

1 **Title:** Maternal provisioning interacts with incubation temperature to affect hatchling mercury
2 exposure in an oviparous reptile

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13
14 **Abstract**

15 The thermal environment experienced by developing embryos can influence the utilization of
16 maternally-provisioned resources. Despite being particularly consequential for oviparous
17 ectotherms, these dynamics are largely unexplored within ecotoxicological frameworks. Here,
18 we test if incubation temperature interacts with maternally-transferred mercury to affect
19 subsequent body burdens and tissue distributions of mercury in hatchling American alligators
20 (*Alligator mississippiensis*). Nine clutches of alligator eggs were collected from a mercury-
21 contaminated reservoir and incubated at either female- or male-promoting temperatures. Total
22 mercury (THg) concentration was measured in egg yolk collected during incubation and in a
23 suite of tissues collected from hatchlings. THg concentrations in residual yolk and blood were

24 higher in hatchlings incubated at cooler, female-promoting temperatures compared to the
25 warmer, male-promoting temperatures. THg concentrations in most tissues were positively
26 correlated with THg concentrations in blood and dermis, and egg yolk THg concentration was
27 the best predictor of THg concentration in many resultant tissues. Our results highlight a hereto
28 unknown role of the developmental environment in mediating tissue specific uptake of
29 contaminants in an oviparous reptile.

30
31 **Keywords:** crocodilian, ecotoxicology, maternal effects

32 33 **Introduction**

34 Maternal provisioning of resources to developing offspring is critical for successful reproduction.
35 In oviparous vertebrates, females deposit nutrients and signaling molecules into eggs, providing
36 both energy and information necessary for the development of the zygote into a free-feeding
37 organism [1–6]. The quality and quantity of these resources are often determined by ecological
38 dynamics including resource availability [7–10], diet [1,11,12], stress status [13,14], and
39 potentially environmental contaminants [15,16]. Thus, maternal provisioning represents a
40 biological pinch point at which prior ecological conditions converge to influence offspring
41 survival and resultant maternal fitness [2,7–9].

42
43 In many non-mammalian vertebrates, the thermal environment experienced by developing
44 embryos influences hatchling phenotypes and, in some cases, sex [17,18]. In the American
45 alligator (*Alligator mississippiensis*), a species with temperature-dependent sex determination
46 (TSD), warmer incubation temperatures (32.5–33.5 °C) result in male development and more

47 efficient conversion of maternal resources into hatchling mass compared to cooler, female-
48 promoting temperatures (29–31 °C) [19–21]. Temperature is known to affect toxicity of external
49 contaminants in developing fish [22] and amphibian [23] eggs, but this phenomenon has not
50 been examined in developing reptiles or in the context of maternally-transferred contaminants.
51 Given that maternal transfer of contaminants into eggs is broadly observed across taxa [24–28],
52 and developing organisms are particularly sensitive to deleterious impacts of contaminants [28–
53 34], this raises fundamental questions as to whether incubation temperature interacts with
54 maternally-derived contaminants to affect body burdens of contaminants and their anatomical
55 distribution within offspring.

56
57 Mercury is a widespread contaminant with well-characterized toxicity [28,30,35–38]. Maternal
58 transfer of mercury to eggs has been demonstrated across a range of taxa [15,26,27,30,39–44].
59 When converted from its inorganic to methylated form, mercury both bioaccumulates within
60 organisms and biomagnifies across trophic levels, making it a contaminant of concern for long-
61 lived, high trophic level predators [45–48]. For these reasons, the American alligator has been
62 used as a sentinel of mercury contamination in the southeastern United States for several decades
63 (Table S1 and citations therein). Herein, we use the alligator as a model system to investigate
64 how incubation temperature influences tissue distribution of maternally-derived mercury in
65 hatchlings from a mercury contaminated site.

66 67 **Methods**

68 We collected nine clutches of alligator eggs from nests located at Par Pond, a mercury-
69 contaminated reservoir on the U.S. Department of Energy’s Savannah River Site, South Carolina

70 [49,50], during the summer of 2021. For more detailed site description and methods on egg
71 collection/incubation, see [51]. In brief, eggs were transported to the University of Georgia
72 Savannah River Ecology Laboratory (SREL) and incubated at 32 °C in programmable incubation
73 chambers (model I36NLC, Percival Scientific, Perry, IA, USA) until Ferguson Stage 15 [52],
74 which immediately precedes the thermosensitive period during which temperature determines
75 sex [53]. Upon reaching Stage 15, clutches were evenly split into either female- or male-
76 promoting temperature groups (FPT [29.5 °C] and MPT [33.5 °C], respectively) until hatch.
77 Since development occurs faster at MPT, individuals were allowed to hatch naturally so they
78 could be sampled at the same developmental stage. Four eggs per clutch were sacrificed at Stage
79 20 to establish baseline levels of maternally-transferred mercury in egg yolks. Hatchlings were
80 raised in living stream tanks (Frigid Units Inc., Toledo, OH, USA) under fasting conditions at the
81 SREL aquatic animal facility.

82
83 At 10 days post-hatch, 1–3 hatchlings per incubation treatment per clutch (n = 24) were
84 sacrificed for tissue collection. Just prior to dissection, blood samples were taken from the post-
85 occipital sinus using sterile 25G PrecisionGlide™ Needles (BD, Franklin Lakes, NJ, USA) and
86 disposable 1 mL luer slip syringes (Resway, Miami, FL, USA). Approximately 1500 µL of blood
87 was placed in lithium heparin Vacutainer® tubes (BD, Franklin Lakes, NJ, USA) and stored at -
88 20 °C until analysis. Hatchlings were then euthanized via decapitation and immediate pithing,
89 and the following tissues were collected for mercury analysis: brain, dermis, fat body, heart,
90 kidney (right only), liver, tail muscle, and residual yolk. Dermis (consisting primarily of
91 keratinous epidermal scales) and tail muscle were collected from the left lateral portion of the
92 tail. We use dermis as a proxy for tail scutes typically collected when marking crocodylians and

93 used in analogous analyses, since it is structurally similar and allowed for comparatively more
94 tissue to be collected. All tissue samples were placed in 58 mL (2-oz.) Whirl-Pak® bags (Nasco
95 Sampling, Madison, WI, USA) and stored at -20 °C until analysis. Blood samples from
96 hatchlings that were not sacrificed (n = 158) were collected 7–10 days post-hatch using the same
97 protocols; hatchlings were subsequently released at their original collection site.

98

99 Total mercury (THg) concentrations were analyzed using a DMA-80 *evo* Direct Mercury
100 Analyzer (Milestone, Shelton, CT, USA) according to U.S. EPA method 7473 and calibrated
101 each day prior to analysis using standard reference materials (TORT-3 and PACS-3, National
102 Research Council of Canada). Yolk from sacrificed eggs and blood samples from non-euthanized
103 individuals were vortexed for ~10 seconds to homogenize and run on a wet weight basis; quality
104 assurance checks for these samples are reported elsewhere [51]. All other samples (except blood
105 from sacrificed individuals, processed the same as prior blood samples) were weighed and
106 freeze-dried prior to analysis. Most freeze-dried samples were run whole; larger samples (liver
107 and residual yolk) were homogenized using a Wig-L-Bug grinder and a subset of ground tissue
108 was analyzed. For quality control, each batch of 10 samples was run with a blank and two
109 different standard reference materials (TORT-3 and PACS-3). For tissue samples that were not
110 run whole (i.e., egg yolk, blood, liver, residual yolk) a sample replicate was also included in each
111 batch, and sample duplicates were averaged. Duplicate samples were considered comparable if
112 the relative percent difference in THg concentration was <10%, and all samples were below this
113 limit. The method detection limit (calculated as threefold the standard deviation of procedural
114 blanks) was 0.193 ng/g of sample, and all samples exceeded this limit. Average relative percent
115 difference between replicate samples was $1.23 \pm 0.35\%$ (n = 8). Mean percent recoveries of THg

116 for the certified reference materials were $109.29 \pm 0.69\%$ ($n = 23$) for TORT-3 and $102.47 \pm 2.28\%$
117 ($n = 23$) for PACS-3.

118

119 Dry weight concentrations were used in analyses to provide more accurate comparisons between
120 tissues and individuals [54,55]; exceptions are for blood and egg yolk samples, for which we
121 only have wet weight concentrations. THg concentrations were compared across tissue and
122 incubation treatment for all tissues, and across incubation treatment and clutch for our larger
123 blood sample set (two-way analysis of variance). Liver to muscle THg ratios were compared
124 between incubation treatments (Wilcoxon test). We built linear models comparing THg
125 concentration in blood and THg concentration in dermis with THg concentration in all other
126 tissues to determine if either blood or dermis could be used as a non-lethal indicator of mercury
127 concentration. THg concentration was log-transformed prior to model building; model residuals
128 were checked for normality (Shapiro-Wilk test) and plotted to visually assess homoscedasticity.
129 To determine factors influencing the distribution of mercury within hatchling tissues, we built
130 predictive linear models for each tissue type incorporating clutch-averaged THg concentrations
131 in egg yolk (reflective of initial maternally-transferred mercury burdens), incubation treatment
132 (reflective of differing metabolic rates [19]), and clutch-averaged hatchling mass (which could
133 affect THg concentrations via a biodilution effect [44]). Full models and all possible iterations
134 were ranked using AICc model selection using the “dredge” function in the package “MuMIn”
135 [56], and results for all supported models ($\Delta AICc < 2$) are reported. All statistical analyses were
136 conducted in R version 4.1.2 [57].

137

138 **Results**

139 Tissue mercury concentrations are reported in Table S1. THg concentration differed significantly
140 with tissue type ($p < 0.001$; Figure 1A), incubation treatment ($p = 0.006$), and the interaction
141 between tissue type and incubation treatment ($F_{7,173} = 5.848$, $p < 0.001$). Post-hoc analysis
142 revealed a significant difference between incubation treatments only in residual yolk ($p < 0.001$).
143 In our larger blood sample set, clutch ($p < 0.001$; Figure 1B), incubation treatment ($p < 0.001$;
144 Figure 1C), and the interaction between clutch and incubation treatment ($F_{8,141} = 2.703$, $p =$
145 0.008) were significantly correlated with THg concentration. Liver to muscle THg ratios
146 averaged 0.425 ± 0.042 for MPT and 0.596 ± 0.054 for FPT, and were significantly different
147 between incubation treatments ($W = 27$, $p = 0.008$).

148

149 THg concentration in most tissues was significantly correlated with THg concentration in both
150 blood (Figure 2A) and dermis (Figure 2B) in our linear models, with the exception of residual
151 yolk (both models) and fat body (dermis model only). Our predictive linear models largely
152 incorporated only clutch-averaged egg yolk THg concentrations in the top model (Table 1). The
153 top predictive models for blood ($\beta = -0.041 \pm 0.019$) and residual yolk ($\beta = -0.427 \pm 0.171$)
154 incorporated negative correlations with incubation at MPT.

155

156 **Discussion**

157 We observed significant effects of incubation temperature on THg concentrations in residual
158 yolk and blood, wherein individuals incubated at FPT had higher THg concentrations compared
159 to those at MPT. The influence of incubation temperature on contaminant body burdens in
160 reptiles has hereto been unexplored, and our findings present the possibility that other aspects of
161 the developmental environment may have additional effects, with downstream impacts on sex

162 and/or size specific mortality. Incubation at FPT produces smaller hatchlings compared to
163 incubation at MPT [19]. This reduction in hatchling mass is thought to result from increases in
164 the energetic cost of development at FPT, with more maternal resources expended on metabolic
165 processes (versus somatic growth) required to complete development [19,58]. Thus, elevated
166 THg concentrations in FPT hatchlings could be due to maternally-deposited mercury
167 representing a higher proportion of hatchling mass in smaller individuals. We are uncertain why
168 we did not observe temperature-related differences in THg concentration in other tissues but
169 speculate this is due to a lack of bioaccumulation time. Since FPT individuals have greater
170 reserves of mercury still being absorbed from residual yolk and blood, we expect differences in
171 THg concentration would develop between presumptive sexes.

172
173 Alligator hatchling tissues varied greatly in THg concentration. As our recovery of certified
174 reference materials during the mercury analysis was slightly higher than expected, we
175 acknowledge that THg concentrations reported here may be overestimated. However, since all
176 tissues were analyzed using the same protocols, results should be comparable within our sample.
177 THg concentrations in liver ranked comparatively low amongst all tissues, contradictory to high
178 liver THg concentrations reported in adults [54,55,59,60,61]. The liver is generally considered
179 the main site of mercury storage and depuration [47,54,55,59,60,62], presenting two possibilities.
180 First, liver THg concentrations observed in this study reflect differences in exposure route, with
181 maternally-deposited mercury being found in different tissues (e.g., muscle) relative to dietary
182 mercury. The second possibility is that depuration processes actively remove detoxified mercury
183 from the liver, decreasing hepatic concentrations in the absence of additional inputs. Liver to
184 muscle THg ratios can be used as indicators of active mercury uptake (ratios >1) or depuration

185 (ratios <1) [54,55,63–65]. Incubation at FPT resulted in higher ratios compared to those observed
186 in individuals incubated at MPT, suggesting that incubation temperature may alter mercury
187 depuration rates in hatchling alligators, although future research is needed to experimentally
188 verify this link.

189

190 Total mercury concentrations in both blood and dermis were significantly correlated with THg
191 concentrations in other tissues, except for residual yolk. There is an impetus for non-lethal
192 mercury sampling [42,62,66,67], and blood has proven a reliable indicator, showing high levels
193 of correlation with internal organs (blood-muscle: $R^2 = 0.9499$ [59]; blood-muscle: $R^2 = 0.82$,
194 blood-liver: $R^2 = 0.79$ [66]). We did not observe such high correlation coefficients, suggesting
195 that although blood could potentially be used as a non-lethal indicator of mercury in hatchlings,
196 this relationship appears stronger in adults. Scutes (proxied by dermis) and egg yolk could also
197 serve as potential indicators, the latter requiring minimal sacrificing without the need to capture
198 hatchlings.

199

200 The developmental environment can play an important yet largely unexplored role in modifying
201 the dynamics of maternally-transferred contaminants. We noted important distinctions when
202 assessing mercury concentrations in neonate alligators compared to adults. Liver THg
203 concentrations in hatchlings are lower compared to concentrations in most other tissues, and
204 blood does not appear to be as reliable an indicator as it is in adults [46,59,66]. Furthermore,
205 incubation temperature influences the dynamics of maternally-derived mercury, potentially
206 mediating developmental cost or depuration. This novel finding is the first case in reptiles that
207 we know of where incubation temperature affects post-hatching tissue concentrations of a

208 widespread contaminant. Our finding is likely to extend to other oviparous species, particularly
209 those reliant upon TSD such as turtles and other crocodylians [21]. Given maternal transfer of
210 other fat-soluble contaminants has been reported [24], this study raises important questions
211 regarding both how the developmental environment mediates these dynamics and their ultimate
212 consequences for affected wildlife.

213

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220

221 **References**

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446 **Figure Captions**

447 **Figure 1.** Distribution of THg concentrations across hatchling tissues (a); letters signify
448 significant differences in THg concentration across tissue types. Blood samples from non-
449 euthanized individuals (b) are shown across clutches along with overall differences between FPT
450 and MPT (c). Boxes show median, first, and third quartiles (inter-quartile range); whiskers
451 extend up to 1.5 times the inter-quartile range, and outliers are shown as individual points.

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453 **Figure 2.** Linear models demonstrating correlations between THg concentration in blood (a) and
454 dermis (b) to THg concentration in other tissues, with model statistics. For (b), blood is reported
455 in wet weight, and all concentrations were log-transformed for analysis.

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Table 1. Summary statistics for predictive models of THg concentration in hatchling tissues. Concentrations were log-transformed for analysis. The full model incorporates clutch-averaged egg yolk THg, incubation treatment, and clutch-averaged hatchling mass (averaged across incubation temperature within clutch). Models for the same tissue are listed in sequential order of support.

Tissue	Supported models	Log-likelihood	AICc	Akaike weight	R²	p-value
Blood	Egg yolk THg + Incubation treatment	3.4	6.8	0.467	0.685	0.002
	Egg yolk THg	1.0	7.0	0.422	0.573	0.003
Kidney	Egg yolk THg	9.6	-10.1	0.833	0.792	<0.001
Fat body	Egg yolk THg	-1.2	11.8	0.552	0.421	0.018
	Egg yolk THg + Incubation treatment	0.5	13.6	0.229	0.525	0.021
Brain	Egg yolk THg	14.3	-19.6	0.535	0.921	<0.001
	Egg yolk THg + Incubation treatment	15.9	-18.2	0.255	0.933	<0.001
Dermis	Egg yolk THg	11.0	-12.0	0.904	0.854	<0.001
Muscle	Egg yolk THg	8.3	-7.5	0.685	0.816	<0.001
Heart	Egg yolk THg	10.6	-12.2	0.557	0.841	<0.001
	Egg yolk THg + Incubation treatment	12.5	-11.2	0.345	0.871	<0.001
Liver	Egg yolk THg	-0.0	9.0	0.453	0.615	0.002
	Egg yolk THg + Incubation treatment	1.9	9.8	0.300	0.691	0.002
	Egg yolk THg + Hatchling mass	1.6	10.5	0.218	0.674	0.003
Residual yolk	Incubation treatment	-1.3	11.6	0.571	0.326	0.031

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