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Monsinjon Jonathan, Girondot Marc, Guillon Jean-Michel

Comment on

Carter AW, Paitz RT, Bowden RM. 2019

The Devil is in the Details: Identifying Aspects of Temperature Variation that Underlie Sex Determination in Species with TSD

Integrative and Comparative Biology 59:1081-1088.

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We read with interest the article by Carter, Paitz, and Bowden entitled "The Devil is in the Details: Identifying Aspects of Temperature Variation that Underlie Sex Determination in Species with TSD" (2019; Integr Comp Biol 59:1081-1088). In their article, Carter et al. (2019) studied the sex ratios produced by the eggs of *Trachemys scripta*, a freshwater turtle with TSD, incubated *in vitro* under various fluctuating temperature regimes. They then explored the explanatory values of metrics for the observed sex ratios, grouping their observations with those described by Carter et al. (2018). Their main result and conclusion were that a new metric – the daily duration with constant-temperature equivalent (DDC) – performed better than the constant temperature equivalent proposed by Georges et al. 1994 (hereafter, Georges' CTE). The DDC is the number of days spent at a CTE superior to the pivotal temperature (which gives both sexes under constant incubation temperatures, Tpiv) during the temperature-sensitive period (TSP), which is the period of development when sex is irreversibly determined. We question the general value of this result.

Carter et al. (2019) claim on several occasions (pp. 1, 2, 5, 6) that Georges' CTE1994 is inaccurate, because it aggregates temperatures across a broad time period spanning the entire TSP. However, the DDC used a different temperature aggregation, namely the aggregation of daily temperature data on the temperature axis. By considering only whether the daily CTE (hereafter, dCTE) is situated above or under Tpiv, this metric loses a considerable amount of information by substituting a quantitative metric (the numerical value of the dCTE) for a qualitative relationship (dCTE > Tpiv or dCTE < Tpiv). Unfortunately, the experimental protocol used to test its predictive value does not allow to properly assess the effects of aggregating temperature data on the temperature axis, as opposed to the effect of aggregating temperature data over time in Georges' CTE.

44 In their experiments, Carter et al. (2018, 2019) incubate eggs of *Trachemys*
45 *scripta* under various fluctuating temperature regimes (Fig. 1a). The authors study sex
46 ratios produced under regimes that differ in two parameters: (i) a primary male-
47 producing mean temperature of 25°C or 27°C (hereafter, *Tm*) and (ii) the number of
48 days (*n*) after the onset of the TSP during which eggs are kept at a female-producing
49 mean temperature of 29.5°C (hereafter, *Tf*) before returning to the original male-
50 producing temperature. Overall, Carter et al. (2019) examine the effect of time spent at
51 the feminising temperature on the sex ratio under specific temperature regimes. A
52 prominent feature of the experimental design is that the feminising temperature
53 (29.5°C) is identical for all incubation treatments.

54 Based on their incubation experiments, Carter et al. (2019) infer that most of the
55 variation in sex ratios can be explained by the fact that the model includes *n* as the only
56 explanatory variable. This result led the authors to conclude: "Our results help direct
57 new sex ratio estimation methods and suggest that the number of days at female-
58 producing temperatures may be a robust metric" (p. 6). This conclusion also suggests
59 that the effect of dCTEs is somewhat additive, because summing the days when
60 dCTE>Tpiv gives "an accurate metric of sex ratios across fluctuating incubation
61 temperatures, even when those conditions have different averages and CTEs" (p. 5).
62 However, new experimental data are needed to assess the generality of these
63 conclusions.

64 Consider the two incubation treatments shown in Fig. 1b and 1c. In Fig. 1b, the
65 female treatment differs from that found in both studies of Carter et al. (2018, 2019),
66 because *Tf*=31°C. In Fig. 1c, the female treatment is not identical during the entire heat
67 wave: here, *n*=16, *Tf*=29.5°C for the first *k* days of the heat wave and then *Tf*=31°C for *n*-

68 *k* days, with *k*=8. Following the conclusions drawn by Carter et al. (2019), the sex ratios
69 produced under the temperature regimes depicted in Fig. 1b and 1c could be predicted
70 based on the sole value of *n*. Incubations regimes shown in Fig. 1a, 1b, and 1c would
71 yield similar sex ratios, because *n* remains the same. However, several observations
72 found in the literature challenge this view.

73 Study of the literature shows that different all-female-producing temperatures
74 have different effects on sex determination in freshwater turtles. An article by Bull et al.
75 (1990) entitled "Sex-determining potencies vary among female incubation temperatures
76 in a turtle" showed that different sex ratios are obtained when eggs of the freshwater
77 turtle *Graptemys ouachitensis*, which belongs to the same subfamily (Deirochelyinae) as
78 *Trachemys scripta*, are shifted from *Tm*=26°C to *Tf*=31°C or from *Tm*=26°C to *Tf*=32°C. A
79 follow-up article by the same authors (Wibbels et al. 1991a) confirmed in *Trachemys*
80 *scripta* itself that "shifting eggs from *Tm*=26°C to *Tf*=32.5°C produced significantly more
81 females than shifts to *Tf*=31°C" (p. 373). The same article also reported that "shifting
82 eggs from *Tf*=31°C to *Tm*=23°C produced significantly more males than shifts to
83 *Tm*=26°C" (p. 373). In keeping with these results, Wibbels et al. (1991b) showed
84 strikingly different effects of estradiol application when eggs of *Trachemys scripta* were
85 incubated at *Tm*=26°C or *Tm*=28°C. This led Wibbels et al. (1998) to state: "[…] in
86 *Trachemys scripta* […] temperature appears to exert a 'dosage effect' on sex
87 determination. The dosage effect depends on the 'potency' of the temperature (i.e., the
88 warmer or cooler the temperature, the more potent it is in producing females or males,
89 respectively) […]" (p. 410). Similar results were found in a Crocodilidae (Alligator
90 mississippiensis; Lang and Andrews 1994). Another support for this conclusion comes
91 from enzymatic studies of Desvages and Pieau (1992): these authors measured the
92 activity of aromatase (the enzyme converting testosterone into estradiol) in the gonads

93 of developing embryos of *Emys orbicularis*, a freshwater turtle of the same family
94 (Emydidae) as *Trachemys scripta*. They found that the gonadal aromatase activity during
95 the TSP increased in eggs incubated at *Tf*=35°C compared to T*f*=30°C. In agreement with
96 these results, the gonads of embryos incubated at *Tf*=35°C are structurally different
97 from the gonads of embryos incubated at *Tf*=30°C (Pieau 1978).

98 These different well-established effects of varying *Tf* cannot be detected in the
99 study of Carter et al. (2019), because they used the same *Tf* in all of their incubation
100 treatments. According to current knowledge, we may expect that both thermal regimes
101 shown in Fig. 1b and 1c would yield a higher proportion of females than the thermal
102 regime shown in Fig. 1a, meaning that *k* and *Tf* would appear along with *n* in the best
103 predictive model. Experimental tests would prove useful to test whether these
104 expectations are verified.

105 The approach followed by Carter et al. (2019) suffers from another limitation. If
106 *Tf* varies during the TSP as in Fig. 1c, the embryonic growth rate will also vary
107 accordingly (faster growth at high temperature). The amount of development at
108 different temperatures, not only its duration, is a major determinant of sexual
109 determination. A temperature regime oscillating symmetrically around a certain mean
110 will yield more females than a constant temperature with the same mean, as long as the
111 growth rate increases with temperature in the range of the oscillation (Georges 2013;
112 Georges et al. 1994). This is because a greater amount of development occurs at high
113 temperatures, and if these high temperatures produce females, then a higher amount of
114 development occurs at *Tf* compared to incubations at the corresponding constant mean
115 temperature. For a primary temperature *Tm*=27°C, the ±3°C oscillations ensure that
116 female-producing conditions are encountered during embryo development. By contrast,

the same oscillations around *Tm*=25°C stay within the range of male-producing conditions. Thus, for the same heat wave duration, incubating at *Tm*=27°C increases the proportion of development at *Tf* during the TSP, compared to incubating at *Tm*=25°C. The result obtained by Carter et al. (2019) that the DDC is a better predictor of sex ratio than Georges' CTE is all the more surprising that the CTE takes this into account, while the DDC does not.

Finally, a prominent feature of all incubations performed by Carter et al. (2018, 2019) is that the heat wave always begins at the supposed onset of the TSP. For one value of *Tm*, incubation regimes only differ in terms of how many days *n* the eggs incubate at *Tf*, starting from the presumed TSP. We suggest that studying other regimes is warranted, for example, when the beginning of the TSP is cooler than the end (Fig. 1d).

This kind of variation is probably embedded in the 23-year sex ratio data of a natural population in Illinois from which the DDC model receives some support. However, given the high variability of temperature regimes found in nature (Monsinjon et al. 2017), the general value of the results described by Carter et al. (2018, 2019) should be validated across various species and populations. Controlled experiments such as those sugested here are also warranted to validate the model in a broader context.

We hope that our comments and suggestions will help refine potential follow-ups of this promising study.

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Author contributions: All authors contributed equally to this work.

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Legends to Figures:

Fig. 1 Actual and hypothetical regimes of incubation following the experimental design in Carter et al (2019). Eggs are incubated *in vitro* at oscillating temperatures T=25±3°C for the first 25 days and then subjected to a heat wave of mean temperature T. In all examples, the length of the heat wave is n = 16, and k is the time elapsed (in days) since day 25. (a) Example of the temperature regime from Carter et al. (2019) with T=29.5°C for $0 \leq k < 16$; (b), (c), and (d) hypothetical temperature regimes considered for discussion, with (b): T=31°C for $0 \leq k < 16$; (c): T=29.5°C for $0 \leq k < 8$ and T=31°C for $8 \leq k < 16$; (d): T=25°C for $0 \leq k < 25-n$ and T=29.5°C for $25-n \leq k < 25$.