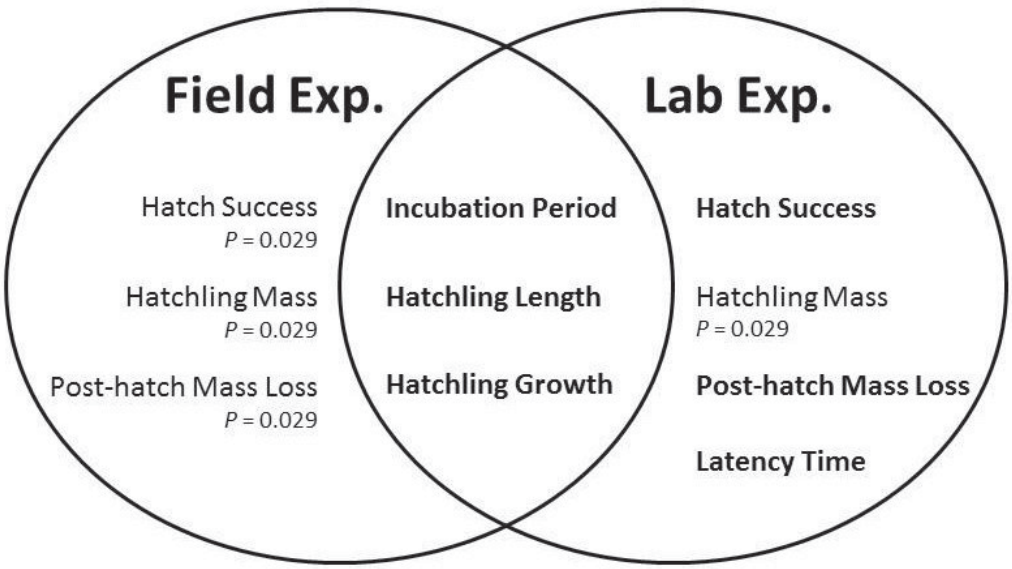

Fig. 2.4 Summary of experimental results of all endpoints measured in this study that were impacted by agricultural treatment or (in one case; hatch success) by the interactive effect of agriculture treatment and mercury treatment. Responses are written in the appropriate location on the VIN Diagram to compare results across our two experiments, significant endpoints are shown in bold and response variables that showed trends below $\alpha = 0.10$ in the same direction in both experiments are shown with associated p-values. Hatchling growth refers to both the percent increase in structural body size during the 21-25 day post-hatch period.

Chapter 3: Agricultural Land Use & Mercury Pollution Interact to Impact Nest Microclimate & Sex Ratios of a Freshwater Turtle

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Abstract

Habitat loss and pollution are two of the greatest global threats to biodiversity. Due to their widespread prevalence, these threats often co-occur, yet their interactive effects on organisms remain poorly understood. Some reptiles are vulnerable to these threats because they have specific microclimate requirements for embryonic development and because pollutants are maternally transferred to their eggs; both incubation temperature and pollutants affect reptile sex determination. In aquatic turtles, females often select nest sites in recently planted agricultural fields but the impact of nesting in polluted agricultural habitats is not understood. We examined the influences of crop agriculture and mercury pollution on nest microclimate and offspring sex ratios of *Chelydra serpentina*. We hypothesized that crop growth in agricultural fields would shade and cool turtle nests, decrease moisture levels, cause male-biased sex ratios, and interact with maternally-derived mercury to impact embryos. As predicted, nests shaded by crops had lower average temperatures (-2.5 °C) and moisture levels (-107 kPa) than control nests. In field and laboratory experiments, agricultural thermal regimens increased the proportion of male offspring in clutches and this effect was intensified in the presence of mercury. Global temperatures are expected to rise within the 21st century and to have a feminizing effect on reptiles with temperature-dependent sex determination. That prediction should be refined to incorporate how the cooling effect of some local habitat conditions (e.g., agricultural fields), and interactions between anthropogenic land-use and common pollutants, will interact with climate change to influence sex ratios of some reptiles.

Keywords: Fluctuating temperatures, mercury, agriculture, nest microclimate, sex ratio

Introduction

Over the last 500 years, anthropogenic changes have led to the loss of over 600 vertebrate species, signaling the onset of the sixth mass extinction event (Ceballos et al. 2015). Among diverse global changes, habitat loss and environmental pollution are two of the greatest concerns for biodiversity (Meiri and Chapple 2016). Despite the fact that pollution and habitat conversion for crop agriculture often co-occur (Matson et al. 1997), very little is known about how they interact to affect biodiversity.

Reptiles are a biologically diverse and highly threatened vertebrate group (Bland and Böhm 2016, Böhm et al. 2016). Most reptiles (~85%) are oviparious and have specific microhabitat, temperature and moisture requirements for successful embryonic development (Deeming 2004). Many reptiles exhibit temperature-dependent sex determination (TSD); the temperature at which eggs are incubated is the primary determinant of the sex of embryos produced (Bull 1980, Janzen 1992) which raises concern that global changes will cause biased offspring sex ratios (Böhm et al. 2016). Additionally, reptiles maternally transfer high levels of environmental pollutants to their eggs that interfere with early development and decreases hatch success (Bishop et al. 1996, Guillette et al. 2000, Schneider 2001).

In this study, we examined the effects of crop agriculture on nest microclimate as well as how agriculturally induced changes in nest microclimate act individually, and interactively with mercury (Hg) pollution, to impact offspring sex ratios of a freshwater turtle with TSD. In numerous species of aquatic turtles, females select nest sites that have a high degree of solar exposure such as sandy, mossy, or rocky patches adjacent to rivers and streams. However, they are sometimes more attracted to areas where humans have disturbed terrestrial substrates and

vegetation (e.g., road shoulders, recently logged areas, or recently tilled agricultural fields), increasing solar exposure and facilitating easier nest excavation (Kolbe and Janzen 2002, Beaudry et al. 2010, Freedberg et al. 2011, Riley et al. 2013, Mui et al. 2015). The costs and benefits of nesting in these disturbed habitats are poorly understood. We focused on co-exposure to Hg because it is one of the most globally widespread contaminants (Driscoll et al. 2013), persists in the environment for hundreds-to-thousands of years (Selin 2009), and has a suite of adverse physiological and developmental effects on vertebrates (Rice et al. 2014). Because hormones are known to influence offspring sex and even reverse TSD predictions in reptiles (Warner et al. 2009, Matsumoto and Crews 2012, Merchant-Larios and Diaz-Hernandez 2012), endocrine disrupting pollutants and early developmental temperatures may interact. Previous studies demonstrated that female turtles bioaccumulate and maternally transfer Hg to their eggs (Hopkins et al. 2013b). However, the effects of Hg on offspring sex ratios and the interactive effects of Hg and agricultural land use have not been studied.

To investigate the individual and interactive effects of Hg and crop agriculture, we used a 2x2 factorial design (presence or absence of agriculture and/or Hg) in coupled field and laboratory experiments. Our objectives were to:

(a) Describe the influence of agricultural land use on turtle nest microclimate (thermal and hydric characteristics),

(b) Determine the individual and interactive effects of agricultural land use and a wide-spread and persistent endocrine disrupting pollutant, Hg, on offspring sex ratios of turtles, *and*

(c) Integrate realistic laboratory simulations with manipulative field experiments to disentangle temperature from other potential mechanisms underlying observed phenotypic effects.

We hypothesized that in the weeks following nesting, rapid growth of crops over turtle nests in agricultural fields would shade and cool nests, reduce water content of nest substrates through evapotranspiration by plants, and cause male biased sex ratios. We also postulated that high concentrations of maternally transferred Hg in eggs would interact with agricultural incubation conditions to exacerbate effects on offspring sex.

Our study advances our understanding of thermal effects of anthropogenic land use on nest microclimate and sex ratios in reptiles with TSD by (1) documenting differences in thermal and hydric profiles of replicated nests in agricultural and non-agricultural conditions while controlling for field specific effects (such as aspect) experimentally, (2) using complex thermal profiles from the field (which incorporated hourly, daily, and seasonal thermal fluctuations) to experimentally manipulate temperatures in the laboratory to evaluate the role of temperature, in isolation of other variables (such as water availability) on offspring sex ratios, (3) controlling for genetic effects in both field and laboratory experiments by splitting-clutches, and (4) investigating both the individual and the interactive effects of a widespread form of habitat modification and a pervasive environmental pollutant on offspring sex ratios (Freedburg et al. 2011, Bowden et al. 2014, Mui et al. 2015).

Methods

Study System. We used the Common Snapping Turtle *(Chelydra serpentina*) as our focal species. Due to their long life span, high trophic-level, and sedentary nature (Hammer 1969, Punzo 1975, Obbard and Brooks 1981), tissue concentrations of Hg in Snapping Turtles can reach high levels (Hopkins et al. 2013a). From mid-May to the end of June, female Snapping

Turtles leave their aquatic habitats to nest, often in agricultural fields (Freedberg et al. 2011, Mui et al. 2015, Thompson et al. 2017). Clutch sizes range from 26-55 eggs (Steyermark et al. 2008). In Snapping Turtles, females are produced at high and low temperatures and males at intermediate temperatures (Bull 1980, Janzen 1992). At constant temperatures in the laboratory, the upper pivotal temperature is 27.8ºC and the lower pivotal temperature is 21.4 ºC; under more variable temperatures in the field, the upper pivotal sex determining temperature may be closer to 25.5 ˚C (Bull 1985).

Our study area was along the South River, Virginia, USA. A manufacturing plant leaked mercuric sulfate into this river from 1929-1959 (Carter 1977). Considerable amounts of Hg remain in the river and the floodplain, and as a result, tissue concentrations of Hg in wildlife living downstream are high (Bouland et al. 2012, Todd et al. 2012, Drewett et al. 2013, Hopkins et al. 2013a).

Our study area is also subject to high agricultural land use. Land use within 200m of the South River upstream and downstream of the Hg source is 61% and 68% agricultural, respectively (2011 National Land Cover Database; Multi-Resolution Land Characteristics Consortium, NLCD 2011).

We collected 43 entire clutches of eggs from nests located upstream (n = 20) and downstream (n = 23) from the initial Hg point source on the South River (Virginia DGIF Permit No. 048080, VT IACUC No. 14-110) from May-June, 2014. We found nests mostly in agricultural fields (88%, n = 38/43). Other nests were found along stream banks (5%, n = 2/43), in commercial nursery properties (5%, n = 2/43), and in a lawn (2%, n = 1/43). Clutches were collected immediately following oviposition or from newly constructed nests (for details see

Chapter 2). We marked each egg with a unique identification number, weighed it to the nearest 0.01g, and measured it to the nearest 0.01 mm.

Nest Microclimate Experimental Design. We used experimental treatment plots to investigate the effects of crop growth on turtle nest microclimate. We constructed treatment plots in a field that was planted with corn in early May 2014 and that had been used by Snapping Turtles for nesting the previous year (> 20 nests in < 6.0 Hectares; Thompson et al. 2017). We used a random sample (n = 10) of the 2013 nest sites as the locations of our experimental plots. All plots (n = 5 per treatment) were circles six meters in diameter and assigned to either a control treatment, referred to as 'Open' (crops removed and non-crop vegetation limited to 10% cover), or an agricultural treatment referred to as 'Shade' (crops were allowed to grow).

We constructed one artificial nest at the center of each plot. The top and bottom of artificial nest chambers were 105 mm and 150 mm below the soil surface, respectively (Congdon et al. 1987). We standardized the clutch size in the nests to 34 artificial eggs to represent the average clutch size and mass at our study site (Hopkins et al. 2013b). Eggs were hollow polypropylene plastic balls with a diameter of 25 mm (CIC Ball Company, PN) filled with wire pulling lubricant (ClearGlide^{TM}) which closely mimics the thermal properties of amniotic eggs (Ardia et al. 2010, Coe et al. 2015). We placed Hobo-Temp temperature probes attached to cables (Onset Computer Corporation, Pocasset, Massachusetts) at the top and bottom of artificial nests and recorded temperature at 1-h intervals. We uploaded temperature data regularly throughout the duration of the study without disturbing the artificial nests (the Hobo-Temp logger sits above the soil surface). Turtles release bladder water while constructing their nests;

we therefore sprayed water onto the dirt as we sealed nest chambers (Marchand and Litvaitis 2004). We placed wire mesh predator guards over each artificial nest (Riley and Litzgus 2013).

We measured vegetation growth in our experimental plots every eight days to relate changes in vegetative height, density, and cover to changes in hydric and thermal dynamics of nests. We established two perpendicular transects through each treatment plot and used the line-point intercept method (LPI) to quantify vegetative cover and height (Noon 1981, Pilliod and Arkle 2013). We recorded vegetation height every 40 cm (± 5 cm) along the two 6 m long transects. We calculated vegetative cover as:

$$\% \text{ Cover} = \frac{\left(V1/16\right) + \left(V2/16\right)}{2} * 100$$

Where V1 and V2 are the number of points bisecting vegetation along transect 1 and 2, respectively. We estimated foliar density across each transect using a cover pole, and categorized density at each 10 cm height interval (Noon 1981, Griffith 1998). Density poles were placed at a constant distance from a viewer who visually estimated the percent of each section of the pole obscured by vegetation using density scores. In this study, density scores for each 10 cm interval were categorized as follows: 0= none, 1= 0-20%, 2= 21-40%, 3= 41-60%, 4= 61-80%, 5= 81-100% of visibility obscured, from a constant distance of 6 m and height of 170 cm. Zeros were not included in average height or density estimates; we use the term cover to describe the percent of plots covered in vegetation, but use height and density to describe how tall and thick the vegetation was only where it occurred. Initially both crop and non-crop vegetation were measured in Shade plots but corn quickly dominated all non-crop vegetation. We removed non-crop vegetation from Open plots on August 6th when a low growing forb (< 15 cm tall) began growing and covered > 10% of some plots (Appendix E).

We also determined soil water content and water potential in our treatment plots every eight days. We collected two soil samples from >10 cm away from nests in random locations in plots using a hand held core sampler to the depth that corresponded to the center of our nest chambers (~ 13 cm). We determined water content by weighing soil samples and then drying them to a constant mass in an Isotemp drying oven (Fisher Scientific, Ottawa, ON, Canada); water content corresponds to the difference between these values. We converted water content to water potential using soil-water retention curves that we established using a Wescor Hygrometer/ Psychrometer with a C-52 Sample Chamber for the two soil types present in our experimental plots (Soil Survey Geographic Database, Appendix F). We used data from the National Oceanic and Atmospheric Association (NOAA) to mark the timing and the relative magnitude of rain events.

Offspring Sex Ratios Experimental Design. We used a 2x2 factorial design (presence or absence of agricultural shade and/or Hg) to conduct simultaneous field and laboratory incubation experiments. We randomly assigned whole clutches to either the field incubation experiment (n =18 clutches total; Hg =10, Ref =8) or the laboratory incubation experiment (n = 25 clutches total; Hg =13, Ref =12) as they were collected.

The first factor of our 2x2 design was agriculture treatment. We randomly split clutches collected from Hg and Ref locations and assigned each half to an agriculture incubation treatment (Open or Shade). The second factor of our 2x2 design was a mercury treatment which had two treatment groups: eggs that contained high concentrations of maternally derived Hg, referred to as 'Hg' (eggs collected from within 16 km *downstream* of the original Hg source), and eggs that only contained background concentrations of Hg, referred to as 'Ref' (eggs

collected 6-14 km *upstream* from the Hg source). Mark-recapture studies conducted at these sites for > 10 yrs document that turtles with markings from the Ref sites are not collected in the Hg sites (or vice versa; Hopkins et al. 2013a). We quantified Hg for each clutch from maternal blood or from one randomly selected egg from each clutch. Turtle eggs from the same clutch have similar maternally-derived trace element concentrations (Van Dyke et al. 2013). We used a regression equation to estimate egg Hg concentration from maternal blood concentration as these two variables are strongly and positively related (Hopkins et al. 2013a). We froze tissues at -20 ℃ until Hg analysis. We lyophilized and homogenized the eggs and report their total Hg (THg) concentrations on a dry mass (dwt, in ppm) basis. The percent moisture of eggs was 75.5 ± 0.7% SE (n = 44). Samples were analyzed by combustion-amalgamation-cold vapor atomic absorption spectrophotometry (Direct Mercury Analyzer 80, Milestone, Monoroe, CT, USA) following U.S. Environmental Protection Agency (U.S. EPA) method 7473 (USEPA 1998). Standard reference materials were analyzed alongside experimental samples; mean percent recoveries of THg for the standard reference materials were 104.3 ± 1.0 % SE (n = 14) and 100.3 ± 0.3 % SE (n = 14). All samples were run in duplicate; the average relative percent difference was 6.3% ± 1.2 SE.

Clutches assigned to the field experiment were transplanted into nests in the experimental plots and left undisturbed until retrieval just prior to their hatching. We constructed 3-4 nests in each plot, in cardinal directions 130 cm from the artificial nests in the center of each plot. We used the same methods to construct nests in the field experiment as we used for our artificial nests. However, we used iButton temperature data loggers instead of Hobo loggers to record thermal profiles in nests (DS1923, Embedded Data Systems, KY, USA). Prior to burying eggs, we standardized the clutch size to 34 by adding artificial eggs in order to standardize bias in

thermal gradients within nests and to control for the impact of clutch size on the thermal inertia of nests. We placed wire mesh predator guards over each nest (Riley and Litzgus 2013).

We retrieved eggs from field nests between August 18 and September 27. We then incubated the eggs under constant temperatures corresponding to the average nest temperature recorded in the field during the previous three weeks until they hatched (Open = 23.3℃, Shade = 20.5℃). During egg retrieval, we categorized the eggs by the order they were removed as we dug down into each nest chamber. We classified the first half of eggs removed as the top layer and the second half as the bottom layer. During retrieval, four of the 18 nests in the Open treatment plots had pipped; no eggs in any of the nests in Shade treatment plots had pipped. We used the retrieval date as the last day of incubation for the Open nests that began to hatch in the field. Based on embryonic staging at oviposition coupled with retrieval and hatch dates, all embryos spent > 90 % of their total incubation in the field. Three of the 72 iButtons malfunctioned (4.2%), resulting in a total 69 iButtons used to investigate the effect of our treatments on nest temperature and offspring sex ratios (Top; N=34, Bottom; N=35).

We calculated incubation regimens for the laboratory experiment from temperatures recorded in the ten artificial nests (Fig. 3.1). Because the laboratory incubation experiment was running simultaneously as we generated our thermal profiles from artificial nests, we staggered the laboratory experiment four days behind the date when temperatures were recorded in the field (Chapter 2). Throughout the duration of incubation, we uploaded temperature data from the field every four days and used it to program the next four days of incubation in the lab. We split clutches assigned to the laboratory experiment between Open and Shade incubation treatments in Memmert IPP 55 Plus incubators (n = 4; 2 per treatment, Memmert GmbH+Co.KG, Schwabach, Germany). We calibrated the incubators prior to the study and monitored their accuracy

throughout incubation using two iButton data loggers placed in the center of each incubator. Eggs were incubated in plastic containers (1:1, water: vermiculite) and capped with a perforated lid. We rehydrated and rotated egg containers among shelves every four days (Packard et al. 1987).

In both experiments, pipped eggs and hatchlings were placed in individual Tupperware™ containers with 2 cm of distilled water and kept at 23℃ in an environmental chamber. Six weeks post-hatch, we euthanized and determined the sex of each hatchling by examination of the gonads under a dissecting microscope (Yntema 1968). At six weeks of age, gonadal morphology is well developed and sexes are markedly different. Male hatchlings have smooth, short, opaque, yellowish gonads and females have longer, translucent or white gonads with prominent ovarian follicles and well-developed Müllerian ducts (Appendix G).

Statistical Methods. Offspring sex ratios were analyzed using R software and the lme4 package (R Foundation for Statistical Computing, Vienna, Austria). All other analyses were performed in JMP Pro 11 (SAS Institute Inc., Cary, NC, USA) or Microsoft Excel 2013. In all cases significance was assessed at $\alpha = 0.05$.

We quantified the impact of crops on the thermal and hydric dynamics of field nests throughout incubation using repeated measures ANOVA (RM ANOVA) that included the agriculture treatment as a between subjects effect and sample date as a within subject effect. For analyses involving water content and water potential, our RM ANOVAs used the average of the two soil core samples from plots as the experimental unit. The experimental unit for analyses of thermal responses (average daily temperature and average daily variance in °C) was each

treatment plot (n = 5 plots/trt), calculated as the average of the field nests (n = 3-4) within each plot.

We also tested the effect of our agriculture treatment on average daily temperature, thermal variability (using variance), and CTE (Constant Temperature Equivalent) in field nests during the thermosensitive period (TSP). Half of development occurs above and below the CTE (Georges 1989); CTEs account for faster development at higher temperatures and slower development at lower temperatures. We calculated the TSP as the middle third of the incubation period (Yntema 1979, Yntema and Mrosovsky 1982). Summaries of thermal conditions during the first and final thirds of incubation are not discussed but are provided in supplemental materials (Appendices D & E). Our CTE models were parameterized using T_0 = 16.01 (Freedberg et al. 2011, Georges et al. 2004). Because our dataset included incubation temperatures above and below developmental zero (T_0) we used the following equations to generate CTEs:

$$\text{CTE} = \text{R} \cdot Cos\,(\text{t}') + \text{M} \qquad [1]$$

$$\text{t}' = \frac{t_0}{2} + \frac{R}{2(M-T_0)}\,Sin(t_0) - \frac{R}{M-T_0}\,Sin(t') \qquad [2]$$

$$\text{t}_0 = Cos^{-1}\left[\frac{T_0-M}{\text{R}}\right] \qquad \text{for } T_0 > \text{M} - \text{R}$$

$$\text{t}_0 = \pi \qquad \text{for } T_0 \leq \text{M} - \text{R} \qquad [3]$$

Where R is the maximum difference in temperature from the mean (M), and t' is the time at which the CTE is achieved (Georges et al. 2004). We used the appropriate solution of t_0 from equation 3 (depending on whether nest temperature dropped below T_0 or not) to solve equation 2 through an iterative procedure, using $t'=\pi/2$ as an initial value and the resulting t' was then used

to solve equation 1 (Georges 1989, Georges et al. 2004). We used linear mixed models (LMM) that included lay date as a random effect, and agriculture treatment, mercury treatment, and nest layer as fixed effects. In our LMMs, we estimated variance components using the restricted maximum likelihood (REML) algorithm to account for unbalanced data (Corbeil and Searle 1976). In all cases significance was assessed at $\alpha = 0.05$. Three way interactions were tested but were not significant ($p > 0.20$ in all cases) and were removed from our final models.

We investigated treatment effects on sex ratios using generalized linear mixed models (GLMM) which included the dichotomous response of male v. female as the dependent variable, clutch as a random effect, and the agriculture and mercury treatments as categorical factors. We included nest layer (top or bottom) as a fixed effect in our models of treatment effects on thermal conditions during the TSD in our field incubation experiment. In both experiments, we included lay date as a continuous covariate but it was insignificant and removed from final models. Because the Hg + Shade treatment group in the laboratory incubation experiment was 100% male, we could not directly test interactive effects of Hg and Agriculture and instead, elected to use two models to test (1) the effect of agriculture treatment on sex within Ref clutches and (2) the effect of mercury treatment on sex within Open treatment clutches.

Using a second set of GLMM models, we examined continuous measures of nest temperature (average temperature, CTE, and thermal variance) and sex ratios in our field incubation experiment. Because there was not enough variation in thermal conditions among clutches in the lab experiment to model thermal parameters as continuous predictors we tested average temperature, CTE, and variance only as categorical predictors in the laboratory experiment.

Results

Nest Microclimate & Hg Concentrations. Total egg mercury concentration (THg, in ppm) reported on a dry mass basis (dwt) did not differ between the laboratory and the field experiments (REML: $F_{1, 80}$= 0.9, p = 0.35). The average egg THg in clutches collected downstream of the mercury source was about 12x higher than clutches collected upstream (Hg = 2.8 ± 0.2 ppm, dwt SE, n = 23; Ref = 0.3 ± 0.3 ppm, dwt SE, n = 20).

In our Shade plots, vegetative cover increased from <10% cover and 15 cm height at the start of the study (late May) to over 95% cover, 80% density, and almost 2 m height during and after the average TSP start date (in early July, Fig. 3.2). In our Open plots, vegetation never exceeded 25% cover, 20% density, or 20 cm in height (Fig. 3.2). At the start of the study period (in mid-May), low levels of non-crop vegetation were present in both Shade and Open plots. This vegetation died by the beginning of July and was followed by buckwheat, a common cover crop used to enrich potassium in soils and suppress weed growth (Bjorkman et al. 2008). Early season non-crop vegetation was very sparse but tall enough to obstruct the first band of the density pole (~10-15 cm tall) such that vegetative cover did not increase yet density increased (Fig. 3.2, Graphs B and C). Late season buckwheat sprouts from residual seeds left in the soil from previous years were short (~ 5 cm tall) and grew in sprawling clumps. Consequently, buckwheat growth didn't obstruct the first band of the density pole unless growing directly in front of the pole (in mid-September), yet covered a substantial portion of our plots (up to about 20% cover in come plots) beginning in mid-August (Fig. 3.2, Graphs A and B).

The Open and Shade plots initially had the same soil moisture levels but they diverged by June 19 (RM ANOVA: $F_{1, 13}$ = 3.2, p < 0.001, Table 3.1, Fig. 3.3, Graph A). After June 19, soils in our Open plots consistently contained more moisture than soils in Shade plots (Fig. 3.3, Graph A). Rainfall during the two days before samples was correlated with reduced differences

between Shade and Open plots ($R^2 = 0.75$). When we converted soil moisture levels to water potentials, the interaction between agriculture treatment and sample date remained significant (RM ANOVA: $F_{1, 13} = 2.4$, $p < 0.02$, Table 3.1, Fig. 3.3, Graph B). Dry conditions in Shade plots on June 19 (2.7% soil moisture) precluded our ability to calculate water potential for that date.

Average temperature and thermal variance (s^2 of temperature) in field nests were affected by the interaction between the agriculture treatment and sample date, both at the top and bottom of nests (RM ANOVA: in both cases $p < 0.01$, Table 3.1). Average daily temperature became lower in Shade plots than in Open plots on June 19th at the top of nests, and on June 27th at the bottom of nests (Fig. 3.4, Graphs A and B). For both the top and bottom of nests, thermal variance diverged between Shade and Open plots on June 19th (Fig. 3.4, Graphs C and D).

During the average TSP, the thermal variance, average temperature, and CTEs of our field nests were lower in the Shade treatment than the Open treatment (REML: in all cases $p < 0.001$, Table 3.2, for other developmental periods see Appendix H). Thermal variance at the bottom of nests was lower than at the top (REML: $F_{1,60} = 148$, $p < 0.01$) but nest thermal gradients were larger in the Open than in the Shade treatment. Nest layer interacted with the agriculture treatment to impact CTE: we observed larger differences in CTE between the top and bottom of nests in the Open treatment than in the Shade treatment (REML: $F_{1,60} = 9.8$, $p < 0.01$, Table 3.2).

Actual (laboratory) and target (field) average daily incubation temperatures differed by only 0.30 ± 0.03℃ SE and the average hourly standard deviation between actual and target temperatures was 0.86 ± 0.02 °C SE over the course of the laboratory incubation experiment (Table 3.3). During the TSP in the lab CTEs were lower than the average of the top and bottom of field nests,

by an average of 0.5°C in Open and 0.05°C in Shade plots (Fig. 3.5, for other developmental periods see Appendix I).

Offspring Sex Ratios. In the field experiment, the Shade treatment produced higher proportions of males than the Open treatment (GLMM: $z = -5.9$, $p < 0.001$, Fig. 3.6, Table 3.4) and this difference was greater in the Hg treatment than in the Ref treatment (interaction GLMM: $z = 2.6$, $p = 0.009$). Nest layer also affected hatchling sex; more males were produced at the bottom of nests than at the top (GLMM: $z = 2.1$, $p = 0.033$, Fig. 3.6, Table 3.4). Similarly, cooler temperatures, CTEs, and lower levels of thermal variance decreased the proportion of males produced (in all cases $R^2 \geq 0.34$). Two clutches in the Reference treatment group produced more females than expected based on TSP predictions. When these two clutches were excluded from analysis, R^2 values increased (average increase = 0.20 ± 0.01, Appendix J) and regression analyses showed that the bivariate model with mean temperature and thermal variance explained more variation in sex ratios than the univariate mean temperature and thermal variance models (27 and > 1600 times more likely, respectively). However, the mean temperature and thermal variance model only explained slightly more variation in hatchling sex ratios than the CTE model (1.11 times more likely; AIC weight = 0.4579, Table 3.5).

In the laboratory experiment we were not able to directly test the interactive effect between agriculture and mercury treatments because the Shade + Hg treatment was 100% male. However, patterns were similar to those observed in the field experiment: within the Ref clutches, the Shade treatment produced more males than the Open treatment (GLMM: $z = -2.7$, $p < 0.001$) and within the Open clutches, the Ref treatment produced more males than the Hg treatments although this difference was not statistically significant (GLMM: $z = -1.9$, $P < 0.062$, Table 3.5).

Discussion

The selection of recently planted agricultural fields for nesting by turtles can have substantial impacts on nest microclimate and offspring sex ratios. We demonstrated that: (1) crop growth near turtle nests can decrease moisture, average temperature, and thermal variance inside of nests; (2) the cooling effect of crops causes male-biased offspring ratios; (3) maternally-transferred Hg impacts sex ratios in a turtle with TSD; and, (4) high levels of maternally derived Hg interact with the cooling effect of crops to magnify effects on offspring sex ratios. Differences in water availability also impact sex ratios of turtles (Wyneken and Lolavar 2015) but because our results were consistent between the field and laboratory experiments, we provide strong and novel evidence that the proximate mechanism driving agriculturally-induced changes in offspring sex ratios is lowered incubation temperature and thermal variance.

Endocrine disrupting chemicals (EDCs) are known to impact offspring sex in reptiles with TSD, yet our study provides the first documentation that Hg affects sex in a species with TSD. Reptiles are an understudied group in ecotoxicology (Hopkins 2000) but the effects of some EDCs, mainly dichlorodiphenyltrichloroethane (DDT) and polychlorinated biphenyls (PCBs), on TSD are well studied (Crews et al. 1995, Kohno et al. 2013). In turtles, embryonic exposure to DDT and PCBs can cause abnormal androgen synthesis and lead to sex-reversal to females at male producing temperatures (Bergeron and Crews 1998, Adams et al. 2016). Mercury is also an EDC that affects sex hormone concentrations and impacts the regulation of enzymes involved with steroidogenesis (Tan et al. 2009). The fact that maternally transferred Hg interacted with thermal conditions to influence sex ratios raises important questions about the underlying mechanisms.

We hypothesize two possible mechanisms for the interactive effect between the agriculture and mercury treatments on hatchling sex ratios. First, the thermal effects of the agriculture treatment and the endocrine disrupting effects of mercury may interact to cause a direct change to the pathways of sexual differentiation. In reptiles with TSD, offspring sex is influenced by an interaction between incubation temperature and yolk steroid hormones (Ding et al. 2012, Páez et al. 2015, Sun et al. 2016). Consequently, the interaction between the agriculture and mercury treatments in our study may be related to interactions between incubation temperature, yolk hormones (Flood et al. 2013, Warner et al. 2014), and the influence of Hg on the production of estrogens (Zhu et al. 2000) and thyroid hormones (Wada et al. 2009, Wada et al. 2010, Meyer et al. 2014; but see Wada et al. 2011). An alternate hypothesis for the interaction between our treatments is the differential mortality of embryos based on sex (Sakamoto et al. 2001, Bouland et al. 2012; DuRant et al. 2016), but inspection of hatch success and sex ratios among clutches in our experiments suggest that this explanation alone is unlikely; hatching success was simply too high (> 87% overall) in the field experiment to account for observed differences in sex ratios between treatments. Although the interactive mechanism of Hg and thermal effects on sex ratios is unknown, our results emphasize that major global changes can have complex interactions that affect early vertebrate development in unexpected ways.

Average temperatures in the laboratory closely matched mean hourly temperatures in field nests, but our laboratory study could not simulate the vertical stratification of temperatures within field nests or the range of thermal profiles among field nests. Collapsing variance between top and bottom nest temperatures from the field into nest average temperatures for laboratory simulations proved to have important implications for sex ratios (Fig. 3.5).

Specifically, temperatures in the laboratory were less likely to cycle above and below the pivotal temperature for sex determination than temperatures in the field. Likewise, some nests in the field were always warmer than the average field nest temperature, and within field nests, some consistently had larger thermal gradients than others despite standardized dimensions and clutch sizes. Because crops dramatically lowered thermal variance, the effects of averaging among nests on the Shade laboratory incubation regimen were much less pronounced than effects on the Open laboratory incubation (Fig. 3.5). Therefore, our work demonstrates the value of coupling laboratory and field studies of TSD, and that extrapolating between these settings can be more appropriate for some contexts (e.g., Shade) than others (e.g., Open) based on the magnitude of thermal fluctuations associated with each treatment.

The majority of clutches used in our experiments followed expected TSD patterns although two (Ref) clutches produced more females than expected. These clutches accounted for three of the four reproductive deformities observed and the eggs were lighter than all but one other clutch (42/43, Appendix K). As female turtles grow larger they produce larger clutches and eggs (Congdon et al. 1987, Paterson et al. 2012, Hopkins 2013b). Nutritionally limited or young mothers have been shown to produce female-biased offspring sex ratios relative to older or higher condition mothers in birds, mammals, and reptiles (Nager et al. 1999, Sheldon and West 2004, Warner et al. 2007) and there is age-related variation in yolk hormones in the eggs of Painted Turtles (*Chrysemys picta*; Bowden et al. 2011). Thus, maternal effects associated with small size of the females that produced the two unusual clutches could be related to the female-biased sex ratios and reproductive deformities we observed.

Our results have important implications for understanding the effects of rapid anthropogenic global changes on turtle populations, espeacially in the context of climate change.

Consistently male-biased offspring sex ratios, like those documented here, are likely to negatively impact population growth rates (Schwanz et al. 2010). Turtle population dynamics are more dependent on the number of reproductive females than the number of males, especially because females can store sperm for years and self-fertilize in the absence of a mate (Congdon et al. 1994, Pearse and Avise 2001). Although climate change is predicted to increase global temperatures and have a feminizing effect on reptiles with TSD (Böhm 2016), our results add to a growing body of literature showing that nesting turtles are attracted to human disturbed landscapes that have a masculinizing effect on reptiles with TSD (Janzen 1994, Mrosovsky et al. 1995, Kolbe and Janzen 2002, Kamel and Mrosovsky 2006). Our results thus suggest that the thermal heterogeneity of habitats that attract nesting turtles, especially human modified habitats, add an additional layer of complexity to current climate projections (Sears and Angilletta 2015). Conservation planning can be improved by considering how anthropogenic land-use, common environmental pollutants, and climate change can interact to influence sex ratios and population dynamics of animals with TSD.

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Table 3.1 Summary of Repeated Measures ANOVAs examining impacts of the agriculture treatment on the hydric and thermal characteristics of field plots and nests during the 15 sampling dates from May 26 to September 23, 2014 (spaced eight days apart). For within subject effects, P-values are from univariate Huynh-Feldt epsilon corrected tests.

	Agriculture		Sample Date		Ag.*Sample Date	
Soil Moisture	$F_{1,8} = 6.8$	***P* = 0.03**	$F_{1,13} = 48.5$	***P* < 0.01**	$F_{1,13} = 3.2$	***P* < 0.01**
Soil Water Potential	$F_{1,8} = 2.7$	$P = 0.14$	$F_{1,13} = 39.2$	***P* < 0.01**	$F_{1,13} = 2.4$	***P* = 0.02**
Avg. Temp. (Top)	$F_{1,8} = 121$	***P* < 0.01**	$F_{1,14} = 135$	***P* < 0.01**	$F_{1,14} = 21.1$	***P* < 0.01**
Avg. Temp. (Bottom)	$F_{1,8} = 128$	***P* < 0.01**	$F_{1,14} = 119$	***P* < 0.01**	$F_{1,14} = 20.2$	***P* < 0.01**
Thermal Variance (Top)	$F_{1,8} = 95.9$	***P* < 0.01**	$F_{1,14} = 34.3$	***P* < 0.01**	$F_{1,14} = 12.5$	***P* < 0.01**
Thermal Variance (Bottom)	$F_{1,8} = 126$	***P* < 0.01**	$F_{1,14} = 39.6$	***P* < 0.01**	$F_{1,3\ 4} = 10.5$	***P* < 0.01**

Table 3.2 Temperature variables observed during the TSP in agriculture and mercury treatments, in the top and bottom layers of nests in the field incubation experiment. Responses were calculated using daily averages and are shown in ℃. Results of REML analyses with interactions between effects denoted with an asterisk (*), significant interactions shown in bold, significance was determined at α = 0.05. Samples sizes (clutch/layer/treatment groups) are as follows: Top Layer: Hg/Open: 10, Ref/Open: 8, Hg/Shade: 10, Ref/Shade: 8, Bottom layer: Hg/Open: 9, Ref/Open: 8, Hg/Shade: 9, Ref/Shade: 7.

	Avg. Temp.		Thermal Variance		CTE	
TRT GROUP	Top	Bottom	Top	Bottom	Top	Bottom
Hg/Open	25.3 ± 0.2	25.0 ± 0.2	8.1 ± 0.8	2.4 ± 0.2	27.1 ± 0.2	25.7 ± 0.3
Ref/Open	25.4 ± 0.2	25.1 ± 0.2	9.0 ± 0.7	2.6 ± 0.2	27.3 ± 0.3	26.0 ± 0.3
Hg/Shade	21.7 ± 0.3	21.6 ± 0.3	1.5 ± 0.1	0.5 ± 0.04	26.0 ± 0.3	21.8 ± 0.3
Ref/Shade	21.7 ± 0.3	21.5 ± 0.3	1.6 ± 0.3	0.7 ± 0.2	22.4 ± 0.3	21.7 ± 0.3
TRT EFFECT						
Mercury	$F_{1,60}$= 1.0,	P = 0.32	$F_{1,60}$= 1.5,	P = 0.22	$F_{1,60}$= 0.6,	P = 0.43
Agriculture	$F_{1,60}$= 951,	***P* < 0.01**	$F_{1,60}$= 336,	***P* < 0.01**	$F_{1,60}$= 823,	***P* < 0.01**
Ag.*Mercury	$F_{1,60}$ < 0.0,	P = 0.86	$F_{1,60}$ < 0.0,	P = 0.99	$F_{1,60}$ < 0.0,	P = 0.89
Nest Layer	$F_{1,60}$ = 3.1,	P = 0.09	$F_{1,60}$= 148,	***P* < 0.01**	$F_{1,60}$ = 41,	***P* < 0.01**
Layer*Ag.	$F_{1,60}$ = 0.8,	P = 0.39	$F_{1,60}$= 1.8,	P = 0.18	$F_{1,60}$ = 9.8,	***P* < 0.01**
Layer*Mercury	$F_{1,60}$ = 0.01,	P = 0.93	$F_{1,60}$= 0.4,	P = 0.56	$F_{1,60}$ = 0.2,	P = 0.67

Table 3.3 Summary temperature variables observed during the TSP in the laboratory experiment. All responses are calculated using daily averages and are shown in ℃ $\pm$ **1** $\boldsymbol{SE}$.

	Hg/Open	Ref/Open	Hg/Shade	Ref/Shade
VARIANCE	3.3 ± 0.1	3.4 ± 0.1	0.6 ± 0.02	0.6 ± 0.02
AVG. TEMP.	25.1 ± 0.1	25.2 ± 0.1	21.6 ± 0.2	21.5 ± 0.2
CTE	26.1 ± 0.1	26.2 ± 0.1	22.0 ± 0.2	21.9 ± 0.2

**Avg. TSP dates in the agriculture validation experiment are the dates of temperature recordings used for incubation regimens during the middle third of incubation. Actual TSP dates were 7/13- 8/5 in open and 7/17- 8/12 in shade treatment.*

Table 3.4 Summary of treatment effects and offspring sex ratios (percent male) in the field and laboratory experiments (LS Means ± SE). Sex ratios are presented for the top and bottom layers of nests in the field incubation experiment. Interactions between effects are denoted with an asterisk (*), significant effects are shown in bold, significance was determined by α =.05. P-values show results of logistic regression (GLMM) analyses.

	FIELD EXP.		LAB EXP.
TRT GROUP	Top	Bottom	
Hg/Open	6.0 ± 3.6	26.8 ± 7.0	73.1 ± 3.3
Ref/Open	12.8 ± 7.2	24.7 ± 7.9	84.0 ± 3.2
Hg/Shade	78.7± 5.6	91.6 ± 5.4	100 ± 0
Ref/Shade	58.5 ± 7.0	63.4 ± 7.4	98.9 ± 3.2
TRT EFFECT			
Mercury	z = -2.3, ***P* < 0.001**		z = -1.9, $P < 0.062$
Agriculture	z = -5.9, ***P* < 0.001**		z = -2.7, ***P* < 0.001**
Ag.*Mercury	z = 2.6, ***P* = 0.009**		–
Nest Layer	z = 2.1, ***P* = 0.033**		–
Layer*Ag.	z = -0.9, $P = 0.346$		–
Layer*Mercury	z = 1.5, $P = 0.138$		–

**The effect of Agriculture treatment on sex was tested between Ref clutches only, see text for details.*
***The effect of Mercury treatment on sex was tested between open clutches only, see text for details.*

Table 3.5 Results of logistic regression models investigating temperature effects on sex ratios in the field incubation experiment. Interactions between effects are denoted with an asterisk (*), significant effects are shown in bold, significance was determined by α =.05.

Model	Z value	Test Stat	AICc	delta-AICc	AIC weight	B	S.E.	Odds Ratio
Avg. Temp + Variance	- 4.0, - 2.6	$p < 0.01$, $p = 0.011$	374.9	0	0.5060	-0.5543, - 0.2585	0.1402, 0.1013	0.57, 0.77
CTE	- 8.6	$p < 0.01$	375.1	0.2	0.4579	-0.6933	0.0810	0.51
Avg. Temp	- 8.5	$p < 0.01$	380.2	5.3	0.0358	-0.8428	0.0989	0.44
Thermal Variance	- 7.3	$p < 0.01$	389.7	14.8	0.0003	-0.5881	0.0808	0.51

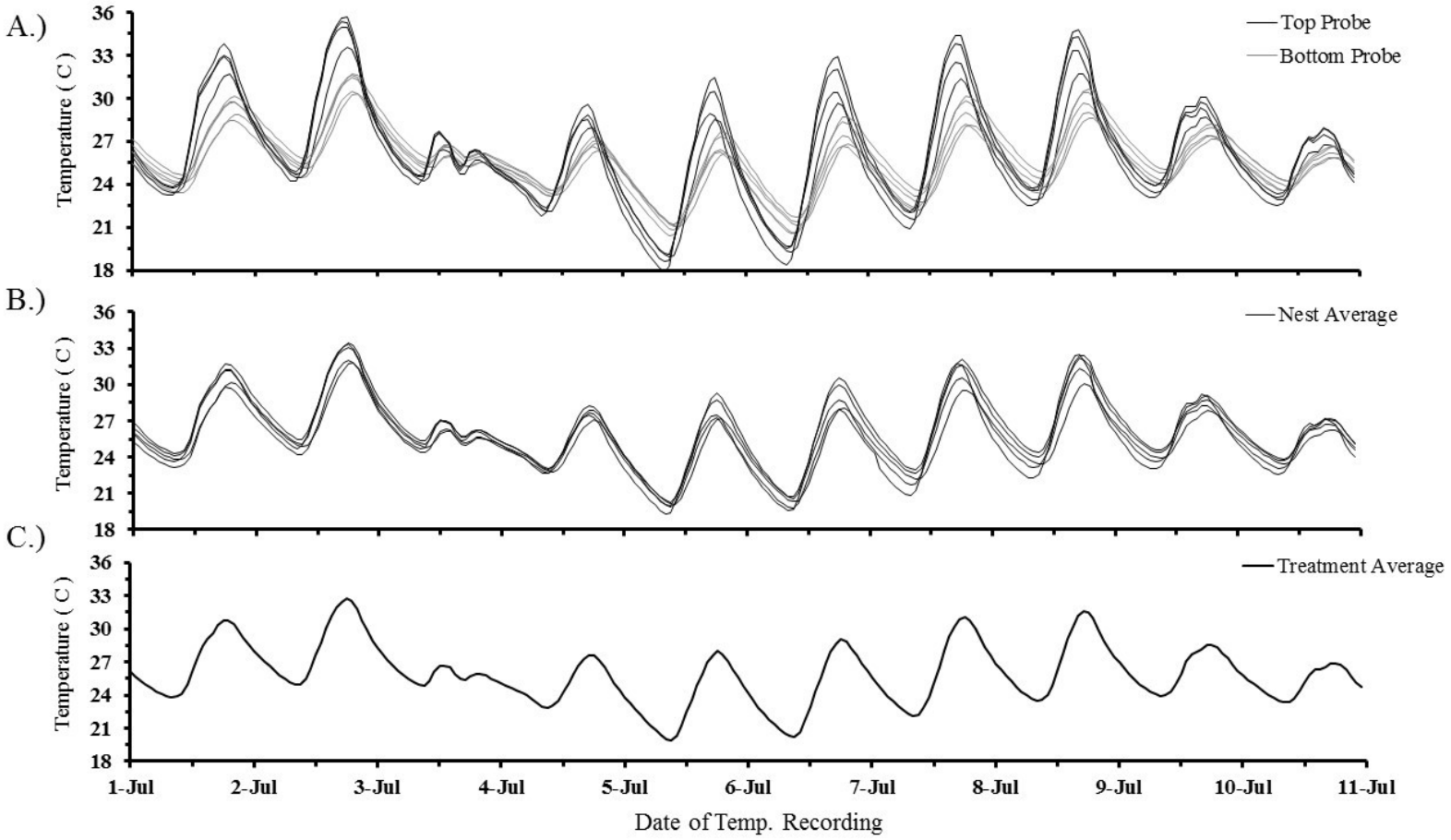

Fig. 3.1 Sampling design used to convert temperatures recorded in the field to a laboratory incubation regime using real data. Graph (A) shows 8 days of temperatures recorded at the top (black) and bottom (grey) of the artificial nests in the Open treatment (N=5). Four days of temperature data were averaged for the top and bottom of each nest, for each hour (B). An overall average was taken from the five treatment replicates (C) and used for incubation in the lab experiment.

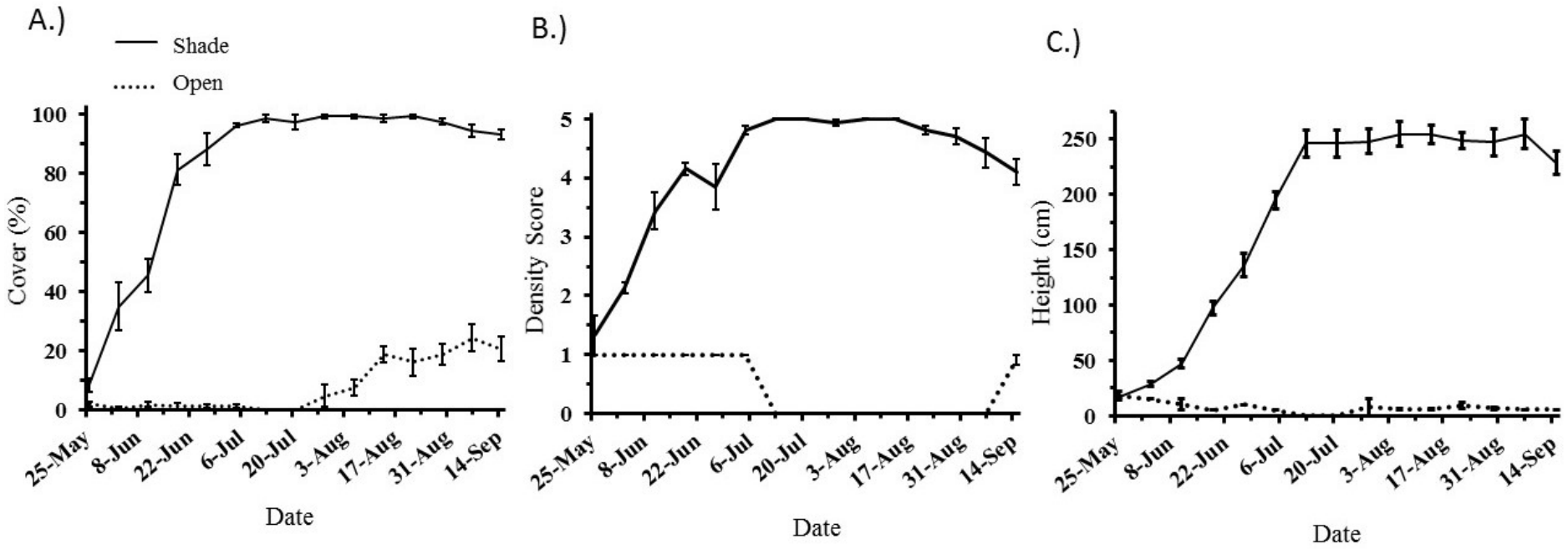

Fig. 3.2 Average vegetative cover (A), density (B), and height (C) for Open (dotted) and Shade (solid) treatment plots throughout the incubation period. Both crop and non-crop vegetation are included in average values.

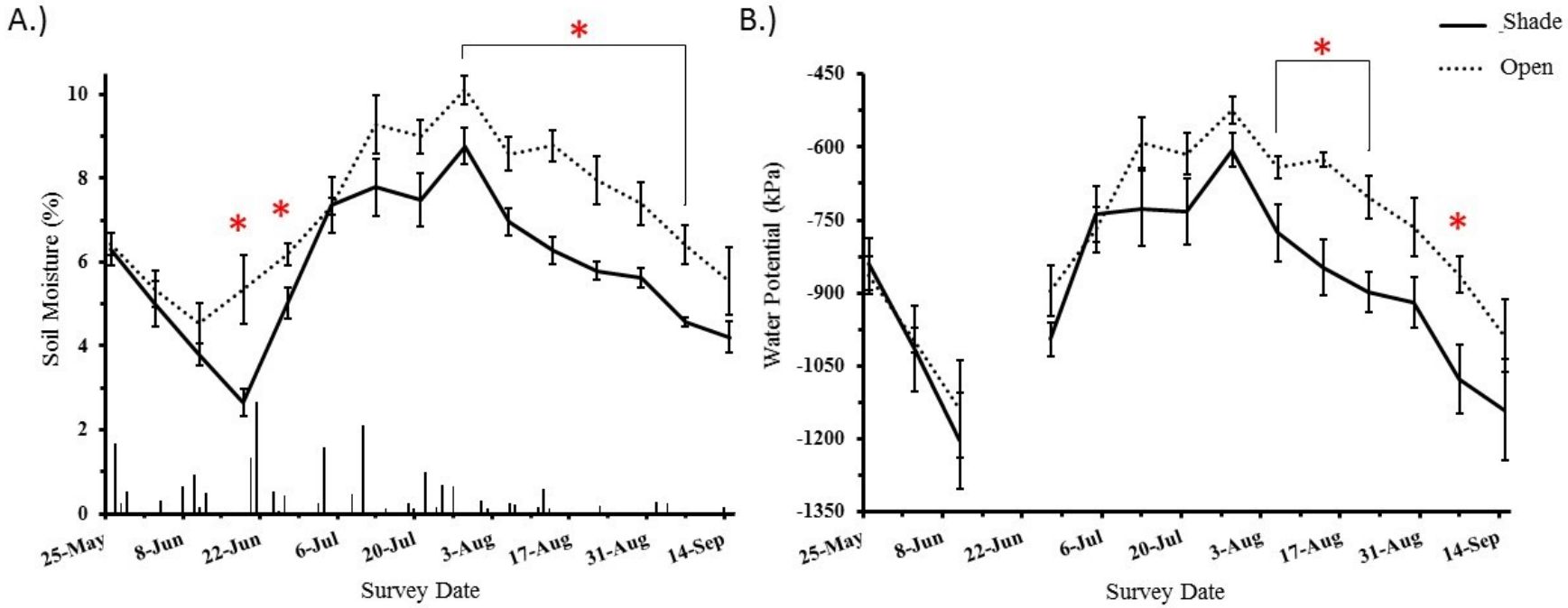

Fig. 3.3 Average percent soil moisture (A) and water potential (B) in Open (dotted) and Shade (solid) plots throughout incubation. Relative levels of daily precipitation are shown in bars along the x-axis of (A). Significant post-hoc tests between Open and Shade plots for each sample date are marked with an asterisks (*), significance was determined at $\alpha = 0.05$. Means ± SE.

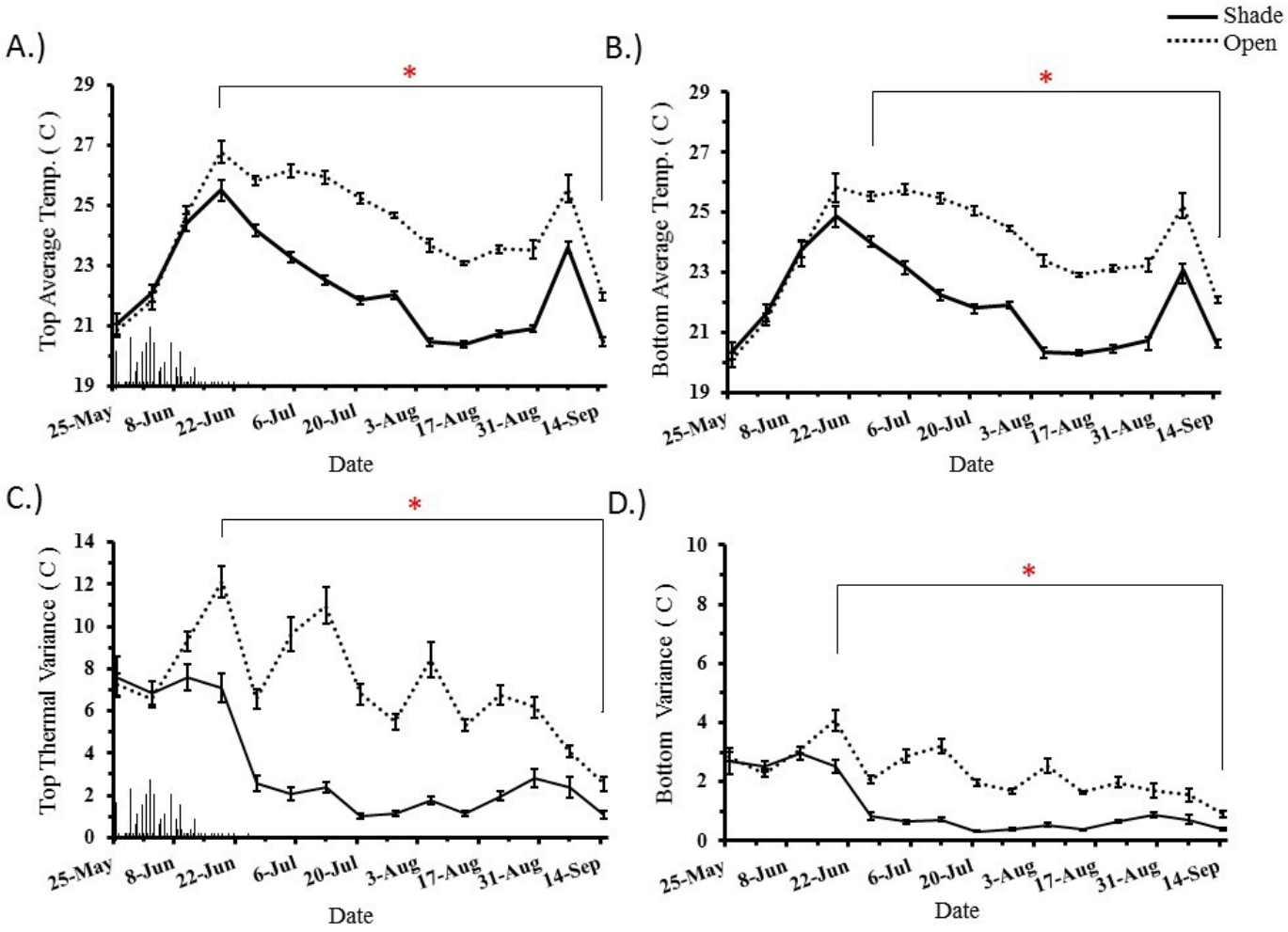

Fig. 3.4 Average daily temperature at the top (A), bottom (B), and average daily thermal variance at the top (C), and bottom (D) of nests in our field incubation experiment. Black bars on the x-axis show the frequency of oviposition along the South River, VA during 2014. Significant post-hoc tests between Open and Shade plots for each sample date are marked with an asterisks (*), significance was determined at $\alpha = 0.05$. Means ± SE.

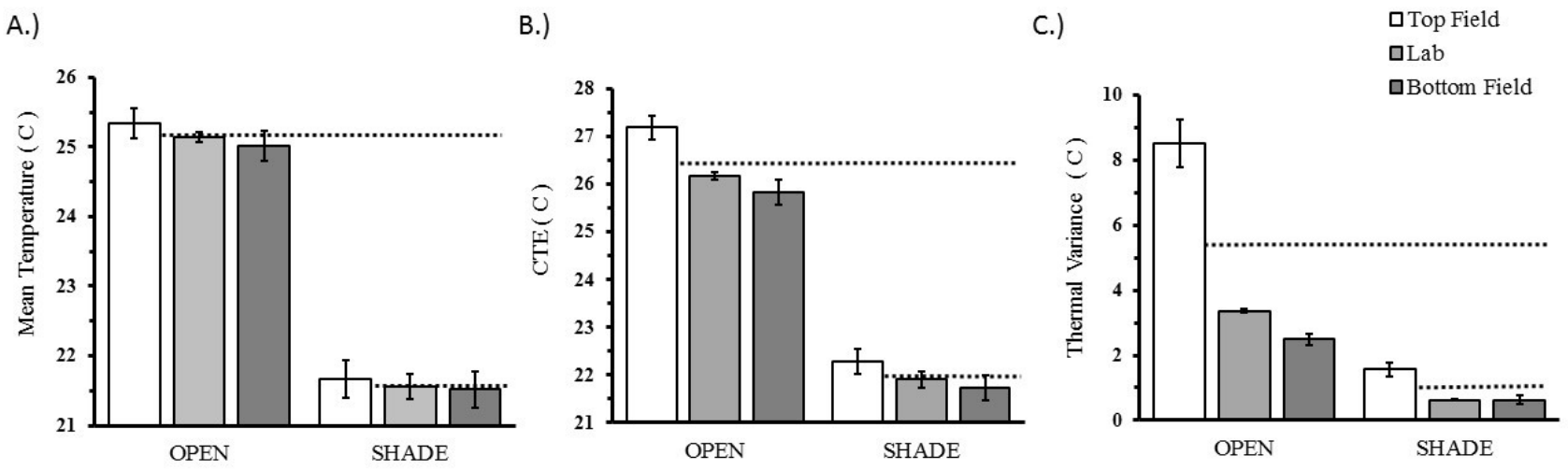

Fig. 3.5 Comparison of temperature variables in the field and the laboratory incubation experiments during the TSP. Temperatures from the lab experiment (light gray) are shown between the average values from the top (white) and bottom (dark grey) of nests in the field plots. The average of the top and bottom of field nests (dotted line) shows that (A) mean temperatures in the laboratory experiment were approximately the same as the mean temperatures in the field, but (B) mean CTEs in the lab were lower than the average of the top and bottom of field nests, and (C) mean variance was 40% lower than the average of the top and bottom of field nests.

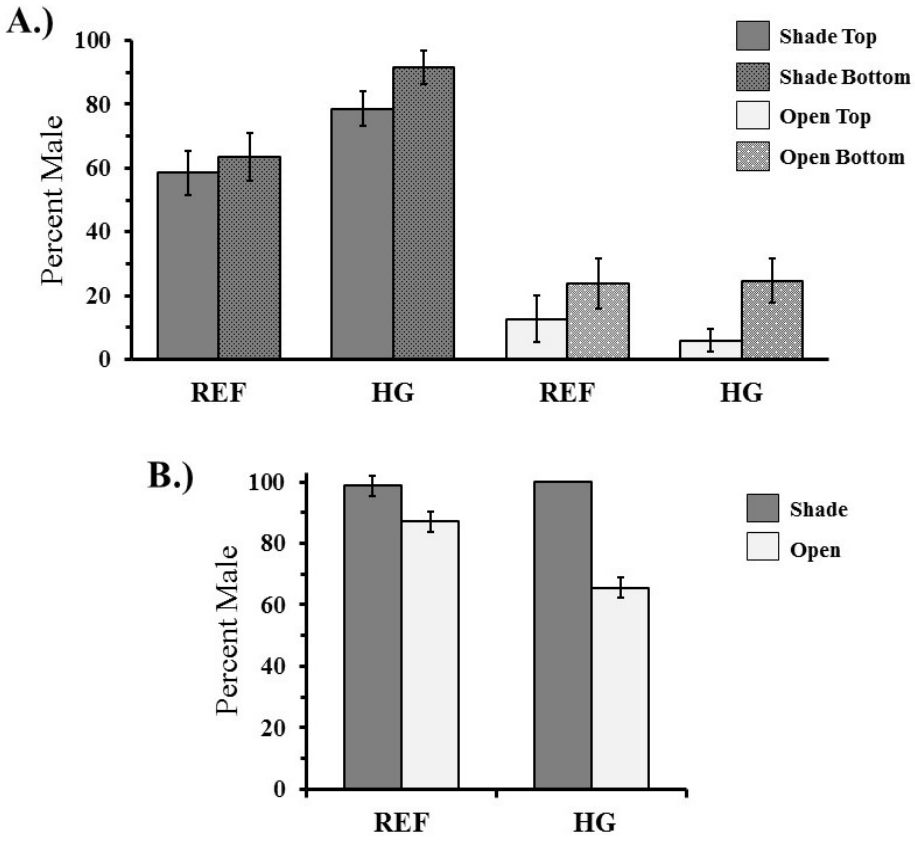

Fig. 3.6 Relationships between treatments and sex ratios in the field incubation experiment (at the top and bottom of nests; A) and in the laboratory experiment (B).

Conclusion

My thesis research provides one of the first documentations of negative interactive effects of mercury (Hg) pollution and habitat quality on early vertebrate development, and it does so using a focal species from an understudied vertebrate group. Because Hg and agriculture are among the most widespread forms of habitat modification in the world (Driscoll et al. 2013, Oakenleaf et al 2015), they are likely to persist and frequently interact with one another to negatively influence organisms on a large spatial scale. Some biological traits, such as temperature dependent sex determination (TSD), are prediced to make reptiles a susceptile taxonomic group to major global changes (Böhm et al. 2016). Yet, only about 40% of reptile species have been assessed by the IUCN red list of endangered species compared to about 99% of bird and mammal species (Meiri and Chapple 2016). Consequently, results from my research help to fill an important research gap in a data deficient taxonomic group; they demonstrate that a major global pollutant can interact with a pervasive form of habitat modification to impact hatch success and sex ratios in a freshwater turtle with temperature-dependent sex determination. Novel findings from my research provide insight into future research avenues that are likely to be valuable to conservation efforts and highlight the importance of examining the combined influence of global changes on biological systems.

Mechanisms of Interactive Effects. My research utilized concurrent laboratory and field studies to show that maternally derived Hg and the cooling effect of agricultural land use can interact to reduce hatch success and alter offspring sex ratios in Snapping Turtles (*Chelydra serpentina*) but did not elucidate the proximate mechanism(s) behind observed interactive effects.

Understanding the proximate mechanism behind the observed negative interactive effects of Hg and agriculture on hatch success, as well as how these negative effects translate to turtle population dynamics, would help to inform management strategies for turtle populations in Hg polluted and human-modified environments. Little natural habitat remains in my study areas and nearly all of the turtle nests found in this study (84%) were located in human disturbed soils. Many studies have demonstrated that low incubation temperatures can negatively impact reptile hatch success (Deeming 2004), maternally-derived Hg can reduce hatch success in turtles (Hopkins 2013) and other vertebrates (Zhu et al. 2000, Bergeron et al. 2011, Bouland et al. 2012). However, my thesis research provides one of the first documentations of negative interactive effects of mercury pollution and habitat quality on hatch success in oviparous vertebrates, and provides novel evidence that Hg contamination can interact with the thermal effects of anthropogenic land use to impact offspring sex ratios in an animal with TSD.

Similarly, the mechanism behind the interactive effect my research showed between the agriculture and mercury treatments on hatchling sex ratios remains an important topic for future research. The effects of incubation temperature and egg yolk hormones on sex determination in reptiles are dynamic (Ding et al. 2012, Flood et al. 2013, Warner et al. 2014, Sun et al. 2016), as are the effects of Hg on the endocrine and reproductive systems of vertebrates (Rice et al. 2014). For example, the effects of Hg on offspring sex ratios may be due to its influence on sex steroids, thyroid hormones, chromosome breakage, and/or realized sex ratios (Figure 1). Consequently, the interactive effect of Hg and agricultural incubation conditions on offspring sex could be explained by several mechanisms; disentangling these possible mechanisms to identify the proximate mechanism(s) would aid in improving predictions of offspring sex ratios of reptiles in human transformed landscapes.

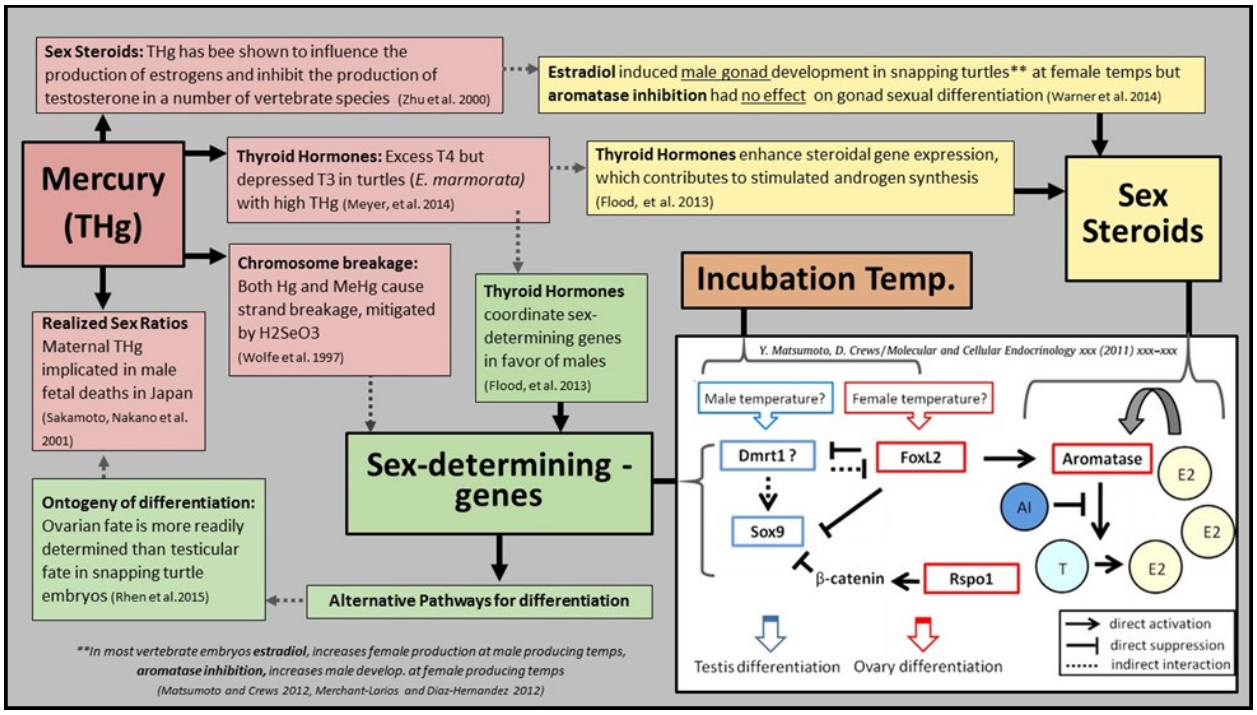

Figure 1. Possible relationships between Mercury and offspring sex ratios through sex steroids thyroid hormones, chromosome breakage, and realized sex ratios.

Population-level Implications. My results are consistent with others who have demonstrated that turtles are attracted to human disturbed soils for nesting which can cause male-biased offspring sex ratios (Mrosovsky et al. 1995, Kolbe and Janzen 2002, Freedburg et al. 2011). Climate change is predicted to result in an increase in global temperatures of about 1.4-4.3°C within the 21st century, and to have a feminizing effect on reptiles with TSD (Böhm et al. 2016). Consequently, my results support recent work suggesting that climate change related impacts to reptile sex ratio predictions should be refined to incorporate an understanding of how microhabitats provide thermal refugia and heterogeneity to landscapes (Sears and Angilletta 2015, Shi et al. 2016).

Species & Latitudinal Differences. In addition to future work that expands upon the novel findings of my research, future studies that replicate my research design over multiple years, latitudinal gradients, and using other focal species would be valuable. Multi-year studies would elucidate how changes in annual mean temperatures, thermal fluctuations, and precipitation levels influence experimental outcomes related to the effects of agriculture and Hg on turtle nests. Both climatic conditions and biological traits of turtles vary along latitudinal gradients; in Snapping Turtles, as latitude increases, the upper pivotal temperature for sex determination decreases (Ewert et al. 2005). Consequently, the effects of agriculture on hatchling sex ratios may vary with latitude both to changes in ambient air temperature and to changes in sex determination patterns. Additionally, during the course of my research, field crew members and I witnessed multiple species of aquatic turtles nesting in agricultural fields (e.g., Stinkpot Turtles, *Sternotherus odoratus*, and Painted Turtles, *Chrysemys picta*) but the thermal effects of agriculture on embryonic development and offspring phenotype have not been directly studied in any species other than the Snapping Turtle. Future studies that replicate my research design over multiple years, over a latitudinal gradient, and/or using multiple species are needed in order to understand the frequency and extent of negative impacts of Hg and agricultural land use on turtle populations.

Conclusion. Over the last 500 years, the onset of the sixth mass extinction event on earth has begun and already has caused extinctions in over 600 vertebrate species (Ceballos et al. 2015). Habitat loss and environmental pollution are two global threats of great concern to species conservation and reptiles are vulnerable to global threats yet understudied compared to amphibians, birds, and mammals (Meiri and Chapple 2016). My thesis results were consistent with other studies that show that turtles are attracted to human disturbed soils for nesting (Kolbe

and Janzen 2002, Beaudry et al. 2010, Freedburg 2011, Paterson et al. 2013). Using concurrent laboratory and field experiments, my research provided new insight into how nesting in human disturbed landscapes can impact turtle nest microclimate, embryonic development, hatch success, and offspring phenotype in an aquatic turtle. My research also provides a novel assessment of the interactive effects of Hg contamination and nest cooling on hatch success and offspring sex ratios in a species with TSD. My results highlight that research on how the cooling effect of agricultural and residential land use, and interactions between anthropogenic land-use and common environmental pollutants, will interact with climate change to influence the survival and sex ratios of reptiles with TSD will be valuable to the conservation of vertebrate biodiversity.

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Appendices

APPENDIX A. Comparison of Common Snapping Turtle (*Chelydra serpentina*) nest predation rates between reference (REF) and Hg sites along the South River, Virginia, USA (2013–2014), and relationships between predation (during the first month of incubation) and habitat characteristics. P-values are for comparisons between reference and Hg sites, and depredated nests versus those that were not depredated (mean ± SE), using logistic regressions.

	REF Sites		HG Sites		DEPREDATION IN HG SITES			
	n	Rate	n	Rate	Veg. Cover	Field Edge	Water Body	South River
DEPREDATED								
2013	0	–	31	65.6%	0	28.0 ± 4.8	39.9 ± 4.2	41.2 ± 7.3
2014	0	–	8	–	2.5 ± 2.5	11.9 ± 1.7	29.4 ± 6.5	26.1 ± 4.7
NOT DEPREDATED								
2013	39	100%	17	35.4%	6.7 ± 6.7	27.1±4.9	31.6 ± 6.8	157.5 ± 46.9
2014	23	–	32	–	0	20.8 ±4.5	38.8 ± 6.4	91.1 ± 29.0
P-VALUE								
2013	–	–	–	–	> 0.999	0.194	0.290	0.101
2014	–	–	–	–	0.998	0.146	0.475	0.290
POOLED					0.146	0.079	0.265	0.001

APPENDIX B. Comparison of habitat characteristics of Common Snapping Turtle (*Chelydra serpentina*) nest sites and randomly selected points in reference and Hg site along the South River, Virginia, USA, 2013–2014.. The number of each type of characteristic measured is shown as n. Within sites, P-values are from univariate logistic regressions. Nest site characteristics (mean ± SE) are compared between reference and Hg sites, using either t-tests or Wilcoxon/Kruskal-Wallis tests.

	REFERENCE SITES					HG SITES					REF-V-HG	
	Nest		Random			Nest		Random			Nest	Random
Variable	n	Mean ± SE	n	Mean ± SE	*P*-value	n	Mean ± SE	n	Mean ± SE	*P*-value	*P*-value	*P*-value
CANOPY	62	0.7 ± 0.6	89	23.7 ± 3.9	0.012	88	1.1 ± 0.8	72	30.0 ± 5.3	0.002	0.746	0.341
BG	62	77.6 ± 3.0	89	23.4 ± 3.4	< 0.001	88	66.9 ± 3.0	72	18.5 ± 3.7	< 0.001	0.009	0.129
FORB	62	4.1 ± 0.6	89	20.1 ± 2.6	< 0.001	88	5.4 ± 1.4	72	17.7 ± 2.4	< 0.001	0.941	0.898
GRASS	62	1.3 ± 3.8	89	26.5 ± 3.8	0.005	88	8.0 ± 2.1	72	36.5 ± 4.4	< 0.001	0.001	0.016
CROP	62	8.9 ± 1.4	89	5.1 ± 2.4	0.339	88	4.1 ± 0.7	72	0.2 ± 0.2	0.002	0.004	0.035
WOOD	62	0	89	1.1 ± 0.4	0.991	88	< 0.05 ± 0	72	1.2 ± 0.4	0.128	0.399	0.905
DETRITUS	62	0.2 ± .1	89	4.5 ± 1.4	0.030	88	1.0 ± 0.3	72	7.6 ± 2.4	0.075	0.079	0.469
TREE	62	0.05 ± 0	89	1.9 ± 1.0	0.390	88	0.2 ± 0.1	72	1.2 ± 0.6	0.171	0.201	0.534
SHRUB	62	0	89	4.6 ± 2.0	0.994	88	< 0.05 ± 0	72	6.7 ± 2.5	0.224	0.399	0.312
ROCK	62	0.6 ± 0.3	89	0.6 ± 0.5	0.997	88	0.8 ± .03	72	0.2 ± 0.2	0.170	0.306	0.494
RIVER	61	37 ± 4	89	54.1 ± 5	0.010	71	71 ± 12	72	65 ± 7	0.636	0.043	0.415
WATER	61	36 ± 3	89	49 ± 4	0.023	77	37 ± 3	72	47 ± 4	0.055	0.935	0.423
FOREST	42	23 ± 3	48	49 ± 6	< 0.001	84	23 ± 4	20	60 ± 7	0.002	0.787	0.206
FIELD	41	21 ± 7	40	47 ± 7	0.001	83	23 ± 4	53	70 ± 11	0.004	0.885	0.085

APPENDIX C. Comparison of habitat characteristics of nest sites of Common Snapping Turtles (*Chelydra serpentina*) and randomly paired random points (mean ± SE) in reference and Hg sites along the South River, Virginia, USA, 2013–2014. The number of nests of each type is shown as n, results are from paired t-tests for all variables except percent water and distance to tributary which were tested using Wilcoxon signed-rank test. Mean paired difference (Diff.) is the percentage difference between nest sites and paired random points, and is relative to the nest site (i.e., nest sites in reference sites had 17.1% less canopy cover than at paired random points).

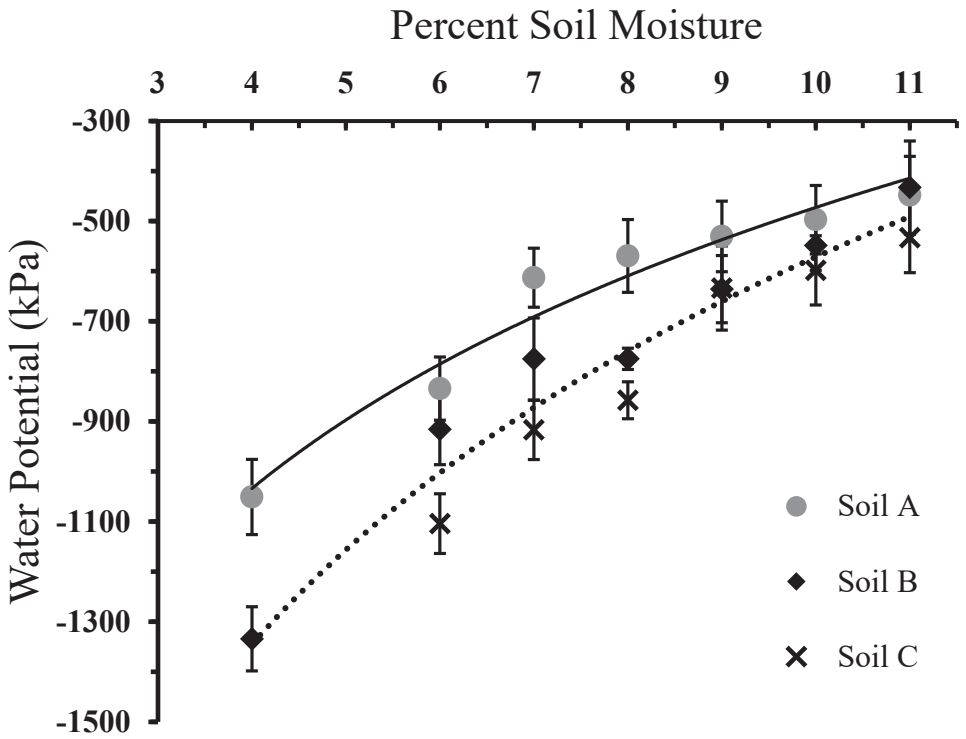
	REFERENCE SITES				HG SITES				REF-V-HG	
Variable	n	Nest mean	Diff.	*P*-value	n	Nest mean	Diff.	*P*-value	Nest *P*-value	Random *P*-value
CANOPY	17	0	-17.1	0.185	29	0.53 ± 0.53	-11.9	0.080	0.468	0.746
WATER	17	8.2 ± 0.6	-0.7	0.313	29	10.3 ± 0.4	0.2	0.639	**0.006**	0.164
BG	17	63.9 ± 6.9	11.4	0.136	29	51.1 ± 4.5	14.3	**0.040**	0.052	0.107
FORB	17	1.1 ± 0.5	-8.9	0.197	29	5.7 ± 2.1	-7.3	0.136	0.294	0.153
GRASS	17	3.1 ± 2.3	1.2	0.222	29	12.9 ± 3.7	-4.6	0.281	0.201	0.213
CROP	17	8.3 ± 1.4	1.8	0.296	29	4.0 ± 0.7	-1.3	0.282	**0.006**	**0.061**
WOOD	17	0	-1.6	> 0.15	29	0	-0.3	0.326	–	0.547
DETRITUS	17	0.14 ± 0.07	1.1	0.308	29	1.5 ± 0.6	-20.1	0.057	0.197	0.761
TREE	17	23.2 ± 6.5	5.0	0.492	29	21.2 ± 3.2	-0.4	0.471	0.341	0.107
SHRUB	17	0	0	–	29	0.3 ± 0.3	-0.5	0.326	0.278	0.468
ROCK	17	0	-7.7	–	29	0.03 ± 0.03	-17.5	0.345	0.447	0.523
RIVER	17	0.14 ± 0.07	0.04	0.580	29	1.4 ± 0.6	50.4	0.441	0.381	0.406
WATER	17	32.9 ± 4.7	.09	0.779	26	83.1 ± 27	7.7	0.217	0.489	0.287
FOREST	17	32.4 ± 4.7	0.2	0.131	26	35.3 ± 5.5	5.2	0.779	0.710	0.760
FIELD	14	19.8 ±5.2	-5.6	0.051	22	16.7 ± 2.1	-5.8	0.296	0.494	0.764

APPENDIX D. Agriculture treatment effects on offspring phenotype in the laboratory and field experiments assessed using a sequential Bonferroni (seqB) procedure; adjusted alpha levels (α_{adj}seqB) and an evaluation of the null hypothesis (*H_o seqB)* with the sequential Bonferroni procedure are provided for each response variable.

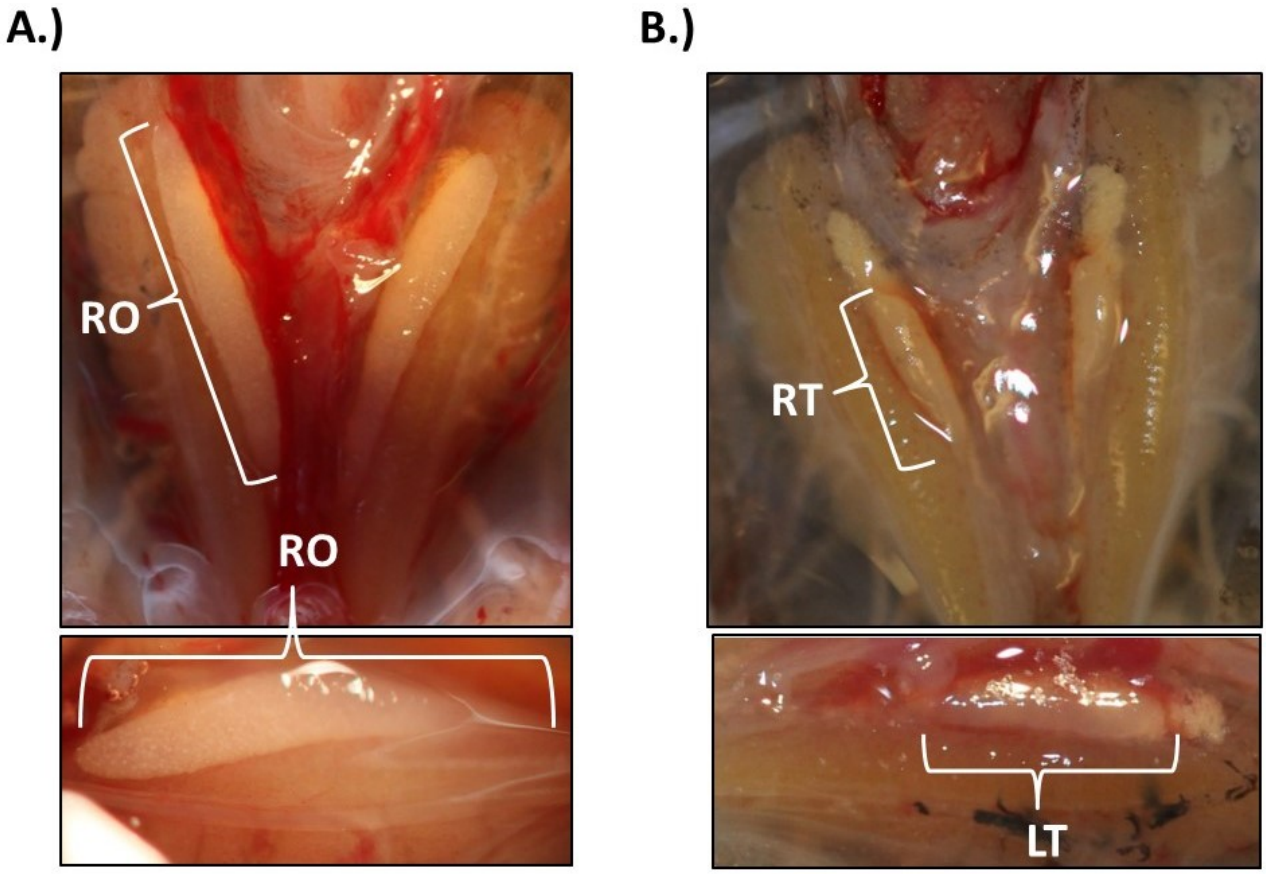
	Field Experiment			Lab Experiment		
Response	*p-value*	α_{adj}seqB	*H_o* seqB	*Agriculture*	α_{adj}seqB	*H_o* seqB
Incubation period (d)	$P < 0.001$	0.0063	**rejected**	$P < 0.001$	0.0063	**rejected**
Hatchling CL (mm)	$P < 0.001$	0.0071	**rejected**	$P < 0.001$	0.0071	**rejected**
Growth CL (%)	$P = 0.001$	0.0083	**rejected**	$P < 0.001$	0.0083	**rejected**
Mass loss (%)	$P = 0.030$	0.0100	retained	$P < 0.001$	0.0100	**rejected**
Hatchling mass (g)	$P = 0.073$	–	retained	$P = 0.029$	0.0167	retained
Latency time (s)	$P = 0.651$	–	retained	$P = 0.002$	0.0125	**rejected**
Mechanical righting (s)	$P = 0.041$	–	retained	$P = 0.525$	–	retained
Terrestrial velocity (cm s^1)	$P = 0.201$	–	retained	$P = 0.903$	–	retained

APPENDIX E. Photos of Open plots in violation of our contention rule for non-crop vegetation (i.e., containing greater than 10% cover by non-crop vegetation). Beginning in the month of August, the small forb seen in the photos above began to growing in our Open plots (A,B,C, see text for details) and a quadrant was used to visually estimate percent cover to manually remove to return to 10% cover (A,B). Wire mesh predator guards can be seen over nests.

APPENDIX F. Soil-water retention curves for the two distinct soil types at the study site. SSURGO characterizes the field as containing three types of fine sandy loams; Chavies (Soil A), Unisom (Soil B), and Buchanan (Soil C). However, we detected no difference in water holding capacity (water potential) among soils B and C, and consequently pooled data to construct one soil-water retention curve for both types (Retention II). A separate retention curve was fit for Soil A (Retention I). Retention I relates soil water moisture to water potential using the equation; $y = 612.59\ln(x) - 1883.1$, with $R^2 = 0.957$. Retention II relates soil water moisture to water potential using the equation; $y = 844.24\ln(x) - 2515.4$, with $R^2 = 0.939$.



APPENDIX G. Photos taken through a dissecting microscope of six week old snapping turtle gonads. The right ovaries (RO) of two female turtles are indicated in Panel A and the right testicle (RT) and the left testicle (LT) of two male turtles are indicated in Panel B. Male hatchlings have smooth, short, opaque, yellowish gonads and females have longer, translucent or white gonads with prominent ovarian follicles which give the gonad a white spotted appearance.



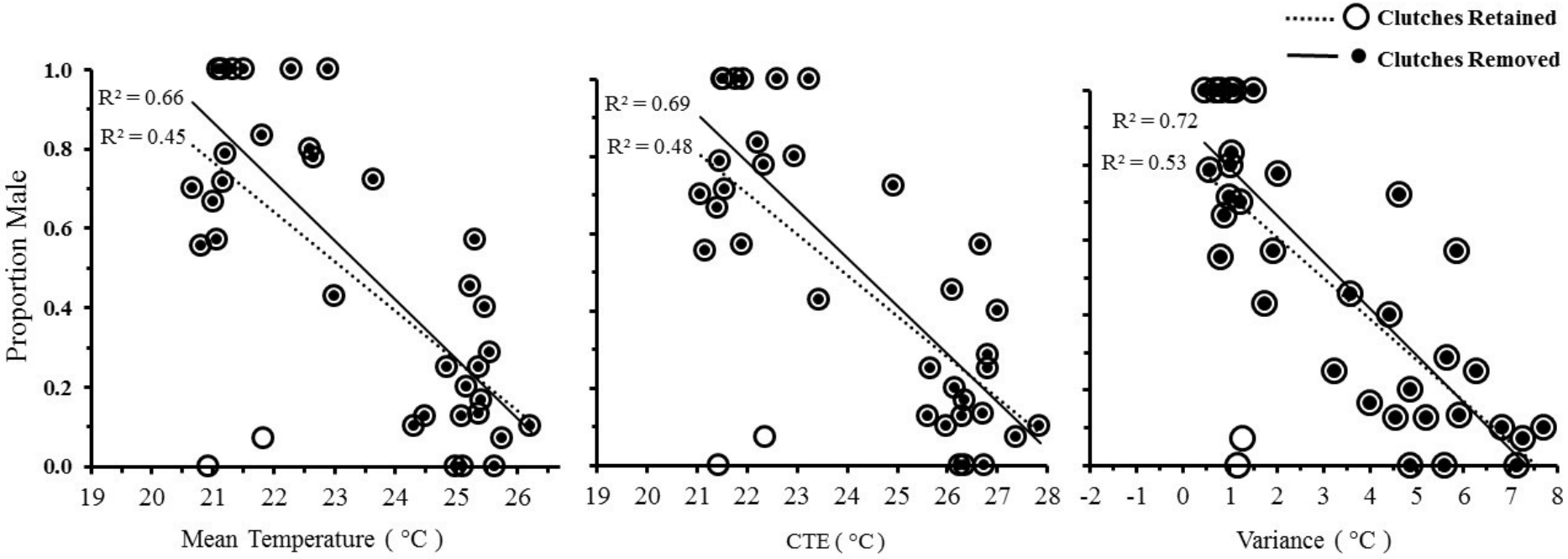
APPENDIX H. Summary temperature variables observed in the top and bottom layers of nests in the field, during the first and final third of incubation. All responses were calculated using daily averages and are shown in ℃. Samples sizes (layer/clutch/ treatment) are as follows: Top Layer: Hg/Open: 10, Ref/Open: 8, Hg/Shade: 10, Ref/Shade: 8, Bottom layer: Hg/Open: 9, Ref/Open: 8, Hg/Shade: 9, Ref/Shade: 7. Means ± SE

	Hg/Open		Ref/Open		Hg/Shade		Ref/Shade	
	Top	Bottom	Top	Bottom	Top	Bottom	Top	Bottom
First Third								
AVG. TEMP.	25.7 ± 0.3	25.2 ± 0.3	25.1 ± 0.3	24.7 ± 0.3	24.1 ± 0.2	23.7 ± 0.2	23.7 ± 0.3	23.3 ± 0.2
VARIANCE	10.0 ± 1.0	3.2 ± 0.3	8.9 ± 1.0	3.0 ± 0.3	4.9 ± 0.6	1.8 ± 0.2	4.8 ± 0.8	1.6 ± 0.3
RANGE	10.6 ± 1.7	7.3 ± 2.3	10.2 ± 2.4	5.3 ± 0.8	6.1 ± 0.4	3.5 ± 0.2	5.6 ± 0.6	3.1 ± 0.4
Final Third								
AVG. TEMP.	23.5 ± 0.1	23.3 ± 0.1	23.7 ± 0.2	23.5 ± 0.1	21.2 ± 0.2	21.0 ± 0.2	20.7 ± 0.3	20.6 ± 0.2
VARIANCE	6.3 ± 0.8	1.9 ± 0.1	7.1 ± 0.5	2.2 ± 0.1	2.0 ± 0.3	0.7 ± 0.1	2.2 ± 0.3	0.7 ± 0.1
RANGE	8.5 ± 1.9	6.1 ± 2.5	9.4 ± 2.3	4.5 ± 0.6	2.8 ± 0.2	2.2 ± 0.1	4.0 ± 0.3	2.2 ± 0.1

APPENDIX I. Summary temperature variables observed during the first third and final third of incubation for agriculture and mercury treatment groups in the laboratory experiment. All responses are calculated using daily averages and are shown in ℃. Samples sizes (layer/clutch/ treatment) are as follows: Samples sizes (clutch/treatment groups) are as follows; Hg/Open: 14, Ref/Open: 10, Hg/Shade: 8, Ref/Shade: 9. Means ± SE

	AGxHg Lab			
	Hg/Open	Ref/Open	Hg/Shade	Ref/Shade
First Third				
AVG. TEMP.	25.2 ± 0.2	25.0 ± 0.2	23.9 ± 0.1	24.1 ± 0.1
VARIANCE	4.6 ± 0.1	4.9 ± 0.1	2.0 ± 0.2	2.1 ± 0.1
RANGE	6.1 ± 0.1	6.2 ± 0.1	4.0 ± 0.1	4.0 ± 0.1
Final Third				
AVG. TEMP.	23.4 ± 0.1	23.4 ± 0.1	20.7 ± 0.1	20.8 ± 0.1
VARIANCE	2.9 ± 0.1	2.9 ± 0.1	0.8 ± 0.1	0.8 ± 0.1
RANGE	5.0 ± 0.1	5.0 ± 0.1	2.6 ± 0.1	2.6 ± 0.1

APPENDIX J. Relationships between sex ratios in the field incubation experiment and mean temperature, CTE, and thermal variance continuous measures of thermal conditions during the TSP. Two clutches from the reference/open treatment deviated from expected TSD patterns and we present R^2 values both with them retained in the dataset (dotted line and hollow circle) and with them removed from the dataset (solid line and circles).



APPENDIX K. Comparison of average egg width (A) and mass (B) between the two reference/open clutches that deviated from expected TSD patterns and all other clutches in the factorial experiments (N=42).