Toward a general explanation for latitudinal clines in body size among chelonians

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For many species, the population-mean body size increases with latitude. Although heat conservation and starvation resistance are frequently invoked to explain latitudinal clines, these explanations seem incompatible with the natural history of turtles, which also increase in size with latitude. We collate the population-mean body size for nearly 100 populations of North American freshwater turtles belonging to seven species. We test the hypothesis that temperature–size relationships in turtles are driven by strong seasonality in the north, which leads to maturation at a larger size and the production of relatively fewer, larger clutches per season. Our results, however, do not support the seasonality hypothesis. We present two new hypotheses that can explain the generality of temperature–size responses in chelonians. First, oxygen consumption is temperature sensitive, placing a premium on small size in warm aquatic environments, because smaller size reduces total oxygen demand during activities requiring submergence. Second, overwintering physiology of turtles might drive size clines, because a decrease in mass-specific metabolic rate with size might result in a disproportionate increase in the capacity of large individuals to buffer lactic acid in anoxic conditions. The pursuit of a general explanation for temperature–size relationships in chelonians would benefit from comparing temperature–size responses between aquatic and terrestrial species.

ADDITIONAL KEYWORDS: Bergmann's rule – cline – oxygen limitation – reproductive cycle – temperature–size rule.

INTRODUCTION

Temperature has strong and predictable effects on body-size evolution (Smith, Betancourt & Brown, 1995; Moran & Woods, 2012; Lawson & Weir, 2014). In general, there is a negative relationship between body size and environmental temperature within many vertebrate and invertebrate clades (Atkinson, 1994; Ashton, Tracy & Queiroz, 2000; Ashton, 2002), and these associations are so strong that they have been described as a series of ecological 'rules': the temperature–size rule (Atkinson, 1994), James's rule and Bergmann's rule (Bergmann, 1847; James, 1970; Blackburn, Gaston & Loder, 1999), relating to observations made within populations, across populations and across species, respectively. Each of these rules has

puzzled life-historians for decades, especially for ectotherms (Perrin, 1995; Angilletta & Dunham, 2003).

The puzzle arises because large adult size confers high fitness (McLaren, 1966; Kingsolver *et al.*, 2001; Rollinson & Rowe, 2015; Armstrong *et al.*, 2018), and large size can be attained by delaying sexual maturity, whereby the individual directs its energy toward growth for a greater period of time before diverting some energy to reproduction. Yet, natural selection will favour a life-history strategy in which energetic investment in somatic or vegetative growth is completely paid back in terms of future reproduction (Kozlowski, 1992). Optimal age and size at maturity must therefore balance the gains in fitness expected from increasing the duration of pre-maturation growth against the risk of mortality across life stages (Charlesworth, 1980; Roff, 2002). Given that the growth rate of ectotherms should be much faster in warm environments compared with cool ones (Forster, Hirst & Woodward, 2011), classical life-history theory predicts the evolution of maturation

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at a larger size in warm environments (Berrigan & Charnov, 1994; Perrin, 1995; Atkinson & Sibly, 1997; Angilletta, Steury & Sears, 2004b; but see Kozlowski, Czarnoleski & Danko, 2004). This prediction opposes the pattern observed in many (but not all) ectothermic clades (Atkinson, 1994; Atkinson & Sibly, 1997).

Although oxygen limitation has been proposed as a mechanism driving negative associations between size and temperature for species that respire in water (Atkinson, Morley & Hughes, 2006; Forster, Hirst & Atkinson, 2012; Horne, Hirst & Atkinson, 2015), few general explanations exist for the same temperature–size relationships observed in air-breathing species (Kozlowski *et al.*, 2004; Atkinson *et al.*, 2006; Forster *et al.*, 2012). In endotherms, for instance, the most popular explanation for James's rule is that large size results in decreased surface area-to-volume ratio, which allows less heat to escape an organism, such that body heat is better retained in cool environments (Mayr, 1956; Osorio-Canadas *et al.*, 2016). Yet, for ectothermic vertebrates, turtles also demonstrate a strong propensity to follow James's rule, whereas lizards and snakes show the reverse pattern (Ashton & Feldman, 2003). For turtles, snakes and lizards, it seems unlikely that surface area-to-volume relationships and heat conservation would drive responses to temperature, as a large ectotherm may retain heat more effectively but acquire it with more difficulty. Furthermore, the disparate responses of temperature–size relationships across birds, mammals and turtles (a negative association) vs. lizards and snakes (a positive association) suggest that the pattern arises, or at least can arise, for reasons that are independent of heat balance.

Many studies on James's rule aim to determine whether a correlation exists between the population-mean or maximal body size and regional temperature means (Ashton *et al.*, 2000; Ashton, 2002; Ashton & Feldman, 2003; Adams & Church, 2008; Berke *et al.*, 2013; Rypel, 2014), but a paucity of empirical work is motivated by a priori hypotheses that seek to understand why body size clines exist in the first place (for exceptions, see: Angilletta *et al.*, 2004a; Litzgus & Smith, 2010; Horne *et al.*, 2015; Osorio-Canadas *et al.*, 2016). Furthermore, most studies do not consider how the joint effects of seasonality and temperature may impact body-size evolution and result in body-size clines (for exceptions, see: Roff, 1980; Blanckenhorn & Demont, 2004; Litzgus & Smith 2010; Horne *et al.*, 2015). Currently, there is to our knowledge only one verbal explanation for James's rule in chelonians (turtles and tortoises) (Litzgus & Smith, 2010; Werner *et al.*, 2016), one that focuses on time-constrained life histories. The premise of the theory is that all freshwater turtles are both iteroparous and oviparous, and eggs are generally large and incubate over a long period of time, often between 60 and 90 days (Ernst & Lovich, 2009). Within most species, the egg-laying period is long in warm climates, where individual females lay sequential clutches over several months; conversely, the egg-laying period is short in cold climates, with most individuals laying one or two clutches per year, generally in the spring or early summer (Moll, 1973; Ganzhorn & Licht, 1983; Mendonca, 1987). The aforementioned pattern is likely to arise because turtles in strongly seasonal environments are subject to a time constraint; turtle embryos are not freeze tolerant and must hatch out of the egg before winter. Given that embryos are subject to time constraints on development, relatively few successful clutches per year are possible when the growing season is short (Edge *et al.*, 2017).

Litzgus & Smith (2010; see also Werner *et al.*, 2016) proposed that, when the time constraint is relaxed or non-existent, as in warm climates, the optimal age and size at maturity are relatively small, because the benefits of delaying reproduction and attaining a larger size are outweighed by the benefits of maturing early and reproducing several times per season. In more northern environments, however, season length limits the number of potentially successful clutches that can incubate in a given year, thereby enhancing the fitness benefits of delaying reproduction so that a large body size can be attained, ultimately allowing the few clutches produced per season to be relatively large (Elgar, 1990; Iverson & Smith, 1993; Iverson *et al.*, 1993; Werner *et al.*, 2016). The result is that annual per capita reproductive output may be similar in northern vs. southern regions despite differences in growing season length, as appears to be the case for spotted turtles (*Clemmys guttata*; Litzgus & Mousseau, 2006). If this hypothesis is correct, then it provides a general explanation for James's rule in freshwater turtles; however, if it is incorrect, then new hypotheses for James's rule need to be proposed and evaluated.

To date, there has been only one restricted test of this hypothesis, which we call the Litzgus–Smith hypothesis. Litzgus & Smith (2010) tested the prediction that relatively strong female-biased sexual size dimorphism (SSD) in *Chrysemys picta* (a freshwater turtle) should evolve in seasonal environments, by virtue of strong upward selection on female size at maturity (and perhaps rates of indeterminate growth; Armstrong *et al.*, 2018). They found that, for this single species, female-biased SSD became significantly less pronounced in seasonal environments (Litzgus & Smith, 2010), which is the opposite of the pattern predicted by the Litzgus–Smith hypothesis. Nevertheless, SSD in turtles can be affected by a broad array of factors (Blanckenhorn *et al.*, 2006), including the extent of

male–male competition for mates and selection for ecological divergence among the sexes when morphospace is ecologically available to be exploited (Shine, 1989; Stephens & Wiens, 2009). It is therefore premature to reject the Litzgus–Smith hypothesis on the basis of this test.

A broader test of the Litzgus–Smith hypothesis requires manipulation of the extent to which time constraints are experienced over a north–south gradient and observing the outcome of body-size evolution in females, all in a phylogenetically explicit context (Felsenstein, 1985). Interestingly, the natural history of a handful of freshwater turtle species lends itself to such a manipulation. Although most species of freshwater turtles are capable of producing multiple clutches per year, four species are known to have reproductive cycles that allow the production of only one clutch per year: snapping turtles (*Chelydra serpentina*), alligator snapping turtles (*Macrochelys temminckii*), Blanding's turtles (*Emydoidea blandingii*) and wood turtles (*Glyptemys insculpta*). Multiple clutching has never been reported in these species, and multiple clutching seems very unlikely because follicular cycles in these three species are amenable to only one clutch per season (Powell, 1967; Steyermark, Finkler & Brooks, 2008; Rollinson, Farmer & Brooks, 2012). For species whose reproductive cycles allow only one clutch per year, the time constraint will be relatively independent of growing season length, as there is no opportunity to increase reproductive success with multiple clutches per season. For single-clutching species, then, the optimal age and size at maturity does not depend on the expected reproductive frequency within a season, and all else being equal, multi-clutching species should exhibit stronger body-size clines than single-clutching species. Furthermore, because of the rarity of single-clutching species, it would not be surprising to observe a general trend of James's rule in freshwater turtles (Ashton & Feldman, 2003), because multiple-clutching species are far more common and, according to the Litzgus–Smith hypothesis, are expected to follow a latitudinal cline.

In the present study, we amass a dataset on adult body size for several populations of seven species of turtle, all of which have broad geographical ranges, and three of which are species that produce single clutches. We compare the strength (slope) of the temperature–size response between single- and multiple-clutching species across populations and latitude, predicting that multiple-clutching species will exhibit relatively strong temperature–size associations. For completeness, we also explore how SSD changes across latitude, following Litzgus & Smith (2010).

MATERIAL AND METHODS

SELECTION OF STUDY SPECIES

To our knowledge, there are four species of freshwater turtle in North America that both produce a single clutch per season and have large geographical range sizes: *M. temminckii*, *Chelydra serpentina*, *G. insculpta* and *E. blandingii*. Body-size data for *M. temminckii* were very scarce, and therefore this species could not be used in this study. Furthermore, *Chelydra serpentina*, *G. insculpta* and *E. blandingii* differ from *M. temminickii* in that their northern geographical range limits extend very far north, into southern Canada (Table 1). Thus, the three single-clutching species used herein all exhibit extensive geographical ranges that extend into very cool thermal environments. To make a fair comparison between single-clutching vs. multi-clutching species, we selected multi-clutching species with large geographical ranges that likewise extend into southern Canada. We found five species that fit these criteria: *Chrysemys picta* ssp., *Apalone spinifera spinifera*, *Clemmys guttata*, *Sternotherus odoratus* and *Graptemys geographica* (Table 1). However, we found very few data for *G. geographica*, such that this species could not be included in our study, leaving four multi-clutching species for analysis.

BODY-SIZE DATA

We used species-specific literature searches to collate data on population-mean body mass (in grams), carapace length (CL; in millimetres) and plastron length (in millimetres), separately for males and females. Unpublished data on several populations and species were also provided by J. Iverson, Ontario Nature and C. Davy. We did not use body-size data from telemetry studies because these data might represent a non-random sample of adults, i.e. those large enough to fit with radio tags.

Different studies varied in how body size was quantified, with most generally reporting some combination of straight-line carapace length (in millimetres) and/or straight-line plastron length (in millimetres) and/or adult weight (in grams). Carapace length was reported most often, although adult mass is likely to be a better metric of body size than carapace length (Lovich & Gibbons, 1992). Where necessary, population-mean female plastron length was converted to population-mean female carapace length or adult mass, and population-mean female carapace length was converted to population-mean female body mass (Supporting Information, Table S1). These conversions were performed on a species-specific basis and a sex-specific basis, with two exceptions. For *A. spinifera*, a species with extreme SSD, we found that a log–log

Table 1. List of (sub)species used in the present study, and the number of populations used in analyses of body mass and sexual size dimorphism

Species	Subspecies	Reprod.	Range (km²)	Minimal latitude	Maximal latitude	N body mass	N SSD
Emydoidea blandingii	–	Single	1 012 873*	37.90°N	46.59°N	10	6
Glyptemys insculpta	–	Single	1 012 873	36.17°N	46.95°N	15	15
Chelydra serpentina	*serpentina*	Single	5 681 234	25.31°N	56.37°N	17	6
Chrysemys picta	*bellii*, *dorsalis*, *marginata*, *picta*	Multiple	5 443 178	28.48°N	57.73°N	21	15
Clemmys guttata	–	Multiple	881 788	28.02°N	47.53°N	9	9
Apalone spinifera	*spinifera*	Multiple	3 597 348	23.77°N	45.92°N	7	5
Sternotherus odoratus	–	Multiple	2 562 525	25.38°N	46.50°N	12	8

*No range size data were available for *E. blandingii*, but its range is similar in size to that of *G. insculpta*, such that the value in the table is approximated from *G. insculpta*. 'Reprod.' refers to the type of reproductive cycle (single vs. multiple clutches); SSD, sexual size dimorphism.

regression fit male and female body size data well ($r^2 = 0.99$), so a single regression was used to convert CL to mass for both sexes. For *S. odoratus*, we used body-size data from Iverson *et al.* (1993) for species in the family Kinosternidae (one data point per species), for species with body sizes similar to *S. odoratus*, to convert female CL to mass ($r^2 = 0.71$). On the whole, most conversions featured high coefficients of determination (mean $r^2 = 0.91$, range 0.71–0.99; Supporting Information, Table S1).

Sample sizes per population averaged 84.6 (range, 5–1113) for female size and 110.7 (range, 5–1637) for male size; however, samples sizes could not always be determined (e.g. data obtained from previous compilations). After data collection was complete, study locations were carefully examined for duplication across studies, and in such cases, the size estimates were averaged across duplicated locations. For each species, we were ultimately left with between seven and 21 (mean = 13.0) estimates of population-mean body mass and between five and 15 (mean = 9.1) estimates of SSD.

TEMPERATURE DATA

The mean annual temperature for each population was collected from the nearest Environment Canada weather station for populations in Canada. The mean annual temperature of populations located in the USA was collected from the nearest National Oceanic and Atmospheric Administration (http://www.noaa.gov/) weather station. In some cases, the exact location of the population was not reported (e.g. for endangered species where collection for the pet trade is a concern). For these cases,

we predicted the mean annual temperature from a regression of latitude (latitude was always known) and the mean annual temperature for species with known locations (Temperature = −0.903(Latitude) + 47.1, $N = 93$, $r^2 = 0.94$, $P < 0.001$). This was done for a minority of observations ($N = 8$ populations). In the main text, we focus on mean annual temperature rather than latitude, as temperature should better reflect growing season length than latitude (e.g. variation in elevation is captured by temperature but not latitude).

PHYLOGENETIC ANALYSES

Owing to large interspecific differences in the species-mean body size, regression slopes of body size over temperature are not comparable across species. We overcame this problem by expressing each datum in terms of its z-score (Zar, 1984), where body size was converted in units of standard deviations, specific to each species, as follows:

$$z = \left(x_{ij} - \mu_j\right)\big/\mathrm{SD}_j \qquad (1)$$

where x is the body size of the ith individual in species j, μ is the mean body size of species j, and SD is the standard deviation in body size of species j.

Having standardized body size for each species, it becomes difficult to perform analyses that simultaneously estimate variation in mean body size across species and the slope of size over temperature (e.g. ANCOVA), because the species-mean body size of all species is zero. Therefore, we first obtained species-specific ordinary least-squares (OLS) regression slopes of standardized size over temperature. Phylogenetic

analysis was introduced after obtaining standardized slopes for each species, because the relevant issue is whether the change in body size with temperature has a phylogenetic signal (see also de Queiroz & Ashton, 2004). A species-level phylogeny of freshwater turtles was obtained from Guillon et al. (2012). We time calibrated the phylogeny following Sanderson (2002), using the penalized likelihood approach. We used the value of λ that minimized the cross-validation score (λ = 100, in this case), having searched across values of λ ranging from 10^{-1} to 10^6. Finally, the tree was pruned to exclude species not in our study (Fig. 1).

We implemented a phylogenetic generalized least-squares (PGLS) model (Martins & Hansen, 1997), comparing the standardized regression slopes of the two groups (single- vs. multi-clutching species), with each species as a data point. However, because the number of populations sampled per species was sometimes small, species-specific OLS regression slopes of standardized size over temperature are likely to be sensitive to outliers. To ensure that the main PGLS result was robust, we explored the sensitivity of our results to potential outliers. First, we used a jack-knife approach, which performs an OLS regression $N - 1$ times for each species (where N is the number of species-specific populations sampled), systematically omitting one population in each regression. We retained the slope estimate for each jackknife run, thereby generating a distribution of plausible slope

values for each species. Next, for 1000 bootstrap runs, we sampled a single slope estimate from the jack-knifed distribution of each species ($N = 7$ data points generated for each bootstrap run, because there are seven species in this study), then used a PGLS model with reproductive cycle (single- vs. multi-clutching species) as a grouping variable to estimate the difference in the standardized slope of single- vs. multi-clutching species, with multi-clutching species set as the reference category. The bootstrapping method resulted in 1000 PGLS estimates of the difference in regression slope between single- vs. multi-clutching species, along with 1000 P-values. If our main PGLS result is robust to the inclusion/omission of outliers, there should be little signature that certain combinations of slope estimates produce results that differ from those in the main analysis (e.g. bimodal distribution of estimates, with the smaller peak approaching statistical significance).

We calculated SSD as follows:

$$\text{SSD} = \left(\text{female size/male size}\right) \qquad (2)$$

We did not subtract a value of one from this ratio (Lovich & Gibbons, 1992), because many species are being compared, such that an SSD value of 1.0 indicates no dimorphism, and values greater than one indicate female-biased SSD. For SSD data, parameters for the OLS regression of each species were calculated, but because there was no general relationship between SSD and temperature (see Results), no bootstrapping comparison of single- vs. multi-clutching species was performed.

All statistical analyses were performed in the R environment (R Core Team, 2016), using the packages *geiger* (Harmon *et al.*, 2008), *phytools* (Revell, 2012) and *caper* (Orme *et al.*, 2013). The PGLS models were fitted with the R package *caper* (Orme *et al.*, 2013), and bootstrapping was performed using the *base* package (R Core Team, 2016).

Figure 1. Phylogeny of seven turtle species, with single-clutching species in bold.

RESULTS

Using OLS regression, we found that five of the seven turtle species exhibited a significant negative relationship between standardized female body mass and temperature (Fig. 2). Only *A. spinifera* and *Clemmys guttata* (both multi-clutching species) exhibited a non-significant relationship between temperature and mass, and in both cases the trend was negative. The OLS results were qualitatively the same whether adult carapace length or adult mass was used as an estimate of size (Supporting Information, Table S2). A two-tailed binomial test suggested that there is a general tendency for turtles to respond negatively to
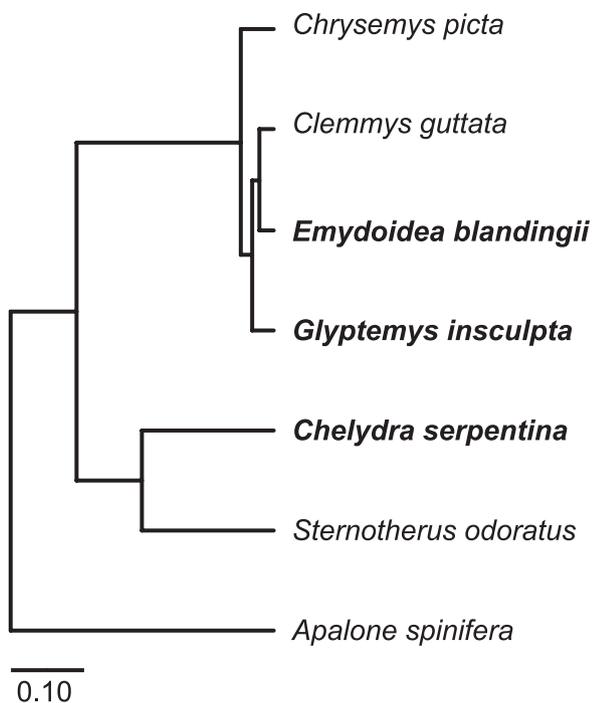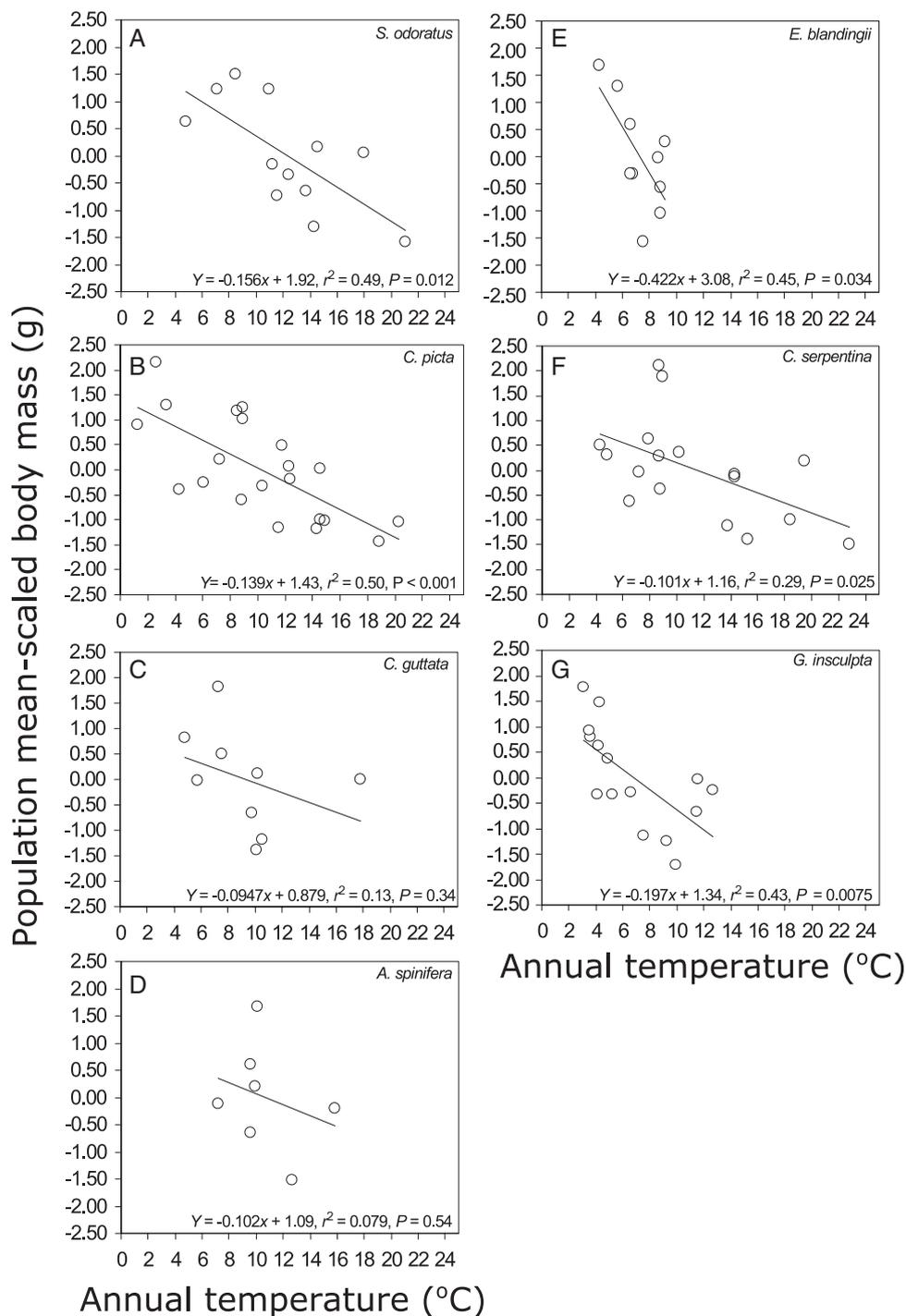
Figure 2. Ordinary least-squares regressions for population-mean female mass (scaled for comparisons across species; see Material and Methods) of seven North American turtle species in relation to mean annual temperature. A–D, multi-clutching species. E–G, single-clutching species.

temperature, as the two-tailed probability that all seven species would exhibit a negative relationship by chance alone was $P = 0.012$.

There was no difference between single- and multi-clutching species in the response of

standardized mass to temperature (PGLS mean difference = -0.117 ± 0.0815, $P = 0.21$, $\lambda = 0$), nor in the response of standardized carapace length to temperature (PGLS mean difference = -0.125 ± 0.0863, $P = 0.21$, $\lambda = 0$). Bootstrap comparison of jackknifed

regression slopes suggested that the main result of our PGLS analysis is robust to the inclusion/exclusion of outlying data points. Specifically, virtually no combination of jackknifed regression slopes produced a parameter estimate in the direction predicted by the Litzgus–Smith hypothesis; in the rare instances that a significant difference occurred, it was in the direction opposite to the Litzgus–Smith prediction (Fig. 3).

In general, SSD was not related to temperature (Fig. 4). Only *S. odoratus* (multi-clutching species) exhibited a significant relationship between SSD and temperature, with female-biased SSD becoming more pronounced with increasing temperature, although this relationship was clearly driven by a single population in the south (Fig. 4A). When carapace length was used as an estimate of size, the SSD of *S. odoratus* was no longer related to temperature, but the female-biased SSD of *Chrysemys picta* increased with temperature (Supporting Information, Table S3), which replicates the result of Litzgus & Smith (2010), who focused exclusively on carapace length.

DISCUSSION

Our results communicate three main findings. First, we confirm that temperature–size responses are generally negative across populations of freshwater turtles
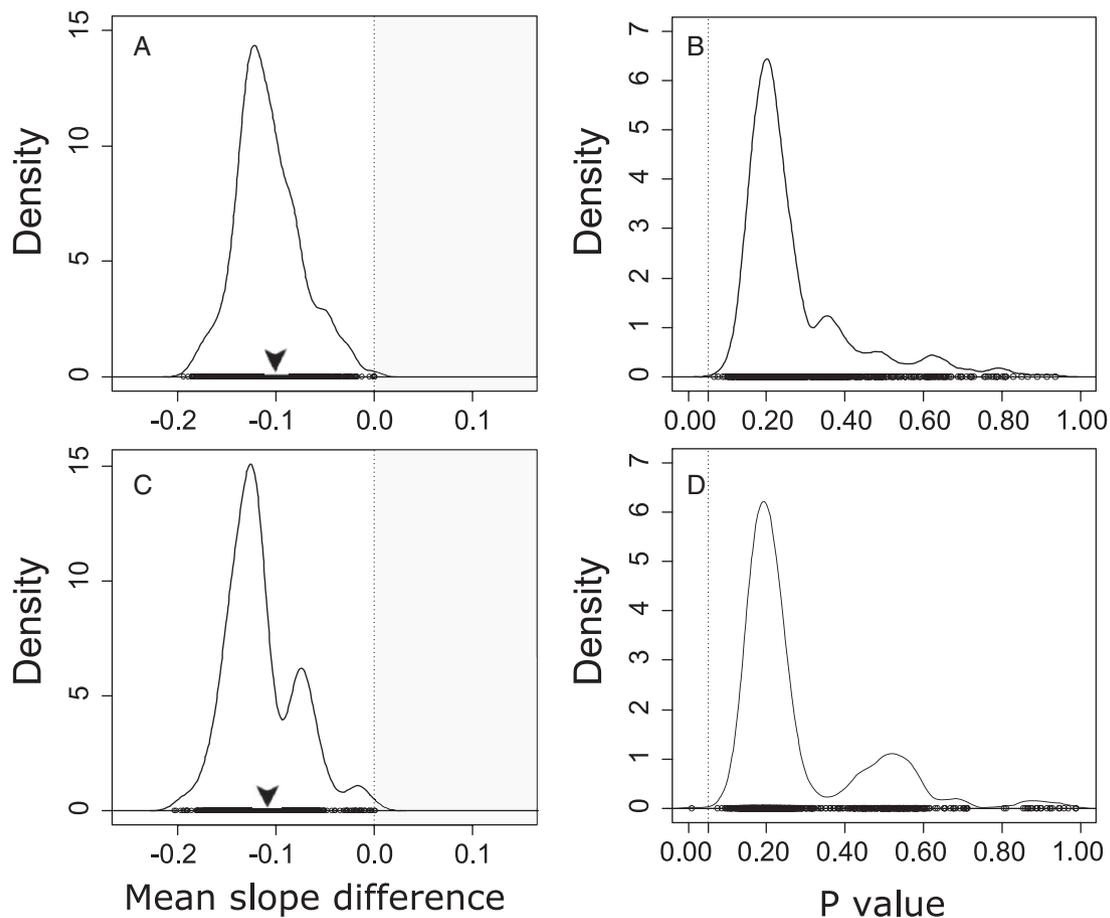

Figure 3. Distribution of estimates and *P*-values from 1000 bootstrapped phylogenetic generalized least-squares (PGLS) analyses. A, distribution of 1000 PGLS differences between single- and multi-clutching species for the mean standardized slope of female body mass over mean annual temperature. Arrowheads indicate the mean difference in the 1000 bootstrap replicates, and dotted vertical lines indicate a difference of zero. Values less than zero on the *x*-axis indicate that, on average, the body size of single-clutching species declines more strongly with temperature than that of multi-clutching species. Values greater than zero in the shaded area indicate that the body size of single-clutching species declines less strongly with temperature than that of multi-clutching species, as predicted by the Litzgus–Smith hypothesis. B, distribution of *P*-values for estimates in A, with dotted vertical line denoting $P = 0.05$. C, distribution of 1000 PGLS differences between single- and multi-clutching species for the mean standardized slope of female carapace length over mean annual temperature. D, distribution of *P*-values for estimates in C.
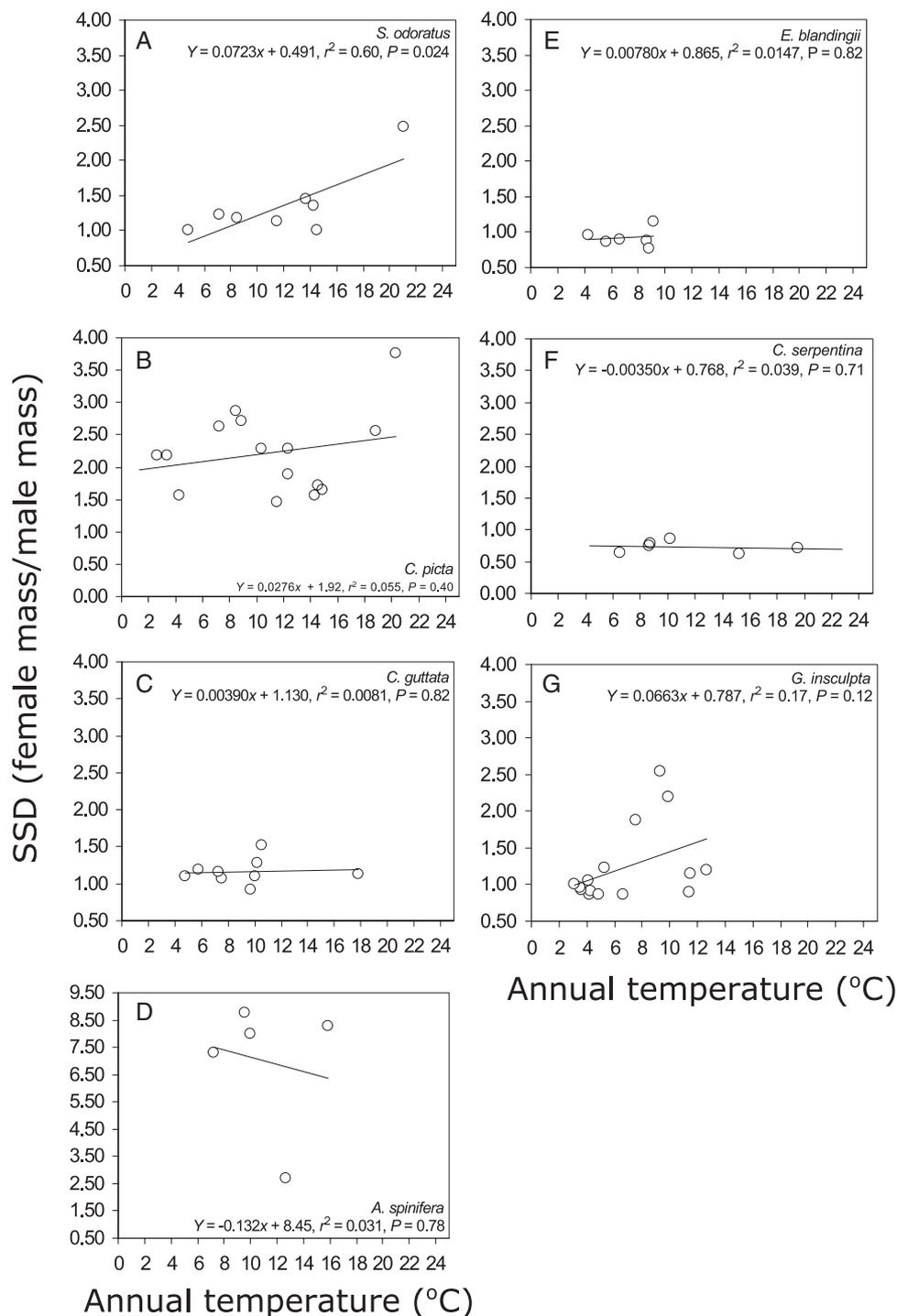
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Figure 4. Ordinary least-squares regressions for the sexual size dimorphism (mean female mass/mean male mass) of seven North American turtle species in relation to mean annual temperature. A–D, multi-clutching species. E–G, single-clutching species. Note the scale difference on the *y*-axis of D.

(for further evidence of this generality, see Ashton & Feldman 2003). Second, temperature–SSD responses are generally absent in freshwater turtles, and those species that demonstrate weak temperature–SSD responses tend to show less female-biased dimorphism in the north compared with the south (see also Blanckenhorn *et al.*, 2006). Third, the type of reproductive cycle is not associated with the strength of

temperature–size responses. We therefore reject the Litzgus–Smith hypothesis, which posits that seasonality and time constraints drive James's rule in freshwater turtles, and we explore alternative explanations for the generality of temperature–size responses in chelonians.

All species in the present study followed James's rule, or at least responded to temperature in the direction consistent with James's rule (see also Ashton & Feldman, 2003). Our findings therefore underline that some general factor governs temperature–size responses in chelonians, regardless of reproductive cycle. To our knowledge, there is currently no working hypothesis that might explain the ubiquity of James's rule in turtles, other than the well-trodden explanations of starvation resistance and heat balance (see also Rodrigues *et al.*, 2018), which seem unlikely for ectothermic animals (Werner *et al.*, 2016). If a new hypothesis is to be developed, then we suggest that it should draw on the observation that close relatives of chelonians, namely squamate reptiles, tend to increase in size as temperature increases, which is the opposite of turtles (Ashton & Feldman, 2003). What key difference between these close relatives results in opposite temperature–size responses across latitude? One possibility is that squamate reptiles tend to be highly terrestrial, whereas chelonians tend to be highly aquatic. In light of the aquatic lifestyle of many chelonians, we offer two non-mutually exclusive hypotheses that can help to explain why representatives of this group become large in cool climates.

First, warmer temperatures result in an increase in oxygen consumption of ectotherms, which results in difficulty regulating oxygen demand and supply (Woods, 1999; Verberk *et al.*, 2011; Verberk & Atkinson, 2013). Given that balancing oxygen supply and demand is facilitated when body size and/or cell size is small (Woods, 1999; Atkinson *et al.*, 2006; Czarnoleski *et al.*, 2015), it has been suggested that the plastic responses of adult size and cell size to temperature, which tend to be negative (Atkinson, 1994), reflect an acclimatization response to both decreased oxygen supply and increased demand in warm environments (Atkinson *et al.*, 2006). There is strong evidence among ectotherms that large adult size and cell size occur when oxygen availability is high (Harrison & Haddad, 2011; Heinrich *et al.*, 2011), and that low temperature exacerbates the benefits of large size by virtue of decreased metabolic rate, hence a further increase in the ratio of oxygen supply to demand (Frazier, Woods & Harrison, 2001). Most importantly, temperature-dependent oxygen limitation is especially pronounced in aquatic environments, where oxygen solubility is strongly temperature dependent and decreases with temperature (Forster *et al.*, 2012;

Horne *et al.*, 2015). Indeed, temperature-dependent oxygen limitation seems to drive James's rule among aquatic invertebrates (Horne *et al.*, 2015; Walczyńska & Sobczyk, 2017) and has also been implicated in negative relationships between temperature and the size of aquatic, ectothermic vertebrates at the macroevolutionary level (Rollinson & Rowe, 2018a, b).

We recognize several characteristics of chelonians that could promote the evolution of size in response to an interaction between temperature and oxygen. First, although temperature-dependent oxygen limitation is generally believed to be most relevant for species that respire in water (e.g. cutaneously or with gills; Atkinson *et al.*, 2006; Rollinson & Rowe, 2018a, b), we note that many aquatic turtles exhibit cutaneous respiration not only in winter conditions, but also during the summer (Ultsch, Herbert & Jackson, 1984). Given that a decrease in body size results in a decrease total oxygen demand, the extent to which cutaneous respiration satisfies oxygen demand in warm conditions could be enhanced when body size is small, although this argument depends on the scaling of skin surface area to turtle volume (e.g. see Rombough, 2007). Second, turtles must hold their breath for protracted periods of time during the active season, given that activities such as foraging, mating and escape from predators (e.g. otters) all occur under water, where pulmonary ventilation is impossible (Ernst & Lovich, 2009). Notably, there is a decrease in the affinity of haemoglobin for oxygen at warmer temperatures, which ultimately facilitates unloading of oxygen at metabolizing tissues but also results in lower oxygen-carrying capacity of the blood (Damsgaard *et al.*, 2013). In warm environments, therefore, turtles might benefit from smaller body and cell size, such that total oxygen demand is reduced and oxygen delivery at the whole organism and cellular (mitochondrial) level is facilitated during activity. Indeed, there is some evidence that the cell size of at least one species of freshwater turtle (*Clemmys guttata*) is positively related to latitude (Litzgus, DuRant & Mousseau, 2004). This intraspecific decrease in cell size with increasing temperature is predicted to be adaptive on theoretical grounds (Woods, 1999) and is consistent with observations of model organisms in laboratory conditions, where joint effects of temperature and oxygen availability have been implicated in decreased cell size (Harrison & Haddad, 2011; Walczyńska *et al.*, 2015).

Second, overwintering physiology might also help to drive the association between body size and temperature. During cold hypoxia/anoxia, metabolic rate decreases and is accompanied by a shift from aerobic respiration to anaerobic glycolysis that produces lactic acid. The acid must be buffered to maintain acid–base homeostasis and prevent metabolic acidosis. In

the extracellular fluid, carbonate (coming from the shell or bones) and bicarbonate act to buffer the lactic acid. The rest of the acid enters the shell and bones and is again buffered by carbonate. Carbon dioxide is produced by these reactions and enters the extracellular fluid, then diffuses into surrounding water (Ultsch, 1989, 2006). During anoxia, therefore, larger individuals have a lower mass-specific metabolic rate, and the lower metabolic rate might ultimately result in proportionately more buffering capacity by virtue of a greater mass of shell and bones relative to acid production. Indeed, anecdotal evidence suggests that larger individuals of a given species can survive longer during anoxia (Ultsch *et al.*, 1984; Reese, Jackson & Ultsch, 2002), and the buffering capacity of a given species has also been implicated in survival duration during cold hypoxia (Jackson *et al.*, 2007). It is therefore possible that large size confers an advantage during cold hypoxia, and James' rule may arise because the duration of cold hypoxia will generally increase with latitude.

Comparing the temperature–size response of aquatic vs. terrestrial turtles across latitude would be a reasonable framework in which to approach both hypotheses, because both hypotheses generally predict that responses to temperature would be more strongly negative in aquatic chelonians compared with terrestrial ones (see also Forster *et al.*, 2012; Horne *et al.*, 2015; Rollinson & Rowe, 2018a,b). Although some terrestrial tortoises do follow James's rule (e.g. Fasola *et al.*, 2007; Werner *et al.*, 2016), it is unknown whether the strength of temperature–size relationships in terrestrial species is weaker than those in highly aquatic species, and this is the crucial test. Furthermore, more nuanced tests of the hypotheses presented herein are also possible. For instance, if overwintering physiology drives James's rule in chelonians, then temperature–size responses in aquatic species that rely heavily on anaerobic respiration should be stronger than those in species that do not. This is because hypoxia is implicated in the advantages of large size, such that species that overwinter in relatively normoxic conditions (e.g. *Apalone*) would not benefit from larger size during overwintering. Indeed, among the weakest temperature–size responses observed in the present study was that of *A. spinifera*, a softshell turtle (Fig. 2D), and this species has a restricted northern range limit compared with all other species examined in the present study, possibly by virtue of its inability to survive well during hypoxia (Dinkelacker *et al.*, 2005).

In summary, our study did not support the hypothesis that time constraints on reproduction drive James's rule in turtles. New explanations for ecogeographical size clines in turtles are therefore required. First, we suggest that temperature-dependent oxygen limitation drives James's rule in turtles, a mechanism that has been implicated in temperature–size relationships in a variety of other groups but has never been applied to turtles. Second, we suggest that temperature–size responses might be related to the overwintering physiology of turtles, by virtue of a decrease in mass-specific metabolic rate with size, resulting in a disproportionate increase in the capacity of large individuals to buffer lactic acid. We suggest that the way forward in the pursuit of a general explanation for temperature–size relationships is to explore and compare temperature–size relationships between aquatic and terrestrial turtles.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Regressions used to covert straight-line plastron length (PL, in millimetres) to carapace length (CL, in millimetres), and CL to mass (in grams).

Table S2. Parameter estimates for ordinary least-squares regressions between standardized female carapace length and mean annual temperature.

Table S3. Parameter estimates for ordinary least-squares regressions between sexual size dimorphism of carapace length (female CL/male CL) and mean annual temperature.