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Hansson, A., & Olsson, M. M. (2018). The influence of incubation temperature on phenotype of Australian Painted Dragons (*Ctenophorus pictus*). Faculty of Science, Medicine and Health - Papers: Part B. Retrieved from <https://ro.uow.edu.au/smhpapers1/983>

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The influence of incubation temperature on phenotype of Australian Painted Dragons (*Ctenophorus pictus*)

Abstract

Incubation temperature is one of the most studied factors driving phenotypic plasticity in oviparous reptiles and has been shown to affect a wide variety of traits including body size, shape, and performance. Thermal regimes during embryogenesis might therefore have direct consequences on fitness, potentially even shaping population trajectories. These effects are likely strongest in short-lived species where even temporary temperature-induced differences in body size or shape might have adaptive significance. We investigated the effects of incubation temperature on the body size and shape of hatchling Australian Painted Dragons (*Ctenophorus pictus*). Eggs incubated at low temperature required a longer incubation period, but produced hatchlings of greater body mass. However, no effect of temperature was found on the structural dimensions of hatchlings. These results might be explained by an increased absorption of water by the developing embryo during the prolonged incubation period. A greater water content might increase early-life desiccation tolerance in this short-lived lizard inhabiting arid and semiarid environments. Egg mass, however, had the strongest effect on hatchling phenotype, with larger eggs producing larger hatchlings. Furthermore, there was a seasonal effect on yolk allocation, with eggs laid earlier being larger than those laid later. Our results indicate that yolk allocation is the most important factor affecting hatchling phenotype in this species, while temperature mainly affects embryo developmental rate and likely has an indirect effect on hatchling water content.

Publication Details

Hansson, A. & Olsson, M. (2018). The influence of incubation temperature on phenotype of Australian Painted Dragons (*Ctenophorus pictus*). *Herpetologica*, 74 (2), 146-151.

1 **Title page**

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3

4 **The Influence of Incubation Temperature on Phenotype of Australian Painted**
5 **Dragons (*Ctenophorus pictus*)**

6

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8

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13 RRH: HANSSON AND OLSSON – INCUBATION TEMPERATURE AND PHENOTYPE IN

14 *CTENOPHORUS*

15

16 ABSTRACT: Incubation temperature is one of the most studied factors driving phenotypic
17 plasticity in oviparous reptiles and has been shown to affect a wide variety of traits including
18 body size, shape and performance. Thermal regimes during embryogenesis may therefore have
19 direct consequences on fitness, potentially even shaping population trajectories. These effects are
20 likely strongest in short-lived species where even temporary temperature-induced differences in
21 body size or shape may have adaptive significance. We investigated the effects of incubation
22 temperature on the body size and shape of hatchling Australian Painted Dragons (*Ctenophorus*
23 *pictus*). Eggs incubated at low temperature required a longer incubation period, but produced
24 hatchlings of greater body mass. However, no effect of temperature was found on the structural
25 dimensions of hatchlings. These results may be explained by an increased absorption of water by
26 the developing embryo during the prolonged incubation period. A greater water content may
27 increase early life desiccation tolerance in this short-lived lizard inhabiting arid and semi-arid
28 environments. Egg mass, however, had the strongest effect on hatchling phenotype, with larger
29 eggs producing larger hatchlings. Furthermore, there was a seasonal effect on yolk allocation,
30 with eggs laid earlier being larger than those laid later. Our results indicate that yolk allocation is
31 the most important factor affecting hatchling phenotype in this species, while temperature mainly
32 affects embryo developmental rate and likely has an indirect effect on hatchling water content.

33

34 **Key words:** Agamidae; Australian lizard; Egg; Hatchling phenotype; Phenotypic
35 plasticity; Seasonality; Water content; Yolk allocation

36

37 ENVIRONMENTALLY induced phenotypic variation (phenotypic plasticity) is common in
38 nature and is proposed to be a property of the genotype (Scheiner 1993), and is often highly
39 specific to both taxa and developmental stages. Plastic responses to environmental conditions can

40 occur at any stage during an organism's life. However, phenotypic plasticity during the egg stage
41 in oviparous reptiles may be of particular importance as embryogenesis and hatching are likely to
42 be under strong selection (Lindström 1999; Shine and Olsson 2003). The hatchling stage being
43 one of those under the strongest selection in the wild, with often more than 80–90% mortality
44 (Olsson and Madsen 2001). One of the most studied environmental factors known to drive
45 phenotypic plasticity at this stage of development is incubation temperature (Deeming 2004),
46 because embryos lack or have very limited ability to behaviorally thermoregulate (but see
47 behavioral thermoregulation in turtle embryos, Du et al. 2011; Zhao et al. 2013). This obviously
48 changes after hatching, when the neonate can exploit ambient thermal heterogeneity to control
49 their body temperature (Stevenson 1985) within relatively narrow ranges (Huey et al. 2012).
50 Eggs, however, are clearly immobile, forcing them to develop at ambient thermal regimes
51 predetermined by the mother's nest-site selection. The site of oviposition may therefore be a way
52 for the mother to indirectly manipulate the offspring's phenotype by exposing the eggs to certain
53 temperatures (Shine and Harlow 1996).

54 There is extensive experimental work on how incubation temperature may induce
55 phenotypic variation in the offspring. Incubation temperature has, in oviparous reptile hatchlings,
56 been shown to modify the expression of several phenotypic traits, such as body size, body shape,
57 sex, locomotor performance, pigmentation, and anti-predator behavior (Deeming 2004). Recent
58 studies even suggest that incubation temperature may affect brain development and thus also
59 cognitive ability (Amiel and Shine 2012; Amiel et al. 2016). All of these phenotypic traits are
60 likely closely linked to the fitness of both hatchling and adult reptiles, and thus subjected to
61 selection. Growing evidence shows that temperature-dependent effects during incubation on
62 hatchling phenotype may be species-specific (Braña and Ji 2000; Shine 2004; Telemeco et al.
63 2010; Ballen et al. 2015), making generalizations difficult. A recent meta-analysis examining the

64 effects of incubation temperature on phenotype and survival in reptiles found that temperature
65 most strongly and widely affected incubation duration and survival (Noble et al. 2017). However,
66 the study also recognized the unpredictability of effects, especially in Squamata, which highlights
67 the importance of examining effects on specific species, rather than generalizing. The choice of
68 experimental incubation temperature should, if possible, be selected based on some form of
69 putative knowledge such as temperature in natural nests, optimal incubation temperature, or one
70 of these two combined with future predictions of climate change within a reasonable time-frame,
71 if the goal is to make evolutionary inferences of natural populations.

72 Here, we used Australian Painted Dragons (*Ctenophorus pictus*) to examine the variation
73 in hatchling phenotypes associated with incubation temperature, with the aim of identifying its
74 effects on incubation duration, hatching success, hatchling size and hatchling shape. No previous
75 study has examined effects of incubation temperature on any phenotypic trait except sex (Harlow
76 2004; Uller et al. 2006) in this species. However, based on similar studies in other species,
77 incubation duration is expected to decrease with temperature and hatchling success may either
78 decrease with temperature or be unaffected (Chen and Ji 2002; Lu et al. 2009; Esquerré et al.
79 2014; reviewed in Noble et al. 2017). The phenotypic traits examined in this study were chosen
80 because previous work has identified their links to components of fitness (Flatt et al. 2001;
81 Rodríguez-Díaz et al. 2010).

82

83

MATERIALS AND METHODS

84

Study Species and Husbandry

85

86

87

Australian Painted Dragons are small, 8–16 g, 65–95 mm snout–vent length (SVL),
agamid lizards occurring in open sandy habitats and low vegetation, with a range from central
and western New South Wales to Western Australia. Male Painted Dragons are polymorphic with

88 respect to head color, and typically occur in three different head color morphs (red, orange and
89 yellow). These lizards are short-lived, with only approximately 10% live past their first year, and,
90 especially red males, are highly territorial (Olsson et al. 2007). Adult Painted Dragons used in
91 this study were caught by noose or by hand in Yathong Nature Reserve, New South Wales,
92 Australia (145°35'E; 32°35'S; datum = GDA94). Lizards were housed in mating pairs in cages
93 (50 L x 40 W x 35 H cm) with a sand substrate and a 40 W spotlight at one end and rocks to
94 allow thermoregulation and shelter. The room temperature fluctuated daily between 15°C and
95 25°C to simulate natural conditions. Additionally, a small 'sand dune' was added to all cages and
96 was continuously moistened to act as a favorable oviposition site. They were fed with a variation
97 of crickets and meal worms dusted daily with calcium and weekly with multivitamins, and misted
98 daily. Males were constantly kept with the females during the entire duration of the experiment as
99 their presence is required for successful fertilization (Uller and Olsson 2005). The study took
100 place between European Autumn 2016 and Spring 2017.

101

102 Egg Collection and Incubation

103 Females produced 1–4 clutches with an average of 1.63 ± 0.20 (mean \pm SE, $n = 19$) clutches with
104 on average 3.67 ± 0.27 (mean \pm SE, $n = 30$) eggs per clutch. Cages were checked daily for
105 recently laid clutches. Laid fertilized eggs ($n = 110$ from 19 females) were cleaned from sand and
106 moisture, weighed to the nearest 0.001 g and placed individually in 125 mL disposable plastic
107 cups half-buried in moist vermiculite (1:5, water:vermiculate volume) filling a third of the cup.
108 The cups were sealed with plastic cling wrap and a rubber band to prevent moisture loss and
109 placed in incubators set at constant 28°C, 30°C and 32°C. There are no data on the thermal
110 profiles of the natural nests of *C. pictus*, and treatment temperatures were chosen based on the
111 previously suggested ideal incubation temperature of 30°C (Olsson et al. 2007). The three

112 incubation temperatures, however, all fall within the natural thermal range of nests in situ (M.
113 Olsson, personal observation). Here, we applied constant incubation temperatures which may be
114 problematic as it has been argued that natural nests experience diel temperature fluctuations
115 (reviewed in Booth 2006). Although while studies have found that fluctuating temperatures may
116 influence hatchlings differently than constant temperatures, these effects are often limited to
117 incubation duration and locomotor performances, rather than morphological traits (Ashmore and
118 Janzen 2003; Ji et al. 2007; Du and Shine 2010; Li et al. 2014), which is the focus of this study.
119 The true temperature in the incubators was measured using HOBO H08 data loggers (Onset
120 Computer Corporation, Bourne, MA) throughout the entire duration of the experiment (191
121 days). We used a split-clutch design in which eggs from a single clutch were allocated equally
122 across the three temperature treatments. The cups were rotated among three shelves in each
123 incubator every two weeks to minimize the effect of thermal gradients inside the incubator.
124 Additionally, water was added to each cup during this rotation to account for the small, however
125 evident, loss of water through evaporation (about 1 g per week). Eggs were then checked daily
126 for pipping and dead eggs were removed.

127

128 Measurement of Hatchling Phenotypes

129 On the day of hatching, hatchlings were blotted dry and brushed clean of vermiculite and
130 weighed to the nearest 0.001 g. The sex of hatchlings was determined by hemipenial
131 transillumination in males (Brown 2009) after a few weeks, as hemepenes were easier to identify
132 at this stage. Hatchlings that died prior to sexing were frozen, however, some deceased offspring
133 could unfortunately not be sexed due to the compromised tissue quality and lack of
134 vascularization on which transillumination sexing depends. Importantly, mortality was not sex-
135 specific (see results) and hence does not explain our results. Size measurements were taken using

136 a digital caliper of total length, SVL, tail length, abdomen length, head width, head length, and
137 right side: forelimb length and hind limb length (Fig. 1), all to the nearest 0.03 mm. The duration
138 of incubation, measured as the number of days to pipping, was recorded for each egg. At the end
139 of the experiment the hatchlings were recruited to the lab population and thus determination of
140 dry mass, tissue composition and residual yolk, requiring euthanasia, were not possible.

141

142 Statistical Analyzes

143 All models were fitted in R v3.3.2 (R Core Team 2016) following a linear mixed model
144 (LMM) and generalized linear mixed model (GLMM) approach using the functions *lmer* and
145 *glmer* from the lme4 package (Bates et al. 2015) with female identity as random effect. Estimates
146 of *P*-values were obtained using parametric likelihood ratio bootstrapping with the *PBmodcomp*
147 function from the pbrttest package (Ulrich and Søren 2014). All bootstrap analyzes were based
148 on 10,000 iterations. The effects of incubation temperature and egg mass on hatching success,
149 incubation duration and phenotypic traits were examined. We also investigated the possibility for
150 sexual dimorphism in the hatchlings and sex effects on incubation duration. The main effects
151 were corrected for the effects of egg mass and temperature, with a *P*-to-enter set at 0.15 (Bursac
152 et al. 2008). All data met the requirements of the statistical tests except a deviation from a normal
153 distribution in the abdomen lengths and incubation duration, which were corrected by log
154 transformations. The incubation temperature of each egg was calculated as an average of the
155 logger data from each incubation period. No interactions were found among incubation
156 temperature, initial egg mass and sex, and were therefore excluded from the analyzes.

157

158

RESULTS

159

Incubation Duration and Hatching Success

160 The actual temperatures in the incubators, measured by temperature loggers, were in the 28°C,
161 30°C and 32°C treatment (mean °C ± SE): 27.9 ± 0.002 ($n = 13,337$), 29.9 ± 0.004 ($n = 10,055$)
162 and 31.8 ± 0.004 ($n = 15,453$), respectively. Incubation duration was strongly affected by
163 incubation temperature (Table 2), with hatchlings emerging earlier at higher temperatures. The
164 average incubation periods differed over two weeks between 28°C and 32°C (Table 1). The
165 duration of incubation was also strongly affected by initial egg mass, with larger eggs hatching
166 later. No significant effect of sex on incubation duration was found (Table 2). Overall hatching
167 success was 60% (66 of 110) and did not differ among incubation temperatures ($\chi^2 = 1.21$, $P =$
168 0.28, $n = 110$) or initial egg mass ($\chi^2 = 0.13$, $P = 0.74$, $n = 110$). Date of oviposition, however,
169 affected hatchling success ($\chi^2 = 6.42$, $P = 0.013$, $n = 110$), with higher egg mortality later in the
170 season. There was a rather high egg mortality, however, it is difficult to differentiate fertile from
171 infertile eggs in Painted Dragons and we have described before that fertility is labile in this
172 species and requires continuous male presence (Uller and Olsson 2005). The degree to which
173 females have responded to male presence is hard to evaluate and was likely the reason for the low
174 fertilization rate. Thus, fertility is hard to establish and the majority of unhatched eggs were in
175 fact probably never fertile in the first place.

176

177 Morphology and Initial Egg Mass

178 Hatchlings incubated at lower temperatures were significantly heavier than those from
179 higher temperatures. One hatchling was excluded from the phenotype measurements as it was
180 born with a deformity (a gaping wound in its chest), and therefore euthanized. Larger eggs, quite
181 expectedly, produced hatchlings with larger body size (Table 1), including a significant effect on
182 body mass, total length, tail length, head length, head width and hind limb length (Table 2). SVL
183 also followed the same trend, however, with a significance value just above the 0.05 threshold.

184 Furthermore, eggs laid earlier in the breeding season were larger than those laid later ($\chi^2 = 23.12$,
185 $P < 0.001$, $n = 110$). The date of oviposition, however, had no effect on any phenotypic trait
186 measured and was therefore excluded from the morphological analyses. Sex did not significantly
187 affect any of the phenotypic traits measured, except forelimb length (Table 2). Post-hatching
188 mortality was not sex-specific ($\chi^2 = 0.32$, $P = 0.60$, $n = 47$).

189

190

DISCUSSION

191 Consistent with studies of other oviparous reptiles (reviewed in Deeming 2004; Deeming
192 and Ferguson 1991), we show that higher incubation temperatures result in shorter incubation
193 durations in Australian Painted Dragons. Variation in incubation duration are most often driven
194 by variation in rate of embryogenesis, easily explained by thermal effects (Q_{10}) on chemical
195 activities, including metabolic rate. Incubation temperature has also been shown to affect
196 hatchling size in many reptiles, with generally ‘larger’ in terms of skeletal dimensions, but not
197 always heavier, hatchlings at lower incubation temperature (reviewed in Deeming 2004; Booth
198 2006). Our results, however, showed a reversed effect, with lower incubation temperature
199 producing heavier, rather than structurally larger, hatchlings. It is possible that this deviation
200 reflects a species-specific response associate to the particular ecological contingencies of the
201 desert-like habitat occupied by *C. pictus*. The majority of studies on developmental biology in
202 reptiles are focused on reptiles living in habitats with more abundant resources (e.g. reviewed in
203 Booth 2006). In the case of *C. pictus*, selection may favor less developed hatchings, in terms of
204 structural size, with a larger residual yolk reserve working as a buffer against desiccation during
205 early life in dry environments (Packard 1991). Although we did not examine residual yolk mass,
206 residual yolk was released from the cloacae during measurements of hatchlings from the high

207 temperature treatment. This was not observed in the low temperature hatchlings, suggesting a
208 higher abundance of residual yolk in high temperature hatchlings.

209 The limited effect of incubation temperature on the morphology and hatching success in
210 this species indicate a robustness to variations in ambient temperature and thus possibly against
211 climate change. However, morphology is only one factor that may be influenced by incubation
212 temperature. It would be necessary to examine the effects of incubation temperature on sex ratio,
213 locomotor performance, and post-hatch growth, survival and reproductive success to determine
214 how this lizard may cope with climate change.

215 The body mass of all hatchlings exceeded the initial egg mass at oviposition, indicating
216 that eggs absorbed water during the embryonic development. This has been suggested to be the
217 case in many small lizard eggs (Tracy 1980; Vleck 1991) and clear evidence for this has been
218 recorded (Ji and Braña 1999; Booth et al. 2000; Ji and Zhang 2001). The reproductive strategy of
219 producing eggs with lower water content will enhance maternal fitness: (1) in habitats where
220 water is a limiting resource for adults, but available for absorption by eggs; (2) in species where
221 bulky eggs would result in a decrement in maternal fitness; and/or (3) allowing for the production
222 of large clutches without the gravid female requiring a great volume (Tracy 1980). These features
223 are congruent with the life-history of Australian Painted Dragons, which inhabits relatively arid
224 niches, where moisture can be absorbed by eggs from the soil. It produces clutches of a size that
225 clearly impedes motility, where larger eggs with a higher water content would likely result in a
226 decrement in maternal fitness. The increased mass of hatchlings in this study, compared to the
227 initial egg mass indicate that water absorbed by the egg is not only used for embryo development,
228 but is incorporated within the hatchling, effectively increasing its body mass (Ji and Braña 1999).
229 Longer incubation periods allow for more water to be absorbed by the egg (Booth et al. 2000),
230 which has been shown to increase the water content and thus also the body mass of hatchlings

231 (Packard et al. 1988; Finkler 1999). The increased body mass in hatchlings incubated at low
232 temperature may therefore simply be due to a greater water content (Ji and Braña 1999) as an
233 effect of longer incubation periods and thus an indirect effect of incubation temperature.
234 Unfortunately, water content can only be analyzed by assessing dry mass which was not done in
235 this study because the fate of the hatchlings was not euthanasia, but recruitment. Nevertheless,
236 hatchlings with greater water content will, when water is scarce, be able to survive longer and
237 loose more water before being adversely affected by desiccation (Finkler 1999). This additional
238 water content may be of major importance for this lizard inhabiting semi-arid environments,
239 typically with hot summers and unpredictable rainfall.

240 Regardless of possible temperature effects on hatchling phenotype, the strongest
241 contributor to hatchling morphology remains maternal yolk allocation (Ferguson and Snell 1986;
242 Deeming and Ferguson 1989; Nelson et al. 2004), affecting size and shape of hatchlings. The
243 yolk has a fundamental influence on the development from zygote to complete hatchling, not
244 only determining how much resources are available for growth and maintenance during
245 embryogenesis, but any surplus may affect hatchling survival when taking the form of residual
246 yolk and fat reserves (reviewed in Congdon 1989). There was also a significant effect of
247 oviposition date on egg mass and hatchling success, with larger eggs and higher embryo survival
248 earlier in the season. Eggs laid earlier in the season are likely of better quality than those laid later
249 due to a greater maternal yolk allocation. This may simply be a result of a seasonally declining
250 maternal condition (Olsson and Shine 1997). Similarly, Warner and Shine 2007 found that the
251 fitness of juvenile jacky dragon lizards depends on seasonal timing of hatching, with early
252 hatched individuals experiencing greater survival rates and chance of reproducing in their first
253 year. They argue that early clutches provide greater fitness returns which may create a selection
254 pressure on the mother to reproduce as early as possible in the season and to allocate more

255 resources toward these early clutches. It is possible that a similar selection pressure could be
256 acting on *C. pictus*.

257
258 **Acknowledgments.**—We are grateful for funding support from the Australian Research Council
259 (MO) and the Swedish Science Council (MO). Special thanks to T. Slight for maintenance and
260 feeding of the lizards. AH collected and analyzed the data, and drafted the manuscript. AH and
261 MO designed the experiment and contributed to revisions of the manuscript. This research was
262 conducted under a scientific research permit (identification number: 001066) issued by the
263 Animal Ethics Committee at the University of Gothenburg, Sweden. Two anonymous reviewers
264 provided constructive criticism on an earlier version of the manuscript. We declare no competing
265 interests.

266

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400 TABLE 1.—Descriptive statistics for incubation duration, mass, size and hatching success of
 401 hatchlings incubated at different temperatures and differences between the sexes.^a

Trait	Temperature			Sex	
	28°C (<i>n</i> = 25)	30°C (<i>n</i> = 20)	32°C (<i>n</i> = 20)	Male (<i>n</i> = 14)	Female (<i>n</i> = 33)
Incubation duration (days)	56.6 ± 0.19	47.0 ± 0.27	40.9 ± 0.25	–	–
Egg mass (g)	0.806 ± 0.018	0.781 ± 0.018	0.798 ± 0.016	0.790 ± 0.024	0.801 ± 0.013
Hatchling trait					
Mass (g)	0.969 ± 0.020	0.935 ± 0.021	0.941 ± 0.022	0.961 ± 0.035	0.960 ± 0.016
Total length (mm)	69.75 ± 0.97	69.60 ± 0.77	69.85 ± 1.02	70.27 ± 1.18	69.51 ± 0.78
SVL (mm)	28.01 ± 0.22	27.66 ± 0.21	27.73 ± 0.28	27.66 ± 0.30	27.92 ± 0.19
Tail length (mm)	41.74 ± 0.83	41.94 ± 0.65	42.13 ± 0.80	42.61 ± 1.01	41.59 ± 0.62
Head length (mm)	6.98 ± 0.08	7.01 ± 0.09	6.97 ± 0.06	6.82 ± 0.09	7.00 ± 0.06
Head width (mm)	7.70 ± 0.05	7.67 ± 0.05	7.64 ± 0.04	7.67 ± 0.06	7.66 ± 0.04
Abdomen length (mm)	10.37 ± 0.16	10.60 ± 0.22	10.53 ± 0.16	10.33 ± 0.21	10.49 ± 0.13
Forelimb length (mm)	7.53 ± 0.09	7.33 ± 0.06	7.40 ± 0.07	7.30 ± 0.09	7.49 ± 0.07
Hind limb length (mm)	12.68 ± 0.12	12.50 ± 0.13	12.59 ± 0.14	12.57 ± 0.15	12.66 ± 0.09
Hatching success (%)	67.6 (25/37)	55.6 (20/36)	56.8 (21/37)	–	–

402 ^a Values are expressed as mean ± SE for all parameters but hatchling success, shown by the
 403 percentage of hatched individual (number hatched/total eggs).

404

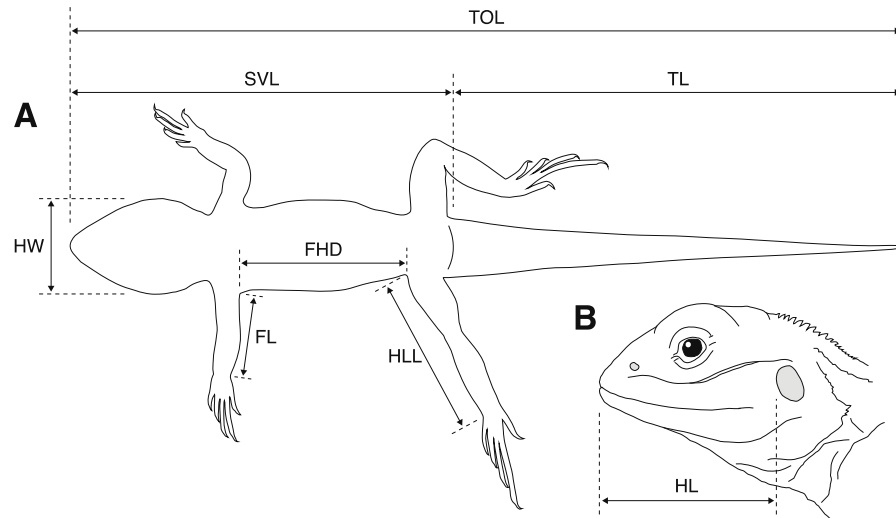
405 TABLE 2.—Results from parametric bootstrapping of linear mixed models, fitted with the *lmer*
 406 function from the lme4 package, to examine the effects of incubation temperature, initial egg
 407 mass and hatchling sex on incubation duration and morphology of hatchlings.^a

Trait	Main effect of temperature (<i>n</i> = 65)		Main effect of egg mass (<i>n</i> = 65)		Main effect of sex (<i>n</i> = 47)	
	χ^2 (df = 1)	<i>P</i>	χ^2 (df = 1)	<i>P</i>	χ^2 (df = 1)	<i>P</i>
Incubation duration	249.01	< 0.001*	6.61	0.017*	0.70	0.427
Morphology						
Mass	4.70	0.032*	44.67	< 0.001*	0.58	0.468
Total length	0.19	0.670	5.22	0.027*	0.02	0.890
SVL	0.47	0.501	4.02	0.056	3.08	0.089
Tail length	0.48	0.501	4.32	0.045*	0.08	0.784
Abdomen length	1.13	0.301	2.93	0.113	0.41	0.537
Head length	0.46	0.513	7.78	0.008*	1.58	0.229
Head width	0.35	0.560	27.11	< 0.001*	0.06	0.819
Forelimb length	1.18	0.283	0.00	1.000	5.01	0.028*
Hind limb length	0.34	0.567	6.29	0.018*	0.31	0.593

408 ^a Initial egg mass and temperature are included as covariates when significant at the 0.15 level.

409 Statistics based on 10,000 bootstrap iterations.

410 * Significant value ($P < 0.05$).



411

412 FIG. 1.—Morphometric measurements used for analysis. (A) Ventral and (B) lateral view

413 of the body plan and head of *Ctenophorus pictus*, showing measurements recorded. Total length

414 (TOL), tip of snout to tip of tail; snout–vent length (SVL), tip of snout to anterior end of vent; tail

415 length (TL), anterior end of vent to tip of tail; fore-hind limb distance (FHD), distance between

416 the inner insertion of the forelimb to the inner insertion of the hind limb, here used as abdomen

417 length; head length (HL), ventral measurement from tip of snout to the anterior end of the

418 external ear; head width (HW), maximum width of head anterior to external ear; forelimb length

419 (FL), from insertion of forelimb to the proximal end of manus; hind limb length (HLL), from

420 insertion of hind limb to proximal end of foot.