

1 **Effects of constant and fluctuating incubation temperatures on hatching success and hatchling traits in**
2 **the diamondback terrapin (*Malaclemys terrapin*) in the context of the warming climate**

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11 **Abstract**

12 As global temperatures continue to rise, so too will the nest temperatures of many species of turtles. Yet
13 for most turtle species, including the estuarine diamondback terrapin (*Malaclemys terrapin*), there is
14 limited information on embryonic sensitivity to elevated temperature. We incubated eggs of *M. terrapin*
15 at three, mean temperatures (31, 34, 37 °C) under two thermal exposure regimes (constant or semi-
16 naturally fluctuating temperature) and measured hatching success, developmental rate, and hatchling
17 size. Hatching success was 100 % at 31 °C and 67 % at 34 °C, respectively; at 37 °C, all eggs failed early in
18 the incubation period. These values were unaffected by exposure regime. The modeled LT₅₀
19 (temperature that was lethal to 50 % of the test population) was 34.0 °C in the constant and 34.2 °C in
20 the fluctuating thermal regime, reflecting a steep decline in survival between 33 and 35 °C. Hatchlings
21 having been incubated at a constant 34 °C hatched sooner than those incubated at 31 °C under either
22 constant or fluctuating temperature. Hatchlings were smaller in straight carapace length (CL) and width
23 after having been incubated at 34 °C compared to 31 °C. Larger (CL) hatchlings resulted from fluctuating
24 temperature conditions relative to constant temperature conditions, regardless of mean temperature.
25 Based upon recent temperatures in natural nests, the *M. terrapin* population studied here appears to
26 possess resiliency to several degrees of elevated mean nest temperatures, beyond which, embryonic
27 mortality will likely sharply increase. When considered within the mosaic of challenges that Maryland's
28 *M. terrapin* face as the climate warms, including ongoing habitat losses due to sea level rise and
29 impending thermal impacts on bioenergetics and offspring sex ratios, a future increase in embryonic
30 mortality could be a critical factor for a population already experiencing ecological and physiological
31 challenges due to climate change.

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33 Key words: Climate change; Global warming; Hatching success; Incubation; Lethal temperature; Thermal
34 tolerance.

35

36 **1. Introduction**

37 Average global temperatures are rising and will likely continue to do so for the foreseeable
38 future unless substantial and immediate actions are taken to curb greenhouse gas emissions (USGCRP,
39 2018). Recent projections suggest that within 80 years, the global average surface temperature will
40 increase by 2 to 5 °C (3.6 to 9 °F; USGCRP, 2018). Ectotherms, which comprise > 99 % of animal species
41 and for which physiological rates vary with body temperature, are particularly responsive to
42 temperature change (see Huey and Kingsolver, 1989; Huey et al., 2012). The rate of change in projected
43 temperatures may be rapid enough that some species, particularly those that have long generation
44 turnover times, will be unable to adapt genetically (e.g. Janzen, 1994; Morjan, 2003). Many reptiles,
45 most notably turtles, are long-lived and slow to mature, conferring them with long generation times and
46 putting them at risk of experiencing reduced fitness in the altered physicochemical environment of the
47 future (Janzen, 1994; Gibbons et al., 2000; Rowe, 2008; Böhm et al., 2016; Refsnider and Janzen, 2016).

48 Depending upon where the upper thermal tolerance threshold exists relative to environmental
49 temperature, and in the absence of rapid evolution of tolerance or acclimatization, ectotherms may
50 express a range of responses to elevated temperature (Huey et al., 2012). Subtle responses to elevated
51 temperature, such as increased metabolic rate, can increase feeding requirements and could alter
52 complex relationships between temperature, resource availability, and competition for resources among
53 individuals (see Harley, 2011; Rowe and Crandall, 2018). Physiological performance (metabolic or
54 otherwise) of ectotherms generally increases with temperature up to an optimum value, after which
55 performance declines. If temperature continues to increase beyond the thermal tolerance limit, heat-

56 induced mortality will result (see Huey and Stevenson, 1979; Schulte et al., 2011; Sinclair et al., 2016).
57 Thus, individuals for which current ambient temperatures are near or above their physiological optimum
58 temperature are at risk of experiencing hindered performance or survival after even relatively small
59 increases in temperature.

60 Recent work on sea turtles (Families Cheloniidae and Dermochelyinae) suggested that current
61 nest temperatures in some rookeries are becoming increasingly closer to embryonic upper thermal
62 tolerance limits. For example, using novel and existing data on nest temperatures and embryonic
63 thermal tolerance limits, Laloë et al. (2017) showed that recent temperatures in nests of loggerhead
64 turtles (*Caretta caretta*) in the Cape Verde islands are within about 1 to 2 °C of the threshold at which
65 embryonic survival (hatching success) declines dramatically. Moreover, several other sea turtle species
66 or populations display similar relationships with temperature (see Tomillo et al., 2012; Howard et al.,
67 2014; Cavallo et al., 2015; Kobayashi et al., 2015; Montero et al., 2018a; Monsinjon et al., 2019),
68 suggesting the strong potential for harmful thermal impacts on embryonic survival in numerous species
69 of endangered reptiles following relatively small changes in the climate. These effects are likely to be
70 greatest for tropical-nesting sea turtles (e.g. Sunday et al., 2011; Huey et al., 2012), because more
71 temperate-nesting sea turtles, such as some *C. caretta* populations, could experience increased hatching
72 success as the climate warms (Pike, 2014; Montero et al., 2018b), assuming that current nest
73 temperatures are below the optimum temperature for successful hatching (e.g. Huey et al., 2012).

74 The diamondback terrapin (*Malaclemys terrapin*; Family Emydidae) is an estuarine turtle that
75 occurs along the Gulf of Mexico and Atlantic coasts of the U.S.A. The diamondback terrapin is the only
76 endemic estuarine turtle in North America, where it is typically a resident of brackish marshes. Adult
77 females additionally require accessible sandy areas, most often near fringing estuarine beaches and
78 shorelines, for nesting. Woodland et al. (2017) projected that within 80 years, about 80 % of
79 historically-used nesting sites of *M. terrapin* in the Maryland portion of the Chesapeake Bay will be

80 inundated due to sea level rise. Over this same time span, brackish marsh habitat is projected to decline
81 by 30 to 50 % on Maryland's Western Shore of the Chesapeake Bay and three- to six-fold on the lower-
82 elevation, Eastern Shore (Woodland et al., 2017) where the bulk of Maryland's *M. terrapin* likely reside.
83 Based upon *M. terrapin*'s habitat requirements and projections for sea level rise, it is likely to experience
84 ecological constraints, reflecting loss of brackish marshes and the low-elevation, undeveloped coastal
85 habitats where it nests (Woodland et al., 2017). It is unknown if climate warming will additionally lead to
86 thermal impacts on the physiology of *M. terrapin* that could exacerbate the effects of habitat loss due to
87 sea level rise.

88 Recent monitoring of temperatures in natural nests of *M. terrapin* in southern Maryland, U.S.A.,
89 during the middle portion of the nesting season (beginning in late June) in 2018 and 2019 showed that
90 the average mid-nest temperatures (± 1 SD) throughout incubation were 28.6 ± 2.8 °C (3 nests) and 29.8
91 ± 2.6 °C (4 nests), respectively (Rowe et al., unpub. data). Yet, due to a lack of data on embryonic
92 thermal tolerance of *M. terrapin*, we are unable to establish a context for these nest temperatures with
93 respect to possible risks of embryonic mortality as global temperatures increase (e.g. Laloë et al., 2017).
94 There are few studies of emydid turtles in the primary literature in which the embryonic, upper lethal
95 temperature was determined or could be approximately inferred, such as through calculation of a
96 comparative metric such as the LT_{50} , the temperature resulting in 50 % mortality in the test population.
97 Reports of increased embryonic mortality at increased temperature in emydids and some other groups
98 (below) provide proof-of-concept of a relationship between incubation temperature and hatching
99 success (e.g. Gutzke and Packard, 1987; Packard et al., 1989), but they are of limited utility for
100 estimating the continuous, thermal response to a range of conditions that may prevail in the future.

101 In sea turtles (Families Cheloniidae and Dermochelyidae), more than other turtle groups,
102 embryonic upper thermal tolerance limits have been well characterized. Howard et al. (2014) reviewed
103 embryonic thermal tolerance studies using sea turtles and concluded that, in general, hatching success

104 declines when incubation temperature throughout the embryonic period exceeds 29 °C. Furthermore, it
105 can be inferred from Figure 1 in Howard et al. (2014), that the embryonic LT₅₀ for the five species
106 considered is about 32 °C. This value is in agreement with the LT₅₀ (~ 32 °C) that we estimated visually
107 from Figure 1 in a more recent review of sea turtles by Hays et al. (2017). In a study of loggerhead (*C.*
108 *caretta*) and green sea turtle (*Chelonia mydas*) nests in Florida, U.S.A., embryonic mortality was strongly
109 influenced by the cumulative incubation time above 34 °C (Bladow and Milton, 2019).

110 As noted above, with few exceptions, data are generally lacking or are inadequate to decipher
111 patterns in embryonic mortality across a range of incubation temperatures for freshwater and terrestrial
112 turtles (see Section 4. Discussion). In particular, comparative indices such as the LT₅₀ have not been
113 calculated or reported for most species, making difficult species- or population-wide comparisons of
114 embryonic thermal tolerance. There is a critical need for more research establishing quantitative
115 relationships between incubation temperature and hatching success in the Emydidae and other under-
116 represented families, given the rapid increase in global surface temperatures projected for the
117 remainder of the 21st century (Najjar et al., 2010; USGCRP, 2018).

118 In local *M. terrapin* nests, mid-nest temperature fluctuates by about 7 to 13 °C per day (Rowe et
119 al. unpub.). A body of work has shown that fluctuations in nest temperature can affect embryonic and
120 hatchling traits in turtles (see, for example, Ashmore and Janzen, 2003; Les et al., 2007, 2009; Refsnider,
121 2013; Horne et al., 2014; Valenzuela et al., 2019). Yet, a paucity of quantitative, comparative estimates
122 of thermal influences on survival or hatchling traits for *M. terrapin* (and most emydid turtles) restricts
123 our ability to assess whether inclusion of fluctuating thermal regimes that capture natural variations
124 during incubation would change estimates of mortality or alter hatchling traits from those based on
125 constant temperature incubations.

126 We conducted this study with the primary goal of determining survival rates for embryonic *M.*

127 *terrapin* across three average incubation temperatures and evaluating outcomes in the context of
128 ongoing climate change. We included both constant and fluctuating temperature regimes to
129 additionally consider whether variability in nest temperatures would influence the results.

130 **2. Material and Methods**

131 In this experiment, we incubated eggs at three constant temperatures (31, 34, 37 °C) as well
132 as in fluctuating temperature regimes in which the average temperature values throughout the
133 incubation period coincided with the constant temperature values (31, 34, 37 °C). The fluctuating
134 temperature treatments reflected weekly mean daily temperature variations measured in natural
135 nests in 2018 during the same portion of the nesting season that laboratory incubations occurred in
136 2019 (below).

137 From June 17 to 19, 2019, we collected freshly-laid clutches of *M. terrapin* at Naval Air Station
138 Patuxent River (NAS PAX), Lexington Park, MD, U.S.A. (38.30600, -76.40208). We collected eggs
139 immediately after the female was observed to finish nesting and transported them to the laboratory
140 within three hours. We collected and incubated six clutches, but two clutches were entirely or mostly
141 non-viable (0/15 and 3/13 hatched), leaving four clutches from which our inferences would be drawn.
142 Since eggs were collected over a three-day period, we maintained them at room temperature (22 to 24
143 °C) until the experiment started (June 20), one day after the last clutch had been collected.

144 Eggs were numbered with graphite pencil and assigned to treatments such that all clutches were
145 represented in all treatments (two to three eggs per clutch per incubator), per an incomplete blocked,
146 randomized design (below; Cochran and Cox, 1957). Incubators each initially received 12 to 13 eggs, but
147 eventual removal of the two non-viable clutches reduced the number of eggs contributing to our data
148 set to eight to nine per incubator. Treatments were three, constant temperatures (31, 34, 37 °C;
149 hereafter “C31”, “C34”, and “C37”) and three fluctuating temperatures with the same mean values as

150 the constant temperature treatments (hereafter “F31”, “F34”, and “F37”). We chose 31 °C as our lowest
151 temperature because previous incubations at 31 °C (constant) resulted in high hatching rates (90 to 100
152 %; Rowe, unpub.).

153 Incubations took place in six, identical, custom-built incubators. Each incubator had its own
154 thermostat (Inkbird ITC-310T-B) that was either set at a constant value or programmed to fluctuate via
155 12, daily temperature set-points. Set-points (2 hr duration each) were derived from the values for hourly
156 fluctuations in three natural nests measured in 2018 as follows. Hourly average temperatures recorded
157 in three natural nests during the same portion of the season in 2018 (June 20 to August 9) were
158 averaged, then subtracted from 31 °C. The resulting difference was then added to each data point in the
159 data set, to bring the mean to 31 °C. The same operation was done to adjust the data set for 34 and 37
160 °C treatments. A similar method was employed by Valenzuela et al. (2019) when replicating natural nest
161 temperature patterns in the laboratory. Each week, the thermostats on the fluctuating incubators were
162 re-programmed to the average values for the next seven days. As a result, the fluctuating incubators
163 included the daily variation in natural nests, averaged weekly (Figure 1). This approach provided a more
164 realistic pattern in variation than is achieved in typical, fluctuating-temperature incubation protocols
165 that employ a symmetric, sinusoidal daily temperature pattern (see Georges et al., 2005, Bowden et al.,
166 2014; Christie and Geist, 2017), yet was less reflective of natural nest conditions than a protocol that
167 varied temperature patterns on a daily basis (e.g. Thompson et al., 2018; Valenzuela et al., 2019).
168 Incubator temperatures were recorded at 10 minute intervals with data loggers (Hobo ProV-2, Onset
169 Corp.) for which the sensor probe was placed in the substrate 1 cm deep in the approximate center of
170 each incubation tray.

171 The eggs were placed in a loosely covered, food grade plastic tray in the center of each
172 incubator. Eggs were buried in damp vermiculite (1.1:1 water:vermiculite, v:v) with the topmost surface
173 of the egg level with the substrate surface, but lightly covered in substrate. Every one to two days, the

174 tray was removed, weighed, and misted with water to replace moisture lost to evaporation. Evaporative
175 loss from the substrate ranged from about 0.2 to 0.5 % per day.

176 Eggs were inspected daily. Date of pipping (initial splitting of the egg shell) and hatching
177 (neonate fully outside of egg shell), wet weight (ww), straight carapace length (CL), straight carapace
178 width (CW), and height and diameter of residual, external yolk at hatching were recorded. Hatchlings
179 were subsequently marked and released at the nesting area.

180 While *M. terrapin* possesses temperature-dependent sex determination, the mean incubation
181 temperatures used here exceeded the temperature above which only females are produced (29.6 °C;
182 Burke and Calichio, 2014). We did not verify sex, which would have required euthanasia and necropsy.

183 2.1. Calculations and Statistical analyses

184 To investigate our experimental results in the context of naturally-varying nest temperature
185 conditions in the field, we calculated the number of degree days above 34 °C ($DD_{>34}$) from the data
186 record for each incubator and for the 2018 field nest temperature data. We chose 34 °C as our index
187 because this is the approximate LT_{50} calculated for our study animals incubated under constant
188 temperatures (below). All $DD_{>34}$ index values were calculated as the sum of hourly mean temperature
189 residuals ($n = 24$ per day) that exceeded 34 °C. Hourly mean temperatures at or below 34 °C were
190 assigned a value = 0. The total number of accrued daily hours > 34 °C were divided by 24 hours to yield a
191 final sum standardized to unit days. Finally, we considered two climate change scenarios, a 2 °C and a 5
192 °C temperature increase, by adding 2 °C and 5 °C to the 2018 field temperatures. We then recalculated
193 the $DD_{>34}$ index for each scenario and interpreted these projected conditions relative to current nest
194 conditions and our experimental results.

195 To put our study in the context of the recent, local climate, we used data for daily air
196 temperature maxima at the field site (NAS PAX) from the NOAA NCEI database

197 (<https://www.ncdc.noaa.gov/cdo-web>) to calculate the average maximum daily air temperature (MDAT)
198 value for years 2001 to 2019 during the mid-nesting season coincident with the lab incubation study
199 (June 20 to August 9). Year 2006 was omitted due to limited data availability.

200 Statistical analyses were conducted using R statistical software (R Core Team, 2013). Statistical
201 significance was evaluated at $\alpha = 0.05$. Individual eggs served as the statistical units for analyzing effects
202 of conditions within the incubators for the experiments. Eggs were assigned at random to temperature
203 treatments using an incomplete blocked, randomized design. Clutches served as blocks to control the
204 variations among clutches. Each clutch was split approximately evenly among the six incubators to
205 ensure consistent randomization in the experiments (Cochran and Cox, 1957).

206 To assess hatching success (lived or died), we used a mixed effects logistic regression to
207 compare observed versus predicted mortality rates among treatments. Clutch served as a random
208 effect. Likelihood ratio based χ^2 tests were conducted for the fixed effects of thermal regime (constant
209 versus fluctuating). Temperature entered the model as a continuous variable to estimate the exposure-
210 response relation. The interaction of temperature and thermal regime was removed because its
211 inclusion did not enhance the model. Predicted hatching rates and 95 % confidence intervals were
212 generated using bootstrapping (Bates et al., 2015).

213 Temperature level corresponding to 50 % hatch rate (LT_{50}) and its standard error (SE) were
214 estimated based on a bootstrapping technique. Hatch rate was bootstrapped 499 times at 99 evenly
215 spaced temperatures between 30.7 and 37.3 °C for both constant and fluctuating thermal regimes. The
216 predicted temperature that would result in 50 % hatching success was identified, resulting in a
217 bootstrap sample of 499 LT_{50} estimates. The mean and SE of this sample are reported as the LT_{50}
218 estimate and its SE. Given the high estimated probabilities, a probit link was used instead of the
219 canonical logit link in estimating LT_{50} .

220 Using blocked ANOVA, we compared development (time to pipping and hatching) and hatchling
221 traits (body size, residual yolk size) among treatments. Type II F tests were conducted, given the random
222 clutch factors and potential interactions between temperature and thermal regime. Tukey's pairwise
223 comparisons among treatment levels were conducted after adjustment for multiple comparisons.

224 3. Results

225 Effects of treatments on hatching success are shown in Figure 2. Hatching success was 100 % at
226 31 °C regardless of whether the temperature fluctuated (F31) or was constant (C31). While hatching
227 success declined as mean temperature increased ($\chi^2_1 = 12.05$, $P = 0.0005$), the thermal regime (constant
228 or fluctuating) did not affect this endpoint ($\chi^2_1 = 0.17$, $P = 0.6836$). Hatching was reduced by 33 % in 34
229 °C treatments (F34, C34) relative to 31 °C. At 37 °C, no eggs hatched regardless of whether the
230 temperature was constant (C37) or fluctuated (F37). The eggs in the 37 °C treatments appeared to fail
231 early in the incubation period, as most showed discoloration within the first several weeks of incubation.
232 Dissection of these eggs after having been stored frozen did not reveal discernable embryos. The probit
233 model estimates (± 1 SE) for the LT_{50s} were 34.0 ± 0.5 (constant temperatures) and 34.2 ± 0.6 °C
234 (fluctuating temperatures; Figure 2).

235 Developmental rates evaluated at the time of pipping were affected by temperature ($\chi^2_1 = 6.55$,
236 $P = 0.0105$), thermal regime ($\chi^2_1 = 27.48$, $P < 0.0001$), and the interaction ($\chi^2_1 = 9.36$, $P = 0.0022$). No
237 pipping occurred in 37 °C treatments (F37, C37). Pairwise comparisons revealed that developmental rate
238 to pipping was faster in treatment C34 relative to C31, F31, and F34, and faster in C31 than in the
239 fluctuating treatments (F31, F34; Table 1). On the other hand, developmental rate evaluated up to the
240 time of hatching (which occurred about 1 – 3 days after pipping) was only influenced by thermal regime
241 ($\chi^2_1 = 12.33$, $P = 0.0005$) such that hatching occurred faster in treatment C34 relative to F34 and F31

242 (Table 1). Developmental rates to hatching were not affected by temperature alone ($\chi^2_1 = 0.93$, $P =$
243 0.3355) or the interaction between temperature and thermal regime ($\chi^2_1 = 3.61$, $P = 0.0575$).

244 Degree day ($DD_{>34}$) calculations showed that cumulative thermal exposure during incubation
245 increased with incubator temperature and was greater in fluctuating temperature treatments than in
246 constant temperature treatments (Figure 3). Values for the $DD_{>34}$ index ranged from 0.04 (C31) to 48.3
247 (F37), with the largest within-temperature difference occurring between C34 ($DD_{>34} = 5.9$) and F34
248 ($DD_{>34} = 28.4$). Field conditions in 2018 yielded a $DD_{>34} = 0.8$, roughly equivalent to the C31 and F31
249 incubation treatments. Including future climate scenarios yielded $DD_{>34} = 6.0$ for the 2 °C increase
250 scenario, and $DD_{>34} = 37.5$ for the 5 °C increase scenario. These climate scenarios were most similar to
251 the $DD_{>34}$ index values for treatments C34 and F34, yielding projected accrued thermal conditions equal
252 to or greater than those associated with our modeled LT_{50} values.

253 Dimensional size of hatchlings (CL, CW) was affected by temperature (CL: $\chi^2_1 = 16.02$, $P = 0.0001$;
254 CW: $\chi^2_1 = 15.75$, $P = 0.0001$), being greater at 31 °C relative to 34 °C (Table 1). Hatchling CL was also
255 affected by thermal regime ($\chi^2_1 = 4.92$, $P = 0.0265$), such that hatchlings in fluctuating treatments were
256 smaller than those in constant temperature treatments. Hatchling CW was not affected by thermal
257 regime ($\chi^2_1 = 0.95$, $P = 0.3304$). Hatchling weight (ww) was independent of treatments (temperature: χ^2_1
258 $= 1.00$, $P = 0.3163$, thermal regime: $\chi^2_1 = 2.34$, $P = 0.1261$), as were external residual yolk height at
259 hatching (temperature: $\chi^2_1 = 1.64$, $P = 0.2002$, thermal regime: $\chi^2_1 = 0.19$, $P = 0.6656$) and external
260 residual yolk diameter at hatching (temperature: $\chi^2_1 = 0.35$, $P = 0.5520$, thermal regime: $\chi^2_1 = 0.13$, $P =$
261 0.7326).

262 Over the period from 2001 to 2019 (2006 excluded), the average MDAT during mid-nesting
263 season (June 20 to August 9) at NAS PAX was 30.4 °C and varied annually such that mid-nesting season
264 MDAT ranged from 28.0 to 33.3 °C (Figure 4). Year 2018, which provided the nest temperature data

265 used in this study, had the 7th warmest average MDAT in the 18-year data set. Year 2019 was the 3rd
266 warmest (Figure 4).

267 Average incubator substrate temperatures (± 1 SD) throughout the experiment were as follows:
268 F31 = 31.00 ± 2.11 , C31 = 30.98 ± 0.29 , F34 = 33.97 ± 2.14 , C34 = 34.16 ± 0.34 , F37 = 36.95 ± 2.10 , C37 =
269 37.00 ± 0.32 °C. Fluctuating temperature incubators displayed total variability (experiment-wide) of 6.64
270 to 7.26 °C and constant temperature incubators varied by 1.61 to 2.23 °C over the seven-week
271 experiment (Table 2). Plots of measured incubator temperatures are provided in Figure 5.

272 4. Discussion

273 Embryonic mortality in response to high incubation temperature in our study population of *M.*
274 *terrapin* occurs above a mean incubation temperature of 31 °C. Statistical models show the LT₅₀ to be
275 34.0 to 34.2 °C for constant and fluctuating temperature treatments, respectively. Recent nest
276 temperatures during the mid-nesting season were 28.6 (2018) and 29.6 °C (2019), suggesting some
277 resiliency (in embryonic survival) to near-term (early to mid-21st century) projected increases in regional
278 average surface temperatures. Yet projections for the late 21st century of up to 5 °C increase in average
279 global surface temperatures (USGCRP, 2018) will result in thermal conditions conducive to substantial
280 egg mortality, assuming thermal tolerance does not increase in the future through acclimatization or
281 adaptation. Genetic adaptation increasing the thermal tolerance of the embryos is unlikely due to the
282 rapid rate of environmental change that is occurring and the long-lived, slow-to-mature life history
283 strategy displayed by many turtles (see Janzen, 1994; Gibbons et al., 2000; Rowe, 2008; Böhm et al.,
284 2016; Refsnider and Janzen, 2016).

285 Variation in temperature did not affect hatching success, even at a mean temperature within
286 the lethal zone (34 °C; Figure 2). A study by Valenzuela et al. (2019) reported mortality of embryos of
287 the emydid *Chrysemys picta* when the temperature fluctuated daily by 6 °C around a female-producing

288 temperature, resulting in actual daily temperature ranges as great as 22 °C (e.g. from 18 to 40 °C/day,
289 Figure 2 in Valenzuela et al., 2019). It is likely that such temperature fluctuations were sufficient to place
290 some embryos in a thermal critical zone for a sufficient period that lethality was experienced
291 (Valenzuela et al., 2019). In our study, temperature fluctuations were dampened somewhat from the
292 absolute range measured in the field (13 °C across the entire incubation period in 2018) because we
293 averaged values from three nests, which reduced influences of extreme values. It is clear that large
294 temperature fluctuations can increase embryonic mortality at high temperatures (Valenzuela et al.,
295 2019), but the fluctuations that we employed (maximum range, experiment-wide = 7.3 °C; Table 2,
296 Figure 5) were insufficient to do so. If, in our population of *M. terrapin*, future conditions bring about
297 fluctuations in nest temperatures of the magnitude of those that induced mortality in *C. picta*
298 (Valenzuela et al., 2019), elevated mortality rates might be expected.

299 Quantitative, comparative values for thermal tolerance (e.g. the LT₅₀) in turtles are scant in the
300 primary literature. We were able to estimate the LT₅₀ for hatching success in sea turtles from the multi-
301 species reviews by Howard et al. (2014) and Hays et al. (2017) and found that the average LT₅₀ for the
302 species reviewed (Families Cheloniidae, Dermochelyidae) is about 32 °C. The LT₅₀ calculated here for *M.*
303 *terrapin* is about 34 °C. Relative to sea turtles, however, emydid turtles are under-represented in the
304 literature regarding lethal upper temperatures to embryos, and it is therefore difficult to establish a
305 quantitative comparison of our LT₅₀ value for *M. terrapin* with other emydid turtles. The only published study
306 we have identified that reports an LT₅₀ for an emydid turtle was that by Christie and Geist (2016) who
307 reported that the upper LT₅₀ = 40.3 °C for *Emys marmorata*, which was much higher than nests would be
308 expected to consistently experience (mean natural nest temperatures were 24 to 30 °C; Christie and
309 Geist, 2016).

310 Other studies of emydid turtle embryos exposed to high temperature do confirm that mortality
311 increases when average incubation temperatures increase above an optimal value, although this

312 optimum likely is species- and population-specific (e.g. Sinclair et al., 2016), as are other, thermally-
313 dependent traits such as hatchling sex ratios (see Burke and Calichio, 2014). *Chrysemys picta* and
314 *Trachemys scripta* displayed ~ 80 % hatching success when incubated at a mean temperature of 31 °C
315 (the highest temperature tested; Les et al., 2009), suggesting that the embryonic LT₅₀s are somewhat
316 above this value. Another study of *T. scripta* (Packard et al., 1989) noted a 19 % decline in hatching
317 success for eggs incubated at 27.7 to 28.7 °C relative to those incubated at 25.7 to 26.7 °C. A study of
318 Blanding's turtles (*Emydoidea blandingii*) reported that hatching success of declined from 95 % in 26.5
319 °C incubations to 77 % in 31 °C incubations (Gutzke and Packard, 1987).

320 Other groups of turtles have also been evaluated for thermal impacts on embryonic
321 development and survival, with a frequent result being that embryonic lethal upper temperatures were
322 somewhat lower than we observed in *M. terrapin*. A study of the freshwater snapping turtle (*Chelydra*
323 *serpentina*; Chelydridae) showed large declines in embryonic survival between 28.5 and 31 °C when
324 incubated in dry substrates; moist substrates resulted in a much higher survival rate (Packard et al.,
325 1987). A later study of *C. serpentina* revealed that an incubation temperature of about 40 °C resulted in
326 50 % embryonic mortality within 30 minutes (Ewert et al., 2005). The freshwater turtle, *Elusor macrurus*
327 (Family Chelidae), experienced 56 % hatching success when incubated at a constant temperature of 32
328 °C, compared to 89 % hatching success when incubated at 26 or 29 °C (Micheli-Campbell et al., 2011).
329 Another study of the same species (Micheli-Campbell et al., 2012) reported extreme reductions in
330 hatching success (about 5 % hatched) at an otherwise benign temperature (23 °C) when incubation
331 temperature fluctuated +/- 6 °C about the mean (23 °C), similar to the findings of Valenzuela et al.
332 (2019) for *C. picta*. Less extreme variation (23 +/- 3 °C) or constant temperature incubation at 23 °C
333 resulted in about 89 % hatching success rates of *E. macrurus* (Micheli-Campbell et al., 2012).

334 Although the above studies might seem to suggest that *M. terrapin* in our study population are
335 relatively resilient as embryos to high temperature, such tolerance is not atypical. For example, in the

336 tropical flatback sea turtle (*Natator depressus*, Cheloniidae), about 90 % hatching success was observed
337 in 35 °C incubations (Howard et al., 2014). Tortoises (Family Testudinidae) experienced reduced
338 hatching success at incubation temperatures above about 30 °C; hatching success of gopher tortoise
339 (*Gopherus polyphemus*) declined from 63 % at 30 °C to 4 % at 34 °C (Demuth, 2001) and that of the red-
340 footed tortoise (*Chelonoidis carbonarius*) declined from 52 % at 29 °C to 0 % at 33 °C (Hernández-
341 Montoya et al. 2017). Incubations of embryonic desert tortoises (*Gopherus agassizii*) at various
342 temperatures in dry sand revealed a rapid decline in hatching success between 32.8 (93 % success) and
343 35.3 °C (29 % success; Spotila et al., 1994), suggesting an LT₅₀ approximating ours for *M. terrapin*.

344 While reiterating our call for more quantitative, comparative studies specifically focused on
345 embryonic thermal tolerance in groups of turtles that have been poorly-studied in that regard, it must
346 be acknowledged that there are caveats to extrapolating thermal responses among species, not the
347 least of which is population-level variation in the relationship between performance and temperature
348 (the thermal performance curve; see Sinclair et al., 2016; Valenzuela et al., 2019). In fact, it could be
349 argued that the only relevant estimates of thermal effects that can be made for a population would be
350 the empirically derived values from representatives of the study population itself (e.g. Sinclair et al.,
351 2016, Valenzuela et al., 2019). Empirical comparisons of thermal sensitivity (embryonic LT₅₀, for
352 example) among populations of *M. terrapin* across its rather extensive range (from the western Gulf of
353 Mexico to Cape Cod) would provide valuable insight into whether species-level data for *M. terrapin* are
354 sufficiently resolved to predict changes in specific populations as the climate warms. For example, Burke
355 and Calichio (2014) reported differences in the relationship between incubation temperature and
356 offspring sex ratio between New York and Maryland populations of *M. terrapin*.

357 Regional models (MARISA, 2019) project that, in southern Maryland, U.S.A., the number of days
358 per year during which air temperature exceeds 35 °C (95 °F) will increase from four per year (years 1981
359 to 2010) to 29 to 60 per year by the end of this century, depending upon the modeled scenario

360 (MARISA, 2019). Furthermore, average summer temperatures are expected to increase by about 2.5 to >
361 4 °C (4.5 to > 7.5 °F) by year 2099 in the region occupied by our study population (Kunkel et al., 2013a
362 and b). Such elevations in mean temperature and the increased incidence of periods of excessive
363 temperatures (MARISA, 2019) may place the local *M. terrapin* population in jeopardy of experiencing
364 elevated embryonic mortality rates (e.g. Venezuela et al., 2019). Scenario testing (Figure 3) showed that
365 increases in mean nest temperature broadly spanning regional climate warming projections (2 or 5 °C)
366 resulted in accumulated thermal exposure > 34 °C, equivalent to our LT₅₀ conditions. Using 2018 nest
367 temperatures as a baseline, an increase by 2 °C would result in a mean temperature within the zone of
368 little or no temperature-induced, embryonic mortality (below about 31 °C; Figure 2) but the accrued
369 thermal exposure due to natural variability would place the embryos well within the LT₅₀ confidence
370 limits. The disparity between these two potential outcomes underscores the importance of accounting
371 for fluctuating temperatures when predicting metabolic or mortality responses at or near physiological
372 thermal thresholds.

373 Cooler incubation temperature (treatments C31, F31) lengthened development (slowed
374 developmental rate) relative to warmer temperature (C34). Cooler (31 °C) temperature also produced
375 dimensionally larger (CL and CW) hatchlings than the warmer (34 °C) temperature, and fluctuating
376 temperatures (F31, F34) produced larger (CL) hatchlings than constant temperatures (C31, C34). There
377 are numerous examples of incubation temperature influencing developmental rates and hatchling traits
378 in turtles in the literature. For example, in a recent study by Stubbs and Mitchell (2018) on embryonic
379 green turtles (*C. mydas*), developmental rate was faster, but hatchlings were smaller (CL, CW, ww, head
380 length and width) and had greater residual yolk mass at warmer temperature (31 °C) relative to a cooler
381 temperature (27 °C). The results generally agree with predictions by Cavallo et al. (2015) that future,
382 warmer temperatures will produce smaller hatchlings.

383 Our results for yolk size, which did not differ among treatments, contrast with those of Stubbs
384 and Mitchell (2018) who observed increased residual yolk mass in hatchlings from higher temperature
385 incubations. Our observation of reduced hatchling size at elevated temperature is also inconsistent with
386 an earlier study of Maryland's *M. terrapin* incubated at two temperatures (27 and 31 °C), in which
387 higher temperature produced elevated developmental rates and elevated growth rates after hatching,
388 but no effect of temperature on hatchling size was observed (Roosenburg and Kelley, 1996). The lack of
389 a statistical effect on hatchling size (Roosenburg and Kelley, 1996) likely reflected the confounding
390 effects of temperature and hatchling sex (different hatchling sex ratios at different temperatures), as
391 was also the case in the study by Stubbs and Mitchell (2018). Such confounding is not unusual (and is
392 frequently unavoidable) in studies of turtles that display temperature-dependent sex determination. A
393 feature of our study is that all hatchlings should have been female, as the constant temperature range
394 producing mixed male and female clutches spans from 28.9 to 29.6 °C, above which only females are
395 produced (Burke and Calichio, 2014). Thus, the effects of temperature on hatchling traits that we
396 observed should have been independent of possible influences of hatchling sex.

397 While fluctuating temperatures did not differ from constant temperatures with respect to
398 hatching success, developmental rates were reduced (longer time to hatching) when temperature
399 fluctuated about 34 °C (F34) relative to when it remained constant at 34 °C (C34). In fact, developmental
400 rates in both constant treatments (C31, C34) were faster than the corresponding fluctuating treatments
401 (F31, F34). This result is congruent with that of Du et al. (2009) who reported faster development in
402 *Chinemys reevesii* (Geoemydidae) incubated at constant relative to fluctuating temperatures. Elevated
403 developmental rate at constant temperatures is likely a reflection of the difference in cumulative
404 thermal exposure between the constant and fluctuating temperature treatments (e.g. Figure 3), in
405 which constant temperature embryos were not subject to the recurring negative temperature
406 anomalies present under fluctuating conditions as temperature varied about the mean.

407 Fluctuations about a high average temperature can also delay development if the fluctuations
408 exceed a threshold beyond which development is arrested (Les et al., 2009). Temperature fluctuations
409 above an upper limit for development (“high thermal limit, HTL,” Les et al., 2009) result in delayed
410 development relative to corresponding constant temperature treatments (see also Georges et al., 2005).
411 If the rate of increase in development with temperature declines above the HTL, and development is
412 additionally slowed during periods in which temperature fluctuates below the mean value, the net result
413 is a reduced rate of development in the fluctuating temperature treatment relative to constant
414 temperature of the same mean value (see Les et al., 2009). While we cannot define the HTL for *M.*
415 *terrapin* based upon our results, it is perhaps noteworthy that temperatures in treatment F34 frequently
416 exceeded 37 °C (Figure 5), the mean temperature at which all embryos died early in development. We
417 also observed (Table 1) that the reduction in developmental rate (increased time to hatch) in fluctuating
418 versus constant regimes was > 2-fold greater at 34 °C (e.g. time to hatch: F34 - C34 = 5.2 days) than at
419 31 °C (F31 – C31 = 1.9 days), suggesting that temperature likely fluctuated above the HTL in the 34 °C
420 treatments more frequently than in the 31 °C treatments. In fact, the higher DD_{>34} value in the F34
421 treatment (28.4 DD_{>34}) relative to the C34 treatment (5.9 DD_{>34}) demonstrates that the accrued thermal
422 exposure of the eggs to temperatures exceeding our approximate modeled LT₅₀ (~ 34 °C) was five-fold
423 higher in the fluctuating treatment (Figure 3).

424 *4.1. Implications of climate change for M. terrapin in Maryland*

425 Diamondback terrapins possess physiological traits and ecological requirements that confer
426 them with susceptibility to the changing climate. In this study we specifically evaluated the survival
427 response of embryos to incubation temperatures above current mean values in natural nests. It appears
428 that there is sufficient resiliency in the thermal tolerance of *M. terrapin* embryos to accommodate
429 current and predicted near-future (~ 30 years) climate conditions in the Chesapeake Bay region. Yet,

430 progressive increases in nest temperatures within regional ensemble climate projections are likely to
431 lead to increased embryonic mortality within the 21st century.

432 There are, however, other aspects of climate change that are likely to affect Maryland's
433 population of *M. terrapin*, perhaps more immediately than the thermal impacts on hatching success
434 studied here. The ectothermic physiology of *M. terrapin* dictates that body temperature varies with
435 operative temperature (e.g. Bakken et al., 2015), elevating metabolic expenditures on maintenance
436 (standard metabolic rate) as temperature increases within pejus temperature limits (e.g. Dillon et al.,
437 2010; Huey et al., 2012; Schulte, 2015). To avoid energetic deficits due to elevated metabolic activity at
438 elevated temperature, energy (resource) assimilation will have to increase (in the absence of behavioral
439 changes or adaptation). An increased "cost of living" in a warmer environment, and subsequent needs
440 for greater resource supplies, could ultimately restrict performance of individuals (e.g. Dillon et al.,
441 2010).

442 Similar to many other turtles, *M. terrapin*'s offspring sex ratios are thermally labile through TSD,
443 a trait that can confer risks of skewed sex ratios in a warmer future (see Janzen, 1994; Katselidis et al.,
444 2012; Laloë et al., 2016; Pezaro et al., 2017; Venezuela et al., 2019). In *M. terrapin*, TSD follows a pattern
445 in which males are produced at relatively low temperatures and females at higher temperatures. The
446 transitional range in temperatures (TRT), in which both sexes are produced, is < 1 °C (28.9 to 29.6 °C) for
447 Maryland's *M. terrapin* (Burke and Calichio, 2014). Such a narrow TRT suggests that even modest
448 temperature variations could substantially affect offspring sex ratios, ultimately bringing about a
449 demographic shift to a female-biased population with ramifications for future population growth rates
450 (e.g. Janzen, 1994; Katselidis et al., 2012; Pezaro et al., 2017; Valenzuela et al., 2019).

451 Compounding any physiological challenges experienced by *M. terrapin* in the warmer future will
452 be a rapid and widespread loss of foraging and nesting habitats. In the mid-Atlantic region of North

453 America, most climate models project an increase in mean sea level of over one meter within the 21st
454 century (Boesch et al., 2013, 2018). As a result, brackish marshes and undeveloped, low-elevation, sandy
455 areas that are critical for *M. terrapin* populations are in substantial decline in the Maryland portion of
456 the Chesapeake Bay (Woodland et al., 2017). Therefore, any future, thermally-induced effects on
457 hatching rates or physiology will operate within the ecological context of increasing restrictions on
458 habitats that are critical for population maintenance.

459 **5. Conclusions**

460 *Malaclemys terrapin* in Maryland could face a perilous future as the global climate continues to
461 warm. Our investigation revealed that hatching success declined between 31 and 34 °C, regardless of
462 whether the temperature fluctuated about the mean or was constant throughout incubation. The
463 estimated, embryonic LT₅₀s were 34.0 and 34.2 °C for constant- and fluctuating temperature
464 incubations, respectively. Recent mid-season nest temperatures in 2018 and 2019 averaged 28.6 and
465 29.8 °C, several degrees below that at which embryonic mortality was observed. Relative to some other
466 turtles, hatching success of *M. terrapin* in this population appears to be more resilient to warming levels
467 projected for the early- to mid-21st century. On the other hand, by the late 21st century, average nest
468 temperatures could approach or exceed the LT₅₀ for embryos in this population. Other, perhaps more
469 immediate, threats of the changing climate to *M. terrapin* are likely to be associated with habitat loss
470 due to sea level rise (Woodland et al., 2017), and, possibly, thermal influences on sexual differentiation
471 (e.g. Janzen, 1994) and bioenergetics (e.g. Willette et al., 2005; Dillon et al., 2010). The physiological and
472 ecological constraints that Maryland's *M. terrapin* will face as the global climate continues to rapidly
473 change could have significant consequences for the population in the future.

474

475 **Conflicts of Interest**

476 The authors declare no conflicts of interest involving this project or the contents of this manuscript.

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487

488 **Figure Legends**

489 Figure 1. Weekly assignment of temperatures to fluctuating temperature treatments based on observed
490 nest temperature data from the field site in 2018, with an example shown (upper left panel) from 27
491 June 2018 to 3 July 2018 (“2018 Temp_{Obs}”). Weekly field nest temperature traces were detrended,
492 averaged at 10 min intervals to obtain a mean daily temperature cycle for that week, then incrementally
493 increased to yield the target time-integrated mean temperature for each fluctuating experimental
494 treatment (upper right panel; 31, 34, or 37 °C “Temp_{Exp}”). The mean daily temperature trace was applied
495 to that seven-day interval when programming fluctuating-temperature thermostats during the 2019
496 incubation experiments (lower left panel). As a result, the incubation period consisted of seven
497 consecutive, seven-day periods within which daily temperature fluctuations were identical but between
498 which daily temperature fluctuations differed.

499

500 Figure 2. Hatching success versus temperature in constant temperature (light gray) and fluctuating
501 temperature (dark gray) treatments (upper panel). Predicted hatching rates and 95 % confidence
502 intervals (indicated by the colored region) were generated using bootstrapping (lower panel). The
503 modeled LT₅₀ for each treatment is indicated by a vertical line.

504

505 Figure 3. Cumulative degree days exceeding 34 °C (DD_{>34}) plotted for field nest temperatures in 2018
506 (Field, open bar); experimental constant (C) and fluctuating (F) temperature incubators with mean
507 temperatures = 31 °C (blue bars), 34 °C (red bars), and 37 °C (black bars); and climate scenarios (gray
508 bars) of + 2 °C and + 5 °C above 2018 field nest measurements. Numbers above each bar indicate
509 calculated DD_{>34} index values.

510

511 Figure 4. Average maximum daily air temperatures during the mid-nesting season (June 20 to August 9,
512 2001 to 2019) at Naval Air Station Patuxent River, Lexington Park, MD, where the study population
513 nests. Horizontal lines on 2018 and 2019 bars indicate mean nest temperatures during these years. Year
514 2006 was excluded due to limited data. Data are from <https://www.ncdc.noaa.gov/cdo-web>.

515

516 Figure 5. Temperature traces from each incubator throughout the incubation experiment. Top panel:
517 Constant temperature treatments. Bottom panel: Fluctuating temperature treatments. Temperatures
518 were measured at 10 minute intervals within the incubation substrate.

519

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