## EARLY-LIFE PHENOTYPIC AND FITNESS EFFECTS OF FLASH DROUGHT AND HEAT WAVES DURING EMBRYONIC DEVELOPMENT IN TWO FRESHWATER TURTLE SPECIES

By

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### A THESIS

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#### ABSTRACT

Climate change is causing rapid shifts in temperature and precipitation patterns worldwide, including more frequent extreme climate events (ECEs) such as flash droughts and heat waves. ECEs can have severe ecological impacts, as temperature and water availability play critical roles in the survival, growth, and functioning of living organisms. Environmental conditions experienced during embryonic development can have lasting phenotypic and fitness consequences, yet the effects of drought and heat waves at this key life stage are not well defined, particularly in vertebrates. Turtles (order Testudines) are particularly susceptible to early-life impacts of drought and heat waves. Embryos have little protection from unfavorable hydric and thermal conditions in the nest, and overall egg and hatchling mortality is high.

In my thesis, I investigated the effects of flash drought and heat waves during embryonic development on offspring traits and survival in two freshwater turtle species. I chose to focus on common snapping turtles (*Chelydra serpentina*) and painted turtles (*Chrysemys picta*) in order to compare taxa with contrasting life history traits. For my first chapter, I incubated *C. serpentina* eggs under varying hydric conditions to simulate flash drought at different embryonic stages and identified time-dependent effects on incubation time and hatchling size. I then conducted an experimental release in the field, which revealed that flash drought during late incubation decreased hatchling survival. For my second chapter, I examined the independent and interactive effects of flash drought and heat wave events during embryonic development in *C. picta*, finding effects on hatching phenology and body size. Moreover, survival increased with body size during dispersal from the nest. My findings shed light on the potential ecological consequences of extreme climate events in oviparous ground-nesting vertebrates.

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#### **INTRODUCTION**

Developing embryos are fundamentally shaped by their environment. Through developmental plasticity, the environmental variation experienced during embryogenesis, filtered through the genome, gives rise to phenotypic variation (West-Eberhard, 2003; Sommer, 2020). Reproductive strategies play a large part in moderating or amplifying the impacts of this environmental variation on the embryo. For instance, viviparity provides thermoregulatory benefits and protects offspring from extreme fluctuations in their environment. However, the vast majority of animals, including most vertebrates, are oviparous (Andrews & Mathies, 2000). Amniotic eggs provide embryos with nutrients and some protection from external stressors (Deeming & Ferguson, 1991), yet eggs are frequently exposed to fluctuating environmental conditions. Variable temperature, moisture, and gas exchange during embryonic development is central in shaping morphology, physiology, behavior, and survival of offspring across many oviparous taxa (Refsnider & Janzen, 2010).

In the Anthropocene, environmental changes are rapidly altering the conditions that organisms experience at all life stages. Mean global temperatures are rising, while precipitation patterns are becoming more variable (Zhang et al., 2024). Beyond these long-term shifts, climate change is also producing more frequent extreme climate events (ECEs), or periods of extreme deviation from historical climate conditions (Smith 2011; La Sorte et al. 2021). ECEs, such as drought, flooding, and heat waves, can have severe impacts on living systems (Cinto Mejía & Wetzel, 2023).

My thesis focuses on biological impacts of two types of ECEs: flash droughts and heat waves. Flash droughts are characterized by soil moisture depletion that develops rapidly, often within a period of weeks. They generally arise from a combination of low precipitation and high evapotranspiration (Yuan et al., 2023). Heat waves are broadly defined as periods of three or

more days of extreme heat, based on local climate records (Perkins & Alexander, 2013). Flash droughts and heat waves are both on the rise due to climate change (Christian et al., 2021; Perkins-Kirkpatrick & Gibson, 2017). Moreover, compound drought and heat wave events are increasing in frequency and severity worldwide (Tripathy et al., 2023). In studies across a wide range of taxa, including microbes, plants, and invertebrates, heat and drought produce severe and interactive ecological consequences (Bérard et al., 2015; Cohen et al., 2021; Harvey et al., 2023; Schepers et al., 2024). As climate change intensifies, there is a growing need for research on the connections between climate and living systems.

The physiology and life history of reptiles make them particularly vulnerable in the context of climate change (Hayden Bofill & Blom, 2024). Many reptile species are oviparous and lack parental care, leaving embryos more susceptible to changes in the nest environment (Noble et al., 2018). Nest-site choice may provide a favorable microclimate (Refsnider & Janzen, 2010), but conditions can change rapidly over the course of incubation (e.g. Cagle et al., 1993; Thompson et al., 2018). Without the ability to behaviorally thermoregulate (Telemeco et al. 2016), embryos are highly constrained in their capacity to respond to thermal stress. The sensitivity of reptile embryos to hydric stress varies with eggshell structure. While some reptiles lay rigid-shelled, highly calcified eggs that are resistant to water loss, other species lay flexible-shelled eggs that allow considerable movement of water between the egg and its surroundings (Packard et al., 1982). Environmental conditions, particularly water flux, temperature, and gas exchange, substantially affect phenotypic outcomes in reptiles (Ackerman, 1991; Belinsky et al., 2004; Noble et al., 2018; Refsnider & Janzen, 2010).

Soil moisture is a key factor in embryonic development for ground-nesting oviparous reptiles. As described previously, many species of lizards, snakes, turtles, and tuatara lay flexible-shelled eggs, in which the calcareous layer is either minimal, absent, or highly porous

(Packard et al., 1982). Flexible-shelled eggs can readily absorb or lose water based on soil water potential, impacting the water resources available to the embryo (Feder et al., 1982). Previous studies have investigated the phenotypic effects of soil moisture during egg incubation in reptiles using a range of methods. These include incubating eggs in the laboratory using constant or fluctuating water potentials in a variety of substrates (e.g., Packard et al., 1987; Robbins & Warner, 2010), as well as measuring or manipulating soil moisture in natural nests (e.g., Thompson et al., 1996; Bodensteiner et al., 2015; Rivas et al., 2018). Together, these studies provide insight into the relationship between the hydric environment and offspring phenotypes. In the laboratory, constant dry conditions limit embryonic metabolism and growth, decreasing egg mass and hatchling size in lizards (Marco et al., 2004), snakes (Brown & Shine, 2004; 2005), and turtles (Morris et al., 1983; Packard et al., 1991; Janzen et al. 1995), but not tuatara (Thomson et al., 1990). Reptile embryos developing under dry conditions also tend to have shorter incubation times and convert less of their yolk to body mass, both in the laboratory (Packard et al., 1985*a*; Janzen et al., 1990; Booth, 2002) and in natural nests (Christian et al., 1991), even with a small decrease in soil moisture (Tucker et al., 1998). In some cases, hatching success also declines when water is scarce (Packard et al., 1991; Tucker & Paukstis, 2000; but see Janzen et al., 1995), including in natural nests (Thomson et al., 1996). Along with body size, body condition also varies with the developmental hydric environment. For instance, dry incubation conditions decrease muscular strength in hatchling keelback snakes (Brown & Shine, 2004). Turtle hatchlings incubated in constant dry conditions have lower body water content and desiccation tolerance (Finkler 1999), and reduced movement speed (Miller et al. 1987). There is some evidence that the phenotypic effects of hydric stress on turtles are time-dependent, being most substantial during the last two-thirds of incubation (Gutzke & Packard, 1986; Delmas et al., 2008), but this has been disputed (Packard & Packard, 1988).

Many researchers have hypothesized that embryonic water deprivation decreases posthatching fitness in reptiles. As described above, low soil moisture negatively affects traits that are likely associated with fitness, such as strength, speed, and body size (e.g., Miller et al., 1987; Booth, 2002; Brown & Shine, 2004). Survival of juvenile reptiles often increases with body size, supporting the paradigm that "bigger is better" (Ferguson & Fox, 1984; Packard, 1988; Janzen, 1993a; Janzen et al., 2000b; Rollinson & Rowe, 2015; Porter et al., 2024), although exceptions exist and selection on body size may be context-dependent (Congdon et al., 1999; Kolbe & Janzen, 2001; Paterson et al., 2014). If "bigger is usually better," then hatchlings incubated in dry conditions will generally have a lower probability of survival. However, few studies have tested this hypothesis. Two field experiments on freshwater turtles reported no relationship between soil water potential and post-hatching fitness (Janzen, 1993*a*; Filoramo & Janzen, 2002). However, the first study provided hatchlings with water prior to the experimental release (Packard, 1999), and the second study decreased water potential by only a small amount, which may not reflect the more extreme hydric conditions that hatchlings would experience during a flash drought event. The relationship between flash drought and post-hatching survival in reptiles remains unknown.

Thermal conditions also strongly influence embryonic development in reptiles. As with substrate water potential, methods vary for studying the effects of incubation temperature in reptiles. While early laboratory experiments were limited to using constant temperatures throughout incubation, technological advances have allowed researchers to implement diel cycles, as well as more fine-scale fluctuations, which more accurately replicate natural conditions (Carter & Janzen, 2021). Nonetheless, the vast majority of studies to date have used constant temperatures (While et al., 2018). These studies have revealed a wide range of temperature-dependent phenotypic effects. Notably, many reptile species have temperature-

dependent sex determination, meaning that the thermal environment during embryogenesis determines offspring sex ratios (Bull, 1980; Valenzuela & Lance, 2004). Moreover, embryonic development is generally accelerated at higher temperatures, resulting in shorter incubation periods (Deeming & Ferguson, 1989; Warner et al., 2020). Hatching success is typically highest at intermediate temperatures, and declines when incubation temperatures are above or below the optimum range (Gutzke & Packard, 1987; Lewis-Winokur et al. 1995). Temperature can also influence hatchling size in freshwater turtles (Brooks et al., 1991; Janzen, 1993a), sea turtles (Booth et al., 2004), and lizards (Downes & Shine, 1999; Andrews et al., 2000). In some cases, temperature affects offspring behavior and performance, such as striking behavior in snakes (Burger, 1990), predator avoidance and microhabitat use in lizards (Downes & Shine, 1999; de Jong et al., 2022), and righting response and movement speed in turtles (Booth et al., 2004; Fisher et al., 2014; Mueller et al., 2019). These phenotypic responses to temperature suggest potential fitness differences, and indeed, laboratory and field experiments have linked incubation temperature and post-hatching survival in lizards (Andrews et al., 2000; Hare et al., 2004; Steele & Warner, 2020), tortoises (Lewis-Winokur & Winokur, 1995), and freshwater turtles (Bobyn & Brooks, 1994; Janzen, 1995).

While the effects of environmental factors during embryonic development have been widely studied across reptile taxa, much remains unknown. First, the impact of fluctuating thermal and hydric conditions is relatively understudied (Bowden & Paitz, 2018). The majority of studies have incubated eggs under constant water potential, while in natural nests, the hydric environment can vary considerably (e.g., Packard et al., 1985*b*; Cagle et al., 1993; Bodensteiner et al., 2015), particularly as ECEs intensify under climate change. Studies on the effects of hydric and thermal fluctuation during embryonic development in reptiles are limited. For instance, a recent meta-analysis was only able to include 22 relevant studies on temperature

fluctuation in reptiles (Raynal et al., 2022). There is some evidence that the timing of heat stress is important in shaping offspring phenotype (e.g., Breitenbach et al., 2023), but more research is needed. Furthermore, the effects of hydric and thermal stress, as well as their interaction, on post-hatching survival are not well understood. Additional research is needed to place the effects of these stressors in an ecologically relevant context.

To investigate the phenotypic and fitness effects of flash drought and heat wave, I studied two widely-distributed freshwater turtles: the common snapping turtle, *Chelydra serpentina*, and the painted turtle, *Chrysemys picta*. My study site was located on the Mississippi River in Carroll County, Illinois, where both species are prevalent. The central United States is considered a global flash drought hotspot, with an increasing risk of flash droughts (Christian et al., 2021), most of which occur in June and July (Christian et al., 2019). Heat waves are also increasing in this region during the summer months (Lyon & Barnston, 2017). Notably, the peak months for drought and heat waves coincide with the incubation periods for both C. serpentina and C. picta at this study site (St. Juliana & Janzen, 2007; Schwanz & Janzen, 2008). Both species lay flexible-shelled eggs that undergo water exchange in response to hydric conditions in the nest (Feder et al., 1982; Packard et al., 1981). C. serpentina females lay large clutches, sometimes over 50 eggs (Kolbe & Janzen, 2001), at a depth of around 175 mm (Telemeco et al., 2016). The magnitude of hydric variation experienced by eggs is inversely related to depth (Packard et al., 1985b). By contrast, C. picta clutches average about 10 eggs, and nests are around 80-95 mm deep (Morjan, 2003), meaning that eggs are likely exposed to greater hydric and thermal fluctuation over the course of incubation. While C. serpentina hatchlings emerge from the nest and migrate to the water soon after hatching (Kolbe & Janzen, 2001), C. picta hatchlings in the study population overwinter in the nest and emerge the following spring (Murphy et al. 2020). Research is needed to determine whether the effects of the incubation environment persist after

brumation.

My thesis investigates the effects of flash drought and heat waves and their interaction during embryonic development on phenotype and survival in C. serpentina and C. picta. In my first chapter, using C. serpentina as a model, I examine the effects of simulated flash drought events at different times during embryonic development on offspring phenotype and posthatching survival. Within this framework, I evaluate the hypothesis that substrate moisture is most important during the "sensitive period," i.e., the last two-thirds of development (Gutzke & Packard, 1986). I predicted that flash drought during mid- to late embryonic development would reduce egg mass, incubation time, and hatchling body size, and decrease hatching success and survival, while flash drought during early incubation would have minimal or no effect on posthatching traits. To test these predictions, I exposed C. serpentina eggs to simulated flash drought events at different times during incubation in a controlled laboratory experiment, then conducted an experimental release in the field. In accordance with my predictions, egg mass, incubation time, and most metrics of body size decreased with mid- to late drought, and late drought substantially reduced hatchling survival during migration from the nest. In addition to supporting the "sensitive period" hypothesis, my findings suggest that acute hydric stress can influence not only phenotypic traits, but also a key aspect of fitness during a crucial early-life stage.

In my second chapter, focusing on *C. picta*, I assess the interactive effects of flash droughts and heat waves during incubation on offspring phenotype and survival. As *C. picta* hatchlings at my study site spend their first winter in the nest, I also investigate whether environmental effects during embryonic development persist through the overwintering stage. Based on the literature and the results of my first chapter, I predicted that flash drought and heat waves would negatively affect egg mass, incubation time, pre- and post-brumation body size, and pre- and post-hatching survival. To test these predictions, I conducted a fully-factorial

experiment, dividing *C. picta* eggs into 8 treatments representing different combinations of hydric and thermal stress. After overwintering the hatchlings in the laboratory, I again returned to the field to implement an experimental release. As predicted, I detected an interactive effect between flash drought and heat waves on egg mass over time, as well as several phenotypic effects related to flash drought, heat waves, or both. Finally, survival during the experimental release increased with post-brumation body size, supporting the "bigger is usually better" hypothesis (Rollinson & Rowe, 2015; Porter et al., 2024). Together, the results of these two studies suggest that ECEs, such as flash drought and heat waves, during embryonic development can strongly influence offspring traits, that these effects can persist beyond hatching, and that post-hatching survival may be directly or indirectly affected by hydric and thermal stress.

# CHAPTER 1: Timing of flash drought during embryonic development affects body size and early-life survival in the common snapping turtle, *Chelydra serpentina* ABSTRACT

Environmental conditions during embryonic development substantially impact offspring phenotype and fitness. In ground-nesting reptiles, developing embryos are sensitive to hydric conditions in the nest. However, the effects of hydric stress at different times during egg incubation, particularly on offspring survival, are not fully understood. In this study, we exposed common snapping turtle (*Chelydra serpentina*) eggs to simulated flash drought at different stages of embryonic development and examined the effects on egg and hatchling traits. We then conducted an experimental release to assess hatchling survival during migration from the nest. Flash drought during mid-to-late development decreased egg mass, incubation time, and hatchling body size, while flash drought during late development substantially decreased hatchling survival during the experimental release. Our results demonstrate the formative effects of hydric stress, particularly during late development, on offspring phenotype and early-life survival in a ground-nesting reptile.

#### **1.1 | INTRODUCTION**

Soil moisture is a key limiting factor in the survival, growth, and function of a wide range of organisms. The biotic impacts of soil water availability have been widely documented in many taxa, including plants, microbes, and invertebrates (Yan et al., 2018; Pugnaire et al., 2019; Xu et al., 2019; Flórián et al., 2019; Evans et al., 2022), yet less is known about the effects of soil moisture on vertebrates. Ground-nesting oviparous vertebrates frequently experience variable hydric conditions in the nest (e.g., Packard et al., 1985; Rivas et al., 2018). For instance, nest flooding and desiccation often lead to egg mortality in birds and non-avian reptiles (e.g., Cassill & Watkins, 2022; Elas et al., 2023). Embryos that survive hydric stress may respond through

developmental plasticity, leading to phenotypic variation that may impact future survival and fitness (West-Eberhard, 2003; Sommer, 2020).

Climate change is driving an increase in hydric extremes, including unprecedented drought events worldwide (Satoh et al., 2022). Drought is broadly defined as a period of water deficit, typically caused by a combination of low precipitation and high evapotranspiration (Ault, 2020). While droughts can develop over months or years, rapid-onset 'flash droughts,' which occur on a sub-seasonal time scale, are increasingly prevalent (Yuan et al., 2023). Flash droughts can rapidly deplete soil moisture, suggesting that these events have the potential to impact the course of embryonic development in ground-nesting vertebrates.

Turtles (order Testudines) are oviparous and typically nest in soil, making them an excellent vertebrate model system for studying the effects of soil moisture during egg incubation. Many species lay flexible-shelled eggs, which are highly sensitive to changing soil moisture due to a porous mineral layer (Packard et al., 1982; Packard, 1991; Ackerman & Lott, 2004). Even so, the potential impacts of flash drought on offspring phenotype and fitness in turtles remain unknown. Turtles are among the most threatened vertebrate taxa globally, with over half of all species listed as threatened and over one-third listed as endangered under IUCN criteria (Stanford et al., 2020). As flash drought and other extreme climate events intensify under climate change, research is needed to understand how drought-induced changes in soil moisture affect turtle embryonic development and survival.

Previous field and laboratory studies have identified phenotypic effects of substrate moisture during egg incubation in reptiles, although not necessarily in the context of climate change (Morris et al., 1983; Janzen et al., 1995; Packard, 1991; 1999). Flexible-shelled eggs generally absorb water in wetter conditions and lose water in drier conditions, impacting the reserve of water available to the developing embryo (Packard, 1999). Dry incubation conditions

limit embryonic metabolism and growth, thereby reducing egg mass, hatching success, incubation time, and hatchling size (Packard et al., 1981; Morris et al., 1983; Tucker & Paukstis, 2000). Additionally, turtle hatchlings incubated in constant dry conditions have lower body water content and desiccation tolerance (Finkler, 1999), reduced movement speed (Miller et al., 1987; Miller, 1993), and may travel shorter overland distances each day (Finkler et al., 2000).

Soil moisture during turtle embryonic development has been suggested to affect posthatching fitness, but few studies have tested this hypothesis. Turtle hatchling survival often varies positively with body size (Janzen, 1993*a*; Janzen et al., 2000*a, b*; Tucker, 2000; but see Congdon et al., 1999; Kolbe and Janzen, 2001; Filoramo and Janzen, 2002). If "bigger is usually better" (Rollinson & Rowe, 2015), then the smaller body size imparted by constant dry incubation conditions should incur a fitness cost. Hatchlings incubated in dry laboratory conditions are slower and more vulnerable to dehydration, traits which may also be associated with reduced fitness (Miller et al., 1987; Finkler, 1999).

The timing of hydric stress may be an important factor in turtle embryonic development. The hydric environment in natural nests is not constant; soil moisture varies over time in response to rainfall and evaporation (e.g., Packard et al., 1985). In two previous studies, hatchling phenotypes were driven primarily by the hydric environment during the last two-thirds of incubation, possibly due to "compensatory water exchange," or increased water absorption when eggs transitioned from dry to wet conditions during mid-development (Gutzke & Packard, 1986; Delmas et al., 2008; but see Packard & Packard, 1988). As flash droughts become more frequent and intense under climate change, it becomes critical to investigate how short-term substantive reductions in soil moisture impact neonatal turtle development, hatchling phenotype, and early-life fitness.

In this study, we use a combination of laboratory and field methods to determine how

flash drought during embryonic development affects early-life phenotypes and survival in the common snapping turtle, *Chelydra serpentina*. This species lays large clutches of flexible-shelled eggs. For this study, we focused on a *C. serpentina* population that nests in sand prairie habitat along the Mississippi River. The central United States, including our study site, is considered a "global hotspot" for flash drought (Christian et al., 2021). Most flash drought events in this region occur between June and August (Christian et al., 2019), coinciding with the nesting and incubation phenology of *C. serpentina* (St. Juliana & Janzen, 2007). In this system, offspring experience strong selection during early life stages, including high predation during the hatchling dispersal stage as they emerge from the nest and move toward water. Thus, potential fitness differences imposed by the nest environment should be observable during this life stage (Janzen, 1993*a*).

We predicted that experimental flash drought would negatively impact *C. serpentina* embryo growth and hatchling survival. Specifically, we expected that flash drought imposed at any time during embryonic development would negatively affect water flux between eggs and substrate, causing eggs to lose mass. We also predicted that any mass lost during early incubation would be regained by the time of hatching due to compensatory water exchange, while mass lost during mid-to-late incubation would not be regained. Finally, we expected that flash drought during mid-to-late incubation would reduce hatching success, incubation time, body size, and hatchling survival, while increasing time to disperse and mass lost during dispersal from the nest to the water.

#### 1.2 | METHODS

#### Egg collection and incubation

Between 2-6 June 2022, we collected 20 eggs from each of 10 *C. serpentina* nests at Thomson Sand Prairie in Carroll County, IL within a few hours of deposition (N = 200 eggs). We used a

permanent marker to label each egg with a unique code identifying the individual and clutch of origin. We then placed eggs in Styrofoam containers and covered them with a layer of moist sand. On 6 June, we transported all eggs to our laboratory. We weighed eggs to the nearest 0.01 g and randomly assigned them to one of five treatment groups, beginning experimental incubation on 7 June. The treatment groups were exposed to a simulated flash drought event during early, middle, late, or mid + late incubation, while the control group experienced no drought (Table 1). Each clutch was equally represented in all treatments. We placed eggs in covered plastic boxes filled with vermiculite substrate, arrayed in 4 x 5 matrices, then placed the boxes in a Percival incubator set to a daily cycle of 12 h 28 °C / 12 h 31 °C, representing a typical diel range in the center of nests at this site (Kolbe & Janzen, 2001; Telemeco et al., 2016). This temperature regime produces 100% female offspring for this population of *C. serpentina* (Janzen, 2008; Krueger & Janzen, 2022), thereby eliminating gonadal sex as a confounding treatment factor.

Under these experimental conditions, we estimated incubation time to be approximately nine weeks, which we subdivided into three 21-day segments. We maintained the control group in moist vermiculite (water potential = -150 kPa) throughout the incubation period, representing a typical non-drought hydric environment (Packard et al., 1985; but see Ackerman & Lott, 2004). To simulate drought conditions, we placed the other four groups in drier vermiculite (water potential = -850 kPa) for 3-6 weeks during early, middle, late, or mid + late incubation (Table 1).

Each week, we individually weighed eggs to the nearest 0.01 g, rehydrated the vermiculite in boxes to maintain target moisture levels, and rotated boxes within the incubator to minimize thermal gradient effects. After the first hatchlings were observed on 2 August 2022, we monitored boxes daily and recorded pipping and hatching dates for each egg. We weighed hatchlings to the nearest 0.01 g approximately 24 h after hatching, and measured carapace and

plastron length and width to the nearest 0.01 mm. Hatchlings were housed individually in small plastic tubs with a piece of damp paper towel.

Once all hatchlings emerged on 13 August 2022, we divided them into four release groups, such that treatment was equally represented in each group. As *C. serpentina* hatchlings can be identified by their unique plastron markings (Janzen et al., 2000*b*), we photographed the plastron of each hatchling for later identification. Each release group was further identified with a small notch on a marginal scute. We then group-housed hatchlings in four plastic boxes lined with damp paper towels, with each treatment represented equally in each group, and transported them to the original collection site on 15 August 2022.

#### Release experiment

We conducted the release experiment at Thomson Sand Prairie, where eggs were initially collected. We constructed an 80 m drift fence parallel to the Mississippi River using 0.3 m tall aluminum flashing, with 17 pitfall traps placed along the fence at 5 m intervals. To install the pitfall traps, we buried 4.5 L plastic jars with their openings at ground level. We created four release points by digging shallow artificial nest cavities centered at the midpoint of the fence. The release points were 10 m apart and 30 m upslope from the fence, in a sandy patch typical of *C. serpentina* nests at this site (Kolbe & Janzen, 2002; pers. obs.). We released all hatchlings at midday on 16 August 2022, a typical date and time of day for hatchling emergence in this population (Congdon et al., 1999; Delaney & Janzen, 2019). Subsequently, we monitored the drift fence three times a day at 7:00 AM, 1:00 PM, and 7:00 PM and collected all hatchlings captured in the pitfall traps. We identified, weighed to the nearest 0.01 g, and released each recaptured hatchling at the edge of the Mississippi River. After two consecutive days with no hatchlings recovered, we concluded the experiment on 22 August 2022.

#### Statistical analyses

All statistical analyses were conducted using R version 4.4.1 (R Core Team, 2024). Linear and generalized linear mixed models were run using the lme4 package (Bates et al., 2015) unless otherwise noted. To assess changes in egg mass over time, we conducted an analysis of covariance with repeated measures. For this model, treatment and week were fixed effects, initial egg mass was the covariate, and clutch was a random effect. We modeled hatching success as a function of treatment using a logistic regression model, with clutch as a random effect and initial egg mass as a covariate. To model incubation time, we used a linear mixed effects model fit by restricted maximum likelihood (REML), denoting treatment as a fixed effect and clutch as a random effect. Similarly, we modeled hatchling mass, and body size metrics (carapace length, carapace width, plastron length, plastron width) using linear mixed models by REML. In each case, treatment was the predictor variable, initial egg mass was a covariate, and clutch was a random effect.

To analyze hatchling survival during migration from the artificial nests, we first used logistic regression to model recapture as a function of treatment, with hatchling mass as a covariate and clutch as a random effect. We then analyzed survival separately as a function of early, middle, and late drought treatments, again using logistic regression with the covariate and random effect described above. We modeled dispersal time as a function of treatment, with hatchling mass as a covariate, using a linear regression. Dispersal time was not normally distributed, so we used the R package DHARMa (Hartig, 2022) to check that our model met the assumptions of a linear regression. Finally, we modeled mass lost during dispersal as a function of treatment using a linear mixed model fit by REML, with hatchling mass as a covariate and clutch as a random effect. Figures were created in ggplot2 (Wickham, 2016).

#### 1.3 | RESULTS

Egg mass was interactively affected by treatment and time (Fig. 1; Table 2). Overall, eggs in non-drought conditions increased in mass, while eggs in drought conditions decreased in mass. However, eggs in the early drought treatment were able to recover and reach the same average mass as the control group by the end of incubation. By contrast, eggs in the middle, late, and mid + late drought treatments ended incubation at lower mass than the control group, with the mid + late drought treatment being the lowest. At the end of incubation, eggs in the mid + late group weighed 3.93 g (or 23.7%) less on average than the control group eggs.

Hatching success was uniformly high across treatment groups, with an overall 93.5% hatch rate (N = 187). We found no impact of treatment ( $F_{4,194} = 1.354$ , P = 0.251) or initial egg mass ( $F_{1,194} = 0.066$ , P = 0.797) on hatching success. Of the 13 eggs that failed to hatch, two developed to late-term embryos and 11 did not develop. Dissection confirmed that both embryos were gonadal females, consistent with the expected sex under the thermal regime used in this experiment.

Incubation time ranged between 56 to 67 days and varied as a function of treatment (Fig. 2;  $F_{4,182} = 10.85$ , P < 0.001). Eggs in the late and mid + late drought treatments took less time to hatch on average. Eggs in the late drought group hatched 0.9 d earlier than eggs in the control group, while eggs in the mid + late drought group hatched 2.3 d earlier.

We analyzed five metrics of hatchling body size: hatchling mass (g), carapace length, carapace width, plastron length, and plastron width (mm). Each variable was positively correlated with initial egg mass; eggs with greater mass at the start of incubation produced larger hatchlings, regardless of treatment (Table 3). Drought treatment significantly affected all hatchling size metrics except plastron width (Table 3). Hatchling mass was not affected by early drought; however, drought during middle, late, and mid + late incubation substantially limited

hatchling mass (Fig. 3). Hatchlings in the mid + late group had the lowest average mass, at 1.5 g (13.0%) lighter than the control group. Similarly, drought during middle, late, and mid + late development reduced carapace length, carapace width, and plastron length.

We recaptured 121 hatchlings out of 187 released (64.7%). Hatchling survival was adversely affected by drought exposure during the last three weeks of incubation (Z = -2.009, P = 0.045; Fig. 4); recapture rate was reduced by 15.8% compared to hatchlings that did not experience late drought. However, drought in the first 6 weeks of incubation had no effect on survival. Hatchling mass had no substantive effect on survival (Z = -0.595, P = 0.552), though there was a non-significant negative trend (Fig. 4). Hatchling survival was similarly unaffected by carapace and plastron length and width (Table S4).

Hatchlings took 7 h – 4 d to reach the drift fence (straight-line distance = 30 m). Average dispersal time was  $49 \pm 24$  h. Drought treatment influenced dispersal time (F<sub>4,115</sub> = 4.302, P = 0.003). Hatchlings that experienced drought during mid-development took the longest to reach the fence, averaging 63 ± 26 h. Dispersal time was unrelated to hatchling mass (F<sub>1,115</sub> = 0.022, P = 0.881). Recaptured hatchlings lost an average of 9.7% (1.06 ± 0.47 g) of their total body mass during dispersal. Drought treatment significantly affected mass lost (F<sub>4,115</sub> = 3.063, P = 0.019), with hatchlings that experienced mid + late drought losing more mass than the control group relative to body size.

#### 1.4 | DISCUSSION

Flash droughts are increasing in frequency and intensity under climate change (Yuan et al., 2023), and their ecological impacts, particularly on vertebrates, are not well understood. In this study, we investigated the effects of flash drought at different stages of embryonic development in *Chelydra serpentina*, a widely distributed ground-nesting turtle. We found that flash drought during the last two-thirds of embryonic development reduced egg mass, time to hatch, and four

of five body size metrics. During hatchling dispersal from the nest, mid-incubation drought increased dispersal time, mid + late drought increased body mass loss, and late drought reduced survival. These results, which we explore in more detail below, indicate that flash drought, particularly during the final trimester of development, produces substantial and ecologically relevant effects on early-life offspring phenotypes and survival in this species.

Our finding that eggs decreased in size under drought conditions aligns with previous literature for this species (e.g., Janzen et al., 1990) and more broadly for other turtles and squamates with flexible-shelled eggs (Vleck, 1991, Packard, 1999, Delmas et al., 2008). We also found that drought during early incubation did not affect egg mass in the long term. This result supports the findings of some previous studies (Gutzke & Packard, 1986; Delmas et al., 2008; but see Packard & Packard, 1986). Eggs in the early drought group experienced an initial reduction in water potential, but when placed in a wet environment, they absorbed substrate water more rapidly than their counterparts in the control group, likely due to the larger difference in water potential relative to the substrate. This compensatory water exchange allowed them to replace mass lost during the first trimester over the ensuing six weeks (sensu Gutzke & Packard, 1986).

Eggs that experienced drought during late and mid + late embryonic development had a slightly shorter incubation period. This result aligns with previous studies in turtles (Gutzke & Packard, 1986, Packard & Packard, 1988), but the mechanism of this phenomenon is not fully understood. Packard (1999) suggested that water-limited embryos are constrained in their growth, leading them to hatch earlier, while embryos with sufficient water reserves will continue to incubate and grow for a longer period of time. Regardless, the biological impact might be minimal given that treatment differences amounted to about 2 d at most (roughly 3% of overall incubation length).

In general, soil moisture is positively correlated with hatchling size in reptiles with flexible-shelled eggs, because greater water availability increases embryo metabolism and growth (Morris et al., 1983; Packard, 1999). We found that this relationship is mediated by timing of hydric stress in *C. serpentina*. Hatchlings from eggs that experienced drought during mid-to-late embryonic development were smaller than those that experienced early or no drought. We attribute this result to the stages of embryonic development. The second and third trimesters of vertebrate embryogeny coincide with the organogenesis and growth phases of development, respectively (Yntema, 1968). The differentiation of tissue and organ systems, and the subsequent considerable increase in body size, require substantial water intake for protein and calcium anabolism that may not be available in a drought environment (e.g., Janzen et al., 1990). This fundamental biochemistry and physiology may explain why drought during mid-to-late embryonic development produced smaller thatchlings.

In this study, flash drought during late embryonic development reduced hatchling survival during migration from the nest, whereas flash drought during early and middevelopment did not. Our findings differ from two previous field studies that did not detect a relationship between constant embryonic water potential and post-hatching offspring fitness (Janzen, 1993*a*; Filoramo & Janzen, 2002). However, hatchlings in the first study had access to water before the experimental release, while the "dry" treatment in the other study received more water than "wet" treatments in previous studies in the same species (Tucker & Paukstis, 1999), suggesting that hatchlings may not have been experiencing significant water stress. Past studies have concluded that larger neonatal turtles have a survival advantage during dispersal (Janzen, 1993*a*; Janzen et al., 2000*a*, *b*; Janzen et al., 2007; Paitz et al., 2007; Tucker et al., 2008; but see Congdon et al., 1999; Kolbe & Janzen, 2001; Filoramo & Janzen, as hatchling size had no detectable

effect on survival during the release experiment. This outcome suggests that drought might affect offspring survival through another mechanism not considered in this study. One possibility could be differential body water content. Hatchlings migrating from the nest to water rely on the water reserve contained in their body tissues and yolk sac; those with lower body water content are more vulnerable to the negative physiological effects of dehydration (Finkler, 1999). Another possibility could be changes to nitrogenous waste concentrations in the egg during embryonic development. Concentrations of urea are higher in *C. serpentina* eggs incubated in drier conditions (Packard et al., 1984), and hatchlings that experience hydric stress during incubation have higher levels of urea in blood plasma. Even at low levels, urea may inhibit metabolism, though it does not appear to affect hatchling size in this species (Packard & Packard, 1989). Further research is needed to uncover the potential physiological mechanisms linking hydric stress and hatchling survival.

One limitation of our study was the drift fence used in the release experiment. Because the fence was constructed in a straight line, rather than fully enclosing the four release points, a hatchling could have traveled all the way around the fence or moved in the opposite direction and not been recaptured. In past release studies at this site, no hatchlings were recaptured in pits away from the direction of the river when enclosed drift fences were used (e.g., Warner and Mitchell, 2013), or near the terminal pits when linear (e.g., Delaney & Janzen, 2019) or curvilinear (e.g., Paitz et al., 2007) drift fences were used. However, in our study, three out of 121 hatchlings recaptured were found in the terminal pits. We cannot exclude the possibility that other hatchlings traveled farther and bypassed the fence, but this is unlikely to have substantially affected our results, as the other 97.5% of hatchlings recaptured were found in non-terminal pits. Nonetheless, we recommend that future studies employ drift fences >80m to avoid this concern.

Future research could investigate the interactive effects of drought and other abiotic

stressors, such as heat waves, on phenotype and fitness. Research is also needed to determine whether other turtle species, including those with rigid-shelled eggs, respond similarly to the timing of hydric stress compared to *C. serpentina*. Furthermore, studies of natural nests should be conducted in the field, to determine whether the results of our study are relevant in the more variable environmental conditions that occur in the field (Packard & Packard, 2000). Finally, the physiological mechanisms underpinning the observed phenotypic and survival effects of flash drought during embryonic development should be explored. Future studies could examine, for example, which genes are being expressed differently under hydric stress, with a potential focus on those involved in muscle and bone growth.

Turtles are a globally imperiled taxon, and climate change is contributing to their decline (Stanford et al., 2020). Among a litany of detrimental climate change effects, flash droughts are increasing in prevalence and intensity (Christian et al., 2021; Yuan et al., 2023), leading to higher levels of hydric stress for developing turtle embryos. Our findings reveal for the first time that flash drought, particularly during mid-to-late embryonic development, can produce significant deleterious effects, including reduced body size and increased juvenile mortality in the common snapping turtle. Moving forward, future research should continue to investigate how flash drought effects may be mediated by other climate change impacts to influence embryo development and hatchling fitness in this and other species. Conservation and management plans should also consider the ecological impacts of flash drought on threatened turtle populations.

# TABLES

# Table 1.1

Summary of the five treatment groups used in this experiment.

Treatment	T1: Day 1-21	T2: Day 22-42	T3: Day 43-63
Control	—		—
Early	Drought		—
Middle	_	Drought	—
Late	—	—	Drought
Mid + Late	—	Drought	Drought

# Table 1.2

Source of variation	df	Mean square	F-ratio	P-value
Treatment	4	69.8	171.83	<2e-16*
Week	8	21.7	53.50	<2e-16*
Initial egg mass	1	2806.4	6906.36	<2e-16*
Treatment $\times$ week	32	18.7	46.11	<2e-16*

Repeated measures analysis of covariance of change in egg mass during incubation.

# Table 1.3

F-ratios and levels of significance (in parentheses) for analyses of variance on five metrics of hatchling body size.

	Source of variation			
Variable	Treatment	Initial egg mass		
Mass	65.06 (<2e-16)*	497.41 (<2e-16)*		
Carapace length	10.46 (1.18e-07)*	75.30 (2.17e-15)*		
Carapace width	15.87 (3.58e-11)*	70.91 (1.07e-14)*		
Plastron length	2.685 (0.0329)*	70.587 (1.2e-14)*		
Plastron width	0.92 (0.453)	21.97 (5.4e-06)*		

### **FIGURES**



# Figure 1.1

Least squares means of egg mass and 95% confidence intervals over time. Dashed lines indicate timing of treatment implementation. Egg mass increased in normal conditions and decreased in drought conditions. Eggs recovered from drought during early incubation, but not during middle, late, or mid + late incubation.



# Figure 1.2

Incubation time (i.e., days to hatch) was shorter by 0.8 d for eggs that experienced drought during late development, and 2.3 d for eggs that experienced drought during mid + late development (P < 0.001). Letters indicate significant differences.



# Figure 1.3

Least squares mean hatchling mass (g) and 95% confidence intervals by treatment. Hatchling mass varied by up to 1.5 g depending on treatment (p < 0.002). Hatchlings that experienced drought during mid- and late incubation were smaller than those that did not. Letters indicate significant differences.



# Figure 1.4

Trend lines represent the probability of recapture as a function of late incubation treatment predicted by a binomial regression model, with 95% confidence intervals. Points represent individuals, and are arranged along the x-axis in order of hatchling mass (g). Note that survival decreased with late drought and was not affected by hatchling mass.

# CHAPTER 2: Flash drought and heat waves during embryonic development influence early-life traits in the painted turtle, *Chrysemys picta*

#### ABSTRACT

Anthropogenic climate change is driving an increase in extreme climate events (ECEs). One consequence is the increased co-occurrence of drought and heat waves. While many studies have investigated the ecological impacts of hydric and thermal stress, less is known about their compound effects. Oviparous reptiles, including turtles, are innately sensitive to temperature and soil moisture conditions experienced in the nest, making them a valuable model system for studying the early-life effects of hydric and thermal stress. In a fully factorial experiment, we imposed simulated flash drought and heat wave events during embryonic development and examined the effects on egg mass, incubation time, hatchling body size, and early-life survival in the painted turtle (*Chrysemys picta*). We found that flash drought and heat wave events interactively influenced egg mass during embryogenesis. In addition, both drought and heat waves reduced incubation time and post-brumation body mass. Finally, smaller post-brumation offspring had a decreased probability of survival during an experimental release in the field. Our results suggest that as compound drought and heat wave events become more frequent and severe, reptiles may face significant physiological impacts during early life stages.

#### 2.1 | INTRODUCTION

Environmental conditions during embryonic development can have important phenotypic and fitness consequences, particularly in oviparous species with no parental care, as developing embryos must often contend with a variable abiotic environment (Deeming & Ferguson, 1991; Noble et al., 2018). In oviparous reptiles, amniotic eggs provide a buffer against hydric and thermal stress, but many species lay their eggs in relatively shallow terrestrial nests with steep fluctuations in temperature and soil moisture (e.g., Packard et al., 1985*a*; Cagle et al., 1993;

Thompson et al., 1996). Moreover, many reptiles lay flexible-shelled eggs that can absorb and lose water depending on their environment, impacting water availability to the embryo (Packard et al., 1982; Feder et al., 1982). These factors make reptile embryos uniquely sensitive to nest microclimate.

As a consequence of anthropogenic climate change, extreme climate events (ECEs), including flash droughts and heat waves, are becoming more frequent and severe (Smith, 2011; La Sorte et al., 2021; Zhang et al., 2024). Flash droughts are periods of severe water shortage generally lasting several weeks (Yuan et al., 2023), while heat waves are characterized by periods of excessive heat (Perkins & Alexander, 2013). As flash droughts and heat waves become more prevalent, they also co-occur more frequently (Mukherjee & Mishra, 2021). The independent ecological effects of drought and heat waves have been widely studied (Maxwell et al., 2019; Cinto Mejía & Wetzel, 2023), but less is known about the interactive effects of these events, particularly in vertebrates.

Hydric conditions play an important role in reptile embryonic development, particularly in species with flexible-shelled eggs (Belinsky et al., 2004). Low soil moisture limits embryonic growth, producing smaller eggs and hatchlings (Morris et al., 1983; Janzen et al., 1995; Marco et al., 2004; Brown & Shine, 2005), and reducing incubation time (Packard et al., 1985*b*). Hatching success may also decrease in dry conditions (Packard et al., 1991; Thomson et al., 1996; Tucker & Paukstis, 2000; but see Janzen et al., 1995). Though most laboratory experiments to date have used constant water potentials throughout incubation, the effects of hydric stress are likely time-dependent, being most substantial during mid- to late incubation (Gutzke & Packard, 1986; Delmas et al., 2008; but see Packard & Packard, 1988).

The thermal environment during embryonic development is also linked to important phenotypic effects in reptiles (Noble et al., 2018). In species with temperature-dependent sex

determination, offspring sex ratios depend on nest temperatures during the thermosensitive period (Bull, 1980). Furthermore, higher incubation temperatures generally lead to accelerated development and shorter incubation periods (e.g., Deeming & Ferguson, 1989; Warner et al., 2020), reduced hatching success (e.g., Gutzke & Packard, 1987; Lewis-Winokur & Winokur, 1995), elevated morphological abnormalities (e.g., Telemeco et al., 2013), and altered hatchling size, body composition, and behavior (Brooks et al., 1991; Janzen, 1993*b*; Downes & Shine, 1999; Booth et al., 2004; While et al., 2018). Thermal stress can also produce differences in DNA methylation patterns that may be associated with fitness (Yen et al., 2024). However, most studies have been conducted in the laboratory under constant environmental conditions (Carter & Janzen, 2021), though natural nests experience hydric and thermal fluctuations (e.g., Packard et al., 1985*a*; Cagle et al., 1993; Murphy et al., 2020).

Moreover, the interactive effects of hydric and thermal stress are not well understood, particularly in the context of ECEs such as drought and heat wave. In one study, water potential and temperature independently affected turtle egg mass and hatchling size, with eggs and hatchlings from the driest and hottest treatments being the smallest (Gutzke et al., 1987). However, the fitness effects are unknown. Body size is frequently linked to survival in turtles (Janzen, 1993*a*; Janzen et al., 2000*a*, *b*; Paitz et al., 2007; Mitchell et al., 2013; Delaney & Janzen, 2019), and environmental stressors that affect offspring body size are predicted to affect survival, but empirical evidence is limited.

In this study, we chose the painted turtle (*Chrysemys picta*) as a model to study the interactive effects of drought and heat waves during embryonic development. This common and widespread species lays flexible-shelled eggs in shallow terrestrial nests, which are exposed to variable hydric and thermal conditions (Cagle et al., 1993). *C. picta* and other turtle species also experience high mortality during early life stages, potentially leading to strong natural selection

on offspring traits (Janzen et al., 2000*a*), including traits conferred by the nest environment. We studied a *C. picta* population on the Mississippi River in the central United States, where both droughts and heat waves are an increasing concern (Kunkel et al., 2010; Christian et al., 2021). Flash droughts and heat waves in this region generally occur between June and August (Lyon & Barnston, 2017; Christian et al., 2019), concurrent with the incubation period of *C. picta*. Furthermore, *C. picta* hatchlings in our study population overwinter in the nest (Murphy et al., 2020). If flash drought and heat waves affect hatchling physiology, we examined whether these effects would persist through the overwintering stage.

The aim of this study was to examine whether simulated flash drought and heat wave events during embryonic development independently or interactively affected offspring traits and survival in *C. picta*. We predicted that flash drought and heat waves during incubation would reduce egg mass, hatching success, incubation time, and pre- and post-brumation body size. We also expected that turtles that experienced heat waves and drought during incubation would experience higher mortality, take longer to disperse from the nest, and lose more mass relative to their body size during dispersal. To test these hypotheses, we conducted a fully factorial experiment that combined experimental incubation in the laboratory with a release experiment in the field in order to place the effects of hydric and thermal stress in an ecologically-relevant context.

#### 2.2 | METHODS

#### Egg collection and incubation

We collected 8 eggs from each of 20 *C. picta* nests (n=160) at the Thomson Causeway Recreation Area in Carroll County, IL between May 30 and June 3, 2023. After individually marking each egg with a clutch and ID number, we transferred them to Styrofoam containers held at ambient temperature and covered them with moist soil. We then transported the

containers to our laboratory at the W. K. Kellogg Biological Station in Kalamazoo County, MI, where experimental incubation began on June 3.

We weighed each egg to the nearest 0.01 g, then divided them into eight treatment groups (Table 1), with each clutch equally represented in all treatments. Thus, each treatment group comprised 20 eggs, one from each clutch. We placed the eggs in a moist (-150 kPa) vermiculite substrate in covered plastic boxes, with each box containing 20 eggs in a 4 x 5 matrix. We situated the eight boxes in two Memmert incubators set to a daily cycle of 12h 22°C / 12h 26°C, representing a slightly condensed diel thermal range in nests at the field site (e.g., Mitchell et al., 2015). This temperature range yields 100% male offspring (Carter et al., 2019), excluding gonadal sex as a confounding variable. Once a week, we re-weighed each egg, rehydrated the vermiculite in each box, and rotated the boxes within the incubators to minimize thermal differences. During the second and third trimesters, we switched a subset of the eggs to a simulated flash drought treatment (-850 kPa). During the final third of incubation, half the eggs experienced a simulated heat wave event (12h 27°C/ 12h 31°C) consistent with temperatures measured in nests at the field site during heat waves (e.g., Mitchell et al., 2015). The timing of the heat wave, occurring after the thermosensitive period of sex determination (Wibbels et al., 1994), excluded the confounding variable of sex from this experiment.

After eggs hatched, we weighed the hatchlings to the nearest 0.01 g and measured carapace and plastron dimensions to the nearest 0.01 mm. We then randomly assorted hatchlings into 20 covered plastic tubs lined with damp paper towels, such that each clutch and treatment group were approximately evenly represented in each tub. At the field site, hatchling turtles remain dormant in nests until spring (Murphy et al., 2020), so we simulated the brumation period by placing them in a dark environmental chamber at 20°C, then lowering the temperature in the chamber to 5°C by late November 2023. During this time, we monitored tubs once a week and

ensured adequate hydration by misting them with water. We brought the turtles out of brumation by slowly raising the temperature (2°C/week) beginning in early February to a typical spring temperature of 20°C.

#### Release experiment

After brumation, we re-weighed the turtles and marked each individual with an ID code on the carapace using a black permanent marker. We conducted the experimental release at the Thomson Sand Prairie in Carroll County, IL. We constructed a 120 m drift fence parallel to the water's edge, with pitfall traps at intervals of 5 m consisting of buried 4.5 L plastic jars. We released turtles at 1600 h on May 2, 2024 from artificial nests located 30 m upslope from the fence, which is a typical distance of *C. picta* nests from the water at the field site (Harms et al., 2005). We checked traps daily at 1500 h, then weighed all recaptured turtles and re-released them at the slough adjacent to the original nest sites. During the release experiment, we captured 8 unmarked painted turtles that had evidently emerged from at least one natural nest at the site, lending validity to the timing and location of our study. The experimental release concluded on May 15 after 48 h with no turtles recaptured.

#### Statistical analyses

We conducted statistical analyses using R version 4.4.0. We modeled changes in egg mass over time using an analysis of covariance with repeated measures, with egg mass as the response variable; drought, heat wave, and week as fixed effects; initial egg mass as a covariate; and clutch as a random effect. To model hatching success, we used a logistic regression model with drought and heat waves as fixed effects, once again setting initial egg mass as a covariate and clutch as a random effect. We modeled incubation time as a linear mixed effects model fit by restricted maximum likelihood (REML), with drought and heat waves as fixed effects and clutch as a random effect. To model hatching body size metrics (i.e., hatchling mass; carapace

and plastron length and width) we used a linear mixed effects model fit by REML with drought and heat waves as fixed effects, clutch as a random effect, and initial egg mass as a covariate. We used a similar model to analyze post-brumation body mass, with hatchling mass as a covariate instead of initial egg mass. We analyzed survival during migration from the nest using a logistic regression model, with drought and heat waves as fixed effects and postbrumation mass as a covariate. We then transformed the logistic regression coefficient to calculate the selection gradient for post-brumation body mass (Janzen & Stern, 1998). Finally, we modeled time to recapture and change in hatchling mass as a function of drought and heat waves using a linear mixed effects model with post-brumation mass as a covariate and clutch as a random effect.

#### 2.3 | RESULTS

Weekly egg mass varied as a function of drought, heat wave, and their interaction, as well as initial egg mass (Table 2; Fig. 1). Eggs under typical moisture conditions gained mass throughout incubation, while eggs experiencing drought during mid-incubation did not gain mass, and those experiencing drought during late incubation decreased in mass. A heat wave in the final trimester magnified the effects of soil moisture on egg mass, leading to greater increases without drought and decreases with drought.

Hatching success was not substantially affected by drought ( $F_{3,151} = 0.879$ , P = 0.453), heat waves ( $F_{1,151} = 2.443$ , P = 0.120) or initial egg mass ( $F_{1,151} = 3.186$ , P = 0.076). Out of 160 eggs, 149 hatched, for a mean hatch rate of 93.1% across treatments. Eggs took between 69 – 82 d to hatch. Incubation time varied inversely with drought ( $F_{3,140} = 2.931$ , P = 0.036) and heat waves ( $F_{1,140} = 718.8$ , P < 0.001). Mid + late drought decreased incubation time by ~0.9 d, while heat waves decreased incubation time by 6.8 d (Fig. 2).

Hatchling mass, carapace length, carapace width, and plastron width all varied with

drought and initial egg mass, but not with exposure to a heat wave (Table 3). Plastron length varied with initial egg mass only. Hatchlings that experienced mid + late drought had the lowest mass, at 0.15 g (~3%) lower than the control group. Post-brumation body mass was positively related to hatchling mass and decreased with drought and heat wave exposure (Table 4). Turtles that experienced mid + late drought and a heat wave were 0.25 g (or ~5.5%) smaller than the control group at the end of the overwintering period.

All turtles survived the laboratory brumation period. In total, 90 turtles (~60.4%) were recaptured out of 149 released in the field experiment. Hatchling survival was unaffected by drought ( $F_{3,140} = 0.304$ , P = 0.823), heat waves ( $F_{1,140} = 1.413$ , P = 0.237), or their interaction ( $F_{3,140} = 0.351$ , P = 0.789); however, survival during migration from nest to water was positively related to post-brumation body mass ( $F_{1,140} = 4.862$ , P = 0.029). In other words, larger offspring had a higher probability of being recaptured, with the heaviest turtles having roughly twice the survival probability of the lightest ones (Fig. 3). The selection gradient on post-brumation body mass was 0.138 with a standard error of 0.175 (P = 0.041).

On average, turtles took approximately  $5.7 \pm 2.0$  d to reach the drift fence. Recapture times ranged from 2 to 11 d. Heat wave exposure reduced time to recapture ( $F_{1,81} = 4.332$ , P = 0.041), as did the interaction between drought and heat waves ( $F_{3,81} = 3.351$ , P=0.023). On average, individuals that experienced mid + late drought combined with a heat wave during embryonic development were the fastest to reach the fence. Furthermore, recapture time varied positively with time to hatch ( $F_{1,87} = 4.947$ , P = 0.029). Post-brumation body mass did not affect time to recapture ( $F_{1,87} = 0.063$ , P = 0.802).

While some turtles lost body mass during the release experiment, most gained mass. The average net change in mass was  $0.09 \pm 0.21$  g. There was no relationship between mass change and treatment or post-brumation body size. However, mass change was positively

correlated with days to recapture ( $F_{1,86} = 11.35$ , P=0.001).

#### 2.4 | DISCUSSION

In this study, we investigated the interactive and independent effects of flash drought and heat waves during embryonic development on early-life phenotypes and survival in the painted turtle (*Chrysemys picta*), a widespread species in North America that occurs in climatically diverse areas (e.g., Carter et al., 2019; Bodensteiner et al., 2023). As predicted, *C. picta* eggs gained mass in moist conditions and maintained or lost mass in drought conditions, in accordance with previous studies on this species and others with flexible-shelled eggs (e.g., Gutzke & Packard, 1986; Packard et al., 1991; Janzen et al., 1995).

Interestingly, heat waves during late incubation appeared to interactively amplify the effects of the hydric environment. Eggs in moist conditions experienced a greater increase in mass when exposed to a heat wave, while eggs under drought conditions experienced a greater decrease in mass. However, heat waves had no effect on hatchling body size. This implies that an increase or decrease in egg water content, rather than embryo body mass, was responsible for the changes in egg mass. Changes in metabolic processes and egg water balance may explain this unexpected result. The metabolic rate of an embryo increases with temperature, elevating metabolic heat production and water evaporation from the egg (Andrews, 2004; Kearney & Enriquez-Urzelai, 2023), as well as metabolic waste (Packard et al., 1984). A heat wave would presumably increase the rate of evaporation from the egg, exacerbating egg water loss. However, in non-drought conditions, water absorption could potentially increase due to higher concentration of waste solutes in the egg (i.e., higher osmotic potential).

Drought and heat wave events also substantially affected hatching phenology. In particular, eggs that experienced a heat wave during late development hatched almost a week earlier than those that did not. Constant high temperatures have previously been linked to

shorter incubation temperatures in *C. picta* (Gutzke et al., 1987; Warner et al., 2020) and other reptile species (Deeming & Ferguson, 1989; Lewis-Winokur & Winokur, 1995; Booth, 1998), but the strong effect of heat over a short period of time is notable.

While changes in egg mass during incubation were interactively affected by drought and heat wave, hatchling body size was not. Most hatchling body size metrics decreased with drought, but not with heat waves. As noted above, this indicates that heat waves impacted egg water content, rather than embryo growth. After the overwintering period, however, body mass varied inversely with both drought and heat wave. This finding suggests a delayed effect of embryonic heat waves on neonatal body mass, possibly resulting from thermal treatment differences in post-hatching metabolism (e.g., O'Steen & Janzen, 1999) affecting the rate of body mass loss during brumation. Future studies could investigate the physiological mechanisms behind this phenomenon.

Offspring survival, whether measured as hatching, brumation, or dispersal success, was not directly affected by drought or heat wave events in this study. However, dispersal survival increased substantially with post-brumation body size. This finding is consistent with positive size-linked survival during this life stage in prior studies of *C. picta* (Brodie & Janzen, 1996; Tucker, 2000; Paitz et al., 2007; Mitchell et al., 2013; but see Colbert et al., 2022) and often, but not ubiquitously, in offspring of other turtle species (Janzen, 1993*a*; Janzen et al., 2000*a*, *b*; Janzen et al., 2007; Tucker et al., 2008; Delaney & Janzen, 2019; but see Congdon et al., 1999; Kolbe & Janzen, 2001; Filoramo & Janzen, 2002; Paterson et al., 2014).

Time to recapture increased with incubation time, suggesting that early hatching may accelerate dispersal from the nest, as suggested by a previous *C. picta* study (Colbert et al., 2022). Drought and heat waves interactively reduced time to recapture. Turtles that experienced mid + late drought combined with heat wave had the lowest post-brumation body

mass, yet they reached the fence more quickly than any other group. Smaller offspring would typically be expected to be slower compared to their larger counterparts (e.g., Miller, 1993; Janzen et al., 2007), but this was not the case in this experiment. This group may have been more vulnerable to desiccation and could only survive by dispersing quickly. However, heavy rain throughout much of the experimental release makes it unlikely that desiccation was a significant factor. In fact, most turtles gained mass between release and recapture, and hatchlings that took longer to reach the fence gained the most, possibly from rainwater accumulation.

High water availability from precipitation might also explain why we did not observe differential survival among embryonic treatment groups. For instance, turtles that experienced drought during incubation may have been able to replenish their water reserve from rain events during dispersal, increasing their probability of survival. A similar explanation was proffered for the finding that incubation substrate moisture did not affect survivorship in *C. serpentina* in a release experiment at the same field site (Janzen, 1993*a*). In that study, hatchlings were exposed to water during tests of swimming performance prior to their release in the terrestrial field experiment. Regardless, as in this *C. picta* study, larger *C. serpentina* hatchlings exhibited a greater likelihood of dispersal survival independent of incubation treatment. Both studies found selection gradients favoring larger neonatal body size ( $\beta_{avggrad} = +0.138$  for *C. picta*,  $\beta = +0.325$  for *C. serpentina*.

Turtles are among the most imperiled vertebrate taxa worldwide (Stanford et al. 2020), and their nesting biology makes them particularly vulnerable to variation in the hydric and thermal environment. Flash droughts and heat waves are increasing in frequency and intensity under climate change (Perkins-Kirkpatrick & Gibson, 2017; Mukherjee & Mishra, 2021; Yuan et al., 2023), and the ecological consequences of these events are projected to be severe

(Maxwell et al., 2019; Cinto Mejía & Wetzel, 2023). Environmental conditions during embryonic development are an important determiner of offspring traits, particularly in groundnesting reptiles (Noble et al., 2018). In this study, compound drought and heat wave events interactively affected egg mass over time, and produced substantive phenotypic effects that persisted beyond the overwintering period. Furthermore, larger offspring had a survival advantage in the experimental release, providing support for the concept of size-dependent selection (Janzen et al., 2000*b*). This study sheds light on the importance of considering extreme climate events and their potential ecological implications during early life stages.

# TABLES

## Table 2.1

Summary of the eight treatment groups used in this experiment and timing of simulated drought and heat wave events. T1, T2, and T3 correspond to early, middle, and late incubation, respectively.

Treatment	T1: Day 1-24	T2: Day 25-48	T3: Day 49-72
WWW			_
WDW	—	Drought	_
WWD			Drought
WDD	—	Drought	Drought
WWW+H	—	_	Heat wave
WDW+H	—	Drought	Heat wave
WWD+H		_	Drought + Heat wave
WDD+H		Drought	Drought + Heat wave

# Table 2.2

Source of variation	df	Mean square	F-ratio	P-value
Drought	3	29.4	210.49	< 0.001
Heat wave	1	0.7	5.37	0.021
Week	8	17.0	121.44	< 0.001
Initial mass	1	749.8	5369.91	< 0.001
Drought × Heat wave	3	0.5	3.24	0.021
Drought $\times$ Week	24	4.5	32.54	< 0.001
Heat wave × Week	8	0.1	0.39	0.928
Drought $\times$ Heat wave $\times$ Week	24	0.2	1.54	0.045

Repeated measures analysis of covariance of changes in egg mass during incubation as a function of main effects and their interactions.

# Table 2.3

F-ratios and P-values (in parentheses) for analyses of covariance on five body size metrics of hatchling snapping turtles, with initial egg mass as the covariate.

		Source of variation	
Variable	Drought	Heat wave	Initial egg mass
Hatchling mass	4.53 (0.005)	0.96 (0.329)	445.63 (<0.001)
Carapace length	3.35 (0.021)	1.58 (0.210)	147.79 (<0.001)
Carapace width	10.92 (<0.001)	0.58 (0.447)	44.44 (<0.001)
Plastron length	0.72 (0.542)	0.74 (0.391)	164.87 (<0.001)
Plastron width	9.29 (<0.001)	0.81 (0.371)	39.23 (<0.001)

# Table 2.4

Results of analysis of covariance of drought and heat waves on post-brumation body mass, with hatchling mass as the covariate.

Source of variation	df	Mean square	F-ratio	P-value
Drought	3	0.235	11.86	< 0.001
Heat wave	1	0.550	27.76	< 0.001
Hatchling mass	1	29.292	1477.75	< 0.001
Drought x Heat wave	3	0.007	0.35	0.792

## **FIGURES**



## Figure 2.1

Least squares means of egg mass over the course of incubation. Colors and shapes indicate flash drought and heat wave treatments. Dashed vertical lines indicate trimesters of incubation.



# Figure 2.2

Box plot showing incubation times (d) for each flash drought and heat wave treatment. See Table 1 for details of each treatment.



# Figure 2.3

Probability of recapture during experimental release as a function of post-brumation body mass (g).

#### CONCLUSION

Environmental conditions during embryonic development are a strong predictor of offspring traits (Sommer, 2020). In oviparous taxa, including many reptiles, eggs are frequently exposed to fluctuating conditions in the nest (Noble et al., 2018). As climate change drives more variable and extreme climatic conditions (Cinto Mejia & Wetzel, 2023), reptile embryos are likely to experience a corresponding increase in hydric and thermal stress. Most early laboratory studies on reptile eggs used constant temperatures and water potentials throughout incubation (e.g., Morris et al., 1983; Gutzke et al., 1987; Janzen, 1995; Burger, 1990; Hokit & Branch, 2004). These studies yielded many important findings, linking hydric and thermal stress with changes in incubation time, body size, and other factors, but researchers have long noted that abiotic conditions in natural nests are highly variable throughout incubation (e.g., Packard et al., 1985). With technological advances, laboratory studies are beginning to implement more realistic fluctuating thermal cycles (Carter & Janzen, 2021), and field experiments have recorded thermal and hydric conditions in natural nests (Thompson et al., 1996; Kolbe & Janzen, 2002), and even manipulated natural nest environments (Bodensteiner et al., 2015). However, less is known about the effects of extreme shifts in temperature and moisture in a climate change context. The "bigger is better" hypothesis posits that larger individuals have a higher probability of survival (Rollinson & Rowe, 2015; Porter et al., 2024), yet relatively few studies have explicitly tested the effects of incubation conditions on post-hatching survival of reptiles in the field (Janzen, 1993a; Andrews et al., 2000; Filoramo & Janzen, 2002; Bock et al., 2023). The effects of extreme climate events during reptile embryonic development on offspring phenotype and survival remain largely unknown. In this thesis, I aimed to explore (1) the early-life phenotypic and fitness effects of flash drought during incubation in the common snapping turtle, Chelydra serpentina, and (2) the interactive effects of flash drought and heat waves during incubation on

those same traits in the painted turtle, Chrysemys picta.

In my first chapter, I investigated the effects of simulated flash drought during early, middle, and late incubation on offspring traits and survival in C. serpentina. My approach combined experimental incubation in the laboratory with a release experiment in the field. I found that eggs lost mass under drought conditions, but eggs that experienced drought during early incubation gained mass at an accelerated rate in the second trimester, allowing them to "catch up" with eggs that did not experience early drought. However, drought during mid-to-late incubation produced smaller eggs and hatchlings. Eggs also hatched slightly earlier after mid-tolate drought. Finally, hatchlings that experienced drought during mid-to-late development lost more body mass during their migration from the nest, and those that experienced late drought were less likely to survive the journey. Together, these results suggest that the timing of flash drought is important, and that drought during the last two-thirds of embryonic development may have negative impacts on offspring fitness. My findings add to previous research linking water deprivation in turtle embryos to reduced incubation time, egg mass, and body size (reviewed in Packard, 1999), and emphasize the significance of the timing of hydric stress (Gutzke & Packard, 1986; Delmas, 2008). Perhaps most importantly, this study connects flash drought during embryonic development with increased early-life mortality in turtles.

My second chapter built on my previous results by combining flash drought and heat waves to test for interactive effects on phenotype and fitness. By turning my focus to *C. picta*, I was also able to test for more long-term effects of the incubation environment. *C. picta* hatchlings in the study population overwinter in the nest, so their access to water and nutrients is limited until the following spring (Murphy et al., 2020). I used similar methods to the first experiment, except for the addition of a simulated heat wave treatment during incubation and the overwintering of hatchlings in the laboratory. I also incorporated a fully-factorial design,

allowing effective comparison across groups. The study revealed complex interactions between temperature, hydric stress, and time. Eggs gained mass in favorable hydric conditions and decreased or plateaued in mass during flash drought events. Heat waves in the final trimester appeared to amplify the effects of the hydric environment, leading to steeper gradients of mass change. Heat waves also reduced incubation time by a full week. Unexpectedly, heat wave effects did not carry over to hatchling body size in the short term, but at the end of the overwintering period, turtles that had experienced a heat wave during embryonic development weighed less than those that had not. Flash drought also reduced both pre- and post-brumation body size. In other words, these extreme climate events substantially affected offspring phenotypes over nine months after these events occurred. While flash drought and heat waves did not affect survival during migration from the nest in this experiment, survival increased substantially with body size, supporting the hypothesis that "bigger is often better" for early-life survival (Ferguson & Fox, 1984; Janzen, 1993*a*; Janzen et al., 2000*b*; Porter et al., 2024).

The results of my thesis work suggest several potential directions for future research. First, future studies could investigate the effects of ECEs in other turtle species, as well as other reptiles, to determine whether response patterns are taxon-specific. In addition to flash drought and heat waves, studies could focus on other ECE types, such as flooding and cold snaps. Furthermore, research is needed to characterize the physiological mechanisms linking hydric and thermal stress to offspring phenotype and survival. Transcriptomic research could assess the effects of ECEs on gene expression, particularly in genes associated with metabolism and growth. Researchers could also continue to explore DNA methylation signatures of ECEs during embryonic development (Yen et al., 2024), and whether such epigenetic markers are retained in later life. Finally, additional research could examine the effects of drought, flooding, and heat waves on natural nests in the field. Field studies could use experimental manipulations (e.g., rain

exclusion; supplemental watering; electric heating) to replicate ECE conditions outside the laboratory.

In summary, this thesis reveals clear and ecologically-relevant connections between flash droughts, heat waves, developmental plasticity, and survival in two widespread turtle species. ECEs can disrupt embryonic growth, hatching phenology, offspring size, and, in some instances, post-hatching survival. These findings contribute to scientific understanding of how environmental stress during embryonic development shapes offspring phenotype within the context of climate change. With extreme climate events on the rise, their impacts on vulnerable early-life stages should not be overlooked.

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