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A novel hypothesis for the adaptive maintenance of environmental sex determination in a turtle

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Temperature-dependent sex determination (TSD) is widespread in reptiles, yet its adaptive significance and mechanisms for its maintenance remain obscure and controversial. Comparative analyses identify an ancient origin of TSD in turtles, crocodiles and tuatara, suggesting that this trait should be advantageous in order to persist. Based on this assumption, researchers primarily, and with minimal success, have employed a model to examine sex-specific variation in hatchling phenotypes and fitness generated by different incubation conditions. The unwavering focus on different incubation conditions may be misplaced at least in the many turtle species in which hatchlings overwinter in the natal nest. If overwintering temperatures differentially affect fitness of male and female hatchlings, TSD might be maintained adaptively by enabling embryos to develop as the sex best suited to those overwintering conditions. We test this novel hypothesis using the painted turtle (Chrysemys picta), a species with TSD in which eggs hatch in late summer and hatchlings remain within nests until the following spring. We used a split-clutch design to expose field-incubated hatchlings to warm and cool overwintering (autumn–winter–spring) regimes in the laboratory and measured metabolic rates, energy use, body size and mortality of male and female hatchlings. While overall mortality rates were low, males exposed to warmer overwintering regimes had significantly higher metabolic rates and used more residual yolk than females, whereas the reverse occurred in the cool temperature regime. Hatchlings from mixed-sex nests exhibited similar sex-specific trends and, crucially, they were less energy efficient and grew less than same-sex hatchlings that originated from single-sex clutches. Such sex- and incubation-specific physiological adaptation to winter temperatures may enhance fitness and even extend the northern range of many species that overwinter terrestrially.

1. Introduction

Polyphenisms comprise a fascinating form of phenotypic plasticity [1]. Intriguingly, the adaptive significance of these traits is readily evident [2–4] and one of the more spectacular cases of a polyphenism is environmental sex determination (ESD). Successful explanations for the adaptive significance of ESD in short-lived taxa have relied on a theoretical framework [5] that emphasizes differential fitness of male and female hatchlings produced under different environmental conditions [6–8]. Under this Charnov–Bull model [5], the environment must consist of different patch types that elicit phenotypic variation in the offspring and the value of particular phenotypic conditions generated by the patch types differs between the sexes.

ESD (in the form of temperature-dependent sex determination (TSD)) is notably widespread in reptiles, yet its adaptive significance and mechanisms for its maintenance, particularly in long-lived taxa, remain obscure and controversial, despite substantial efforts on both empirical and theoretical fronts [9]. Comparative analyses identify an ancient origin of TSD in turtles, crocodilians and tuatara (all long-lived taxa) [10], suggesting that this trait should be
adaptive (but see [11]). Based on this assumption, as a reasonable starting point, researchers primarily have employed versions of the Charnov–Bull model [5] to examine sex-specific variation in hatching phenotypes and fitness generated by different incubation temperatures. Specifically, the Charnov–Bull model predicts that TSD is adaptive if the fitness of sons is greatest for individuals that hatch from eggs incubated at temperatures that naturally produce males, and the fitness of daughters is greatest for individuals from eggs incubated at temperatures that naturally produce females. A study of short-lived jacky dragons (Amphibolurus muricatus) used hormonal treatments to disentangle sex-specific traits from incubation conditions and provided the first empirical evidence unequivocally supporting this model in reptiles [12].

The longevity of other reptiles with TSD has clouded similar tests of the Charnov–Bull model using lifetime fitness. In most studies, only one or two juvenile traits were measured, yet many morphological, physiological, behavioural and ecological traits interact to affect survival and fecundity (e.g. [13]). Furthermore, some studies find that the sex under stronger selection is produced at incubation temperatures that maximize post-hatching growth (e.g. [14]), whereas other studies yield contrasting results under the same scenario (e.g. [15]). Conflicting findings even occur within the same species (e.g. [16] versus [17]). While dispiriting, the ambiguity may lay in the parameters that are measured rather than embody a rejection of the Charnov–Bull model as a general explanation for the evolution or maintenance of TSD in long-lived reptiles.

The steadfast focus on incubation conditions may be misplaced at least in the many turtle species in which hatchlings overwinter in the natal nest or in other terrestrial circumstances [18,19]. Here, this distinct life stage offers an opportunity for TSD to enhance maternal/hatchling fitness beyond incubation conditions. That is, if overwintering temperatures are predicted by the natal environment and differentially affect fitness of male and female hatchlings, then TSD might be maintained adaptively by enabling embryos to develop as the sex best suited to those overwin\-tering conditions. We test this novel hypothesis using the painted turtle (Chrysemys picta), a species with TSD in which eggs hatch in late summer and hatchlings remain within natal nests until the following spring. This overwintering trait is quite common in turtles with TSD, particularly those with geographical ranges in temperate zones [18,19]. To survive for these many months of terrestrial aestivation, hatchlings rely on maternally provisioned yolk reserves and other sources of stored energy, whose depletion depends on post-hatching thermal conditions [20,21], which may differ between male-producing (generally cooler) and female-producing (generally warmer) nests [22]. To test the overwintering hypothesis, we assessed whether male and female hatchlings performed optimally (minimizing metabolic expenditure and enhancing growth before emerging from the nest in spring) under different overwintering temperature regimes. An adaptive explanation would be supported if we detected a significant sex-by-overwinter temperature interaction such that males were more physiologically efficient (i.e. reduced metabolic rates and yolk catabolism with enhanced growth and/or survival) at cooler temperatures relative to warmer temperatures and vice versa for females.

2. Material and methods

(a) Study species and population
The painted turtle is a common inhabitant of freshwater environments from coast to coast in the northern USA and southern Canada and into parts of the southern USA [23,24]. Terrestrial overwintering of hatchlings is typical of many, if not most, North American turtles with TSD and is apparently absent in all five North American turtles with GSD (i.e. Apalone and Glyptemys) [18,19,22]. Natural nests of painted turtles were studied on an island (the Thomson Causeway) in the Mississippi River near Thomson, Carroll County, IL, USA (41°57′ N, 90°7′ W). The soil was generally a moist sandy loam, similar to nesting substrates used by painted turtles elsewhere (e.g. [25]). The nesting area was surveyed at least every 2 h from 05.00 to 23.00 from late May to early July 2003 for nesting activity. When a nest was identified, the precise location was mapped for subsequent excavation after eggs completed natural development. The nest was carefully opened and the eggs were weighed to the nearest 0.1 g before being placed back into the nest. Measurements of vegetation cover were determined at ground level for each nest with a Model-A spherical densiometer [26]. On 19 September 2003 (autumn), all nests were excavated and hatching turtles were removed from nest cavities and transported to Iowa State University for overwintering treatments (see the electronic supplementary material, Methods).

(b) Temperature regime and experimental design
From the 326 nests constructed at the study site in 2003, we identified 40 nests that were likely to have produced primarily male (cool incubation temperatures, more than 90% male), female (warm incubation temperatures, more than 90% female) and mixed-sex sibships (intermediate incubation temperatures, less than 70% dominant sex). Most painted turtle nests are unisexual and this sex ratio is strongly predictable from densiometer readings (vegetation cover) [26], and summer vegetation cover is also strongly negatively correlated with measures of winter nest temperatures [22]. At the outset, two hatchlings from each clutch were euthanized with an overdose of anaesthetic (0.5 ml of 1:1 distilled H₂O: Nembutal) injected into the pericardial cavity. Sex of the hatchlings was determined by macroscopic examination of the gonads (see the electronic supplementary material, Methods) [27]. Fresh masses of residual yolk and turtle carcases were recorded to the nearest 0.001 g. Residual yolks were stored at −20°C and carcases were fixed in 10% formalin and preserved in 70% ethanol. The remaining members of each clutch were randomly divided into two groups, each maintained at one of two distinct (warm versus cold) overwintering regimes (electronic supplementary material, figure S1). Monthly fluctuating temperature regimes were designed to reflect ‘warm’ and ‘cool’ overwintering conditions similar to those in the field. Critically, ‘cool’ overwintering temperatures were up to 5°C cooler than ‘warm’ overwintering temperatures in the warmer months and fluctuated daily between +1°C and −2°C in January and February, whereas ‘warm’ overwintering temperatures fluctuated between 0.5°C and 2.5°C over the same period (electronic supplementary material, figure S1).

(c) Hatching metabolism
One neonate from each nest was removed from its group overwin\-tering container, weighed and acclimated individually on a damp piece of paper towel in a small (150 ml) tin metabolic chamber for 48 h before the chamber was sealed. The rate of oxygen exchange (VO₂ (µl h⁻¹ g⁻¹)) was measured using closed system respirometry for each neonate during the overwintering period (see the electronic supplementary material, Methods).
supplementary material, table S1)). By contrast, in the warm December) and spring (April and May) (figure 1; electronic supplementary material, table S1) during winter (January and February), when temperatures consistently were at or below freezing (electronic supplementary material, table S1). Mixed-sex nests showed similar sex-specific trends, with males displaying increased metabolic rates compared with females in the warm overwintering regime (electronic supplementary material, table S2), whereas the opposite result occurred in the cool overwintering regime (electronic supplementary material, table S2). Comparing metabolic performances of same-sex hatchlings from single- and mixed-sex nests indicates that the metabolic rates are similar regardless of overwintering regime, except that individuals from predominantly male-biased nests maintained lower metabolic rates than males from mixed-sex nests exposed to the warm overwintering regime (p < 0.03—November, January, February, March).

Reduced yolk catabolism yielded increased body size. Females at warmer temperatures grew, on average, 3% larger than females at cooler temperatures (F1,34 = 79.7, p < 0.001), whereas males at cooler temperatures grew on average 2% larger than males at warmer temperatures (F1,34 = 44.3, p < 0.001) (figure 3a). Males from single-sex clutches grew larger than males from mixed-sex clutches in the cooler regime (F1,25 = 7.7, p = 0.001; figure 3b). Similarly, females

\[ \text{VO}_2 (\mu l \cdot h^{-1} \cdot g^{-1}) \]

Figure 1. \( \text{VO}_2 \) of male (diamonds) and female (squares) hatchling painted turtles in the warm (solid line) and cool (dashed line) overwintering regimes (± s.e.).

3. Results

Nineteen nests were categorized as male dominant, 14 were categorized as female dominant and seven were categorized as mixed. In the cool overwintering regimes, female turtles from female-dominant nests consistently had higher \( \text{VO}_2 \) than males from male-dominant nests in autumn (November and December) and spring (April and May) (figure 1; electronic supplementary material, table S1)). By contrast, in the warm overwintering regime, males from male-dominant nests had higher \( \text{VO}_2 \) levels than females from female-dominant nests over the same periods, except in November (figure 2). \( \text{VO}_2 \) was extremely low and did not differ between treatments (electronic supplementary material, table S1) during winter (electronic supplementary material, figure S1). Mixed-sex nests showed similar sex-specific trends, with males displaying increased metabolic rates compared with females in the warm overwintering regime (electronic supplementary material, table S2), whereas the opposite result occurred in the cool overwintering regime (electronic supplementary material, table S2). Comparing metabolic performances of same-sex hatchlings from single- and mixed-sex nests indicates that the metabolic rates are similar regardless of overwintering regime, except that individuals from predominantly male-biased nests maintained lower metabolic rates than males from mixed-sex nests exposed to the warm overwintering regime (p < 0.03—November, January, February, March).

To assess sex-related fitness differences to test the Charnov–Bull model, we focused on body size and survival through winter. We compared (ANOVA) sex-specific changes in body size (straight carapace length) between autumn and spring from each overwintering period. Moreover, we also compared changes in body size between males from single- and mixed-sex clutches in the cool overwintering regime and did the same for females in the warm overwintering regime. All data were checked for normality and equal variance and log-transformed as appropriate.

(d) Dry mass of residual yolk

At the end of the overwintering period (17–21 May), turtles were euthanized, weighed, measured, dissected and sexed, as described above. Residual yolks were dried to a constant mass in a 60°C oven for 24 h to obtain dry mass. The average dry mass of residual yolk per nest/treatment combination was calculated. Total dry yolk mass used over the winter period was determined by subtracting this estimate from the average dry mass of residual yolk of the two hatchlings from the same nest that were sacrificed prior to commencement of the overwintering portion of the experiment.

(e) Analyses

We directly compared the metabolic rates of male and female turtles (identified at the end of the overwintering period) using separate ANOVAs for each month of the overwintering period (electronic supplementary material, figure S1). Differences in yolk mass between males and females (average mass per nest) in each treatment at the end of the overwintering period were also compared using ANOVA. These physiological parameters provide insight into energetic efficiency leading to potential sex-related differences in fitness at our two overwintering temperatures.

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Sex-specific variation in metabolic rate was reflected in residual yolk use. Males consumed less residual yolk than females in the cool overwintering regime (F1,34 = 58.4, p < 0.01) (figure 2). Forty per cent of females and 18% of males had no discernible reserves remaining at the end of the overwintering period in this treatment. Females consumed less residual yolk compared with males in the warm overwintering regime (F1,34 = 74.3, p < 0.01) (figure 2). Fifty per cent of males and 25% of females had no discernible reserves remaining at the end of the overwintering period in this treatment. Similar trends in yolk utilization were found for turtles from mixed-sex nests.

Reduced yolk catabolism yielded increased body size. Females at warmer temperatures grew, on average, 3% larger than females at cooler temperatures (F1,34 = 79.7, p < 0.001), whereas males at cooler temperatures grew on average 2% larger than males at warmer temperatures (F1,34 = 44.3, p < 0.001) (figure 3a). Males from single-sex clutches grew larger than males from mixed-sex clutches in the cooler regime (F1,25 = 7.7, p = 0.001; figure 3b). Similarly, females

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from single-sex clutches grew larger than females from mixed-sex clutches in the warmer thermal regime \( (F_{1,20} = 5.2, p = 0.03; \text{figure 3}i) \). No mortality occurred during the cooler months of the experiment. Only a small number of individuals died during spring and thus no statistical analyses were conducted, however, during May, 10% of males and 2% of females died.

**Figure 2.** Amount of yolk reserves consumed by male (black) and female (grey) hatchlings from predominantly single-sex nests of painted turtles in the different overwintering regimes (± s.e).

**Figure 3.** (a) Average change in carapace length between winter and spring in male (black) and female (grey) hatchlings from predominantly single-sex nests of painted turtles in the different overwintering regimes (± s.e). (b) Average change in carapace length between winter and spring in hatchlings from single-sex (dark grey) and mixed-sex (light grey) clutches in the cool (males) and warm (females) overwintering treatments (± s.e).
in the warm overwintering treatment and 5% of females and no males died in the cool overwintering treatment.

4. Discussion

Revealing the evolutionary mechanisms by which an individual’s sex is determined is a major challenge. The adaptive significance and maintenance of ESD remain largely unsolved in vertebrates despite 45 years of research (but see [8,12]). Theory predicts that natural selection should favour ESD over genotypic sex determination when the developmental environment differentially influences male and female fitness [5]. Thermal regimes of nests during incubation also positively reflect overwinter temperatures in nest chambers [22], and we found that males and females exhibited different optimal overwintering temperature regimes that relate to environmental properties of the nests. Specifically, we showed that each sex is more efficient in its native overwintering thermal regime in metabolizing energy reserves that, by the time of spring emergence from nests, yielded larger hatchlings with higher survival than same-sex hatchlings in the non-native overwintering thermal regime. Thus, our results provide unequivocal, if unexpected, support for the Charnov–Bull model.

The considerable longevity of most reptiles with TSD has clouded tests of the Charnov–Bull model. While the sex-specific fitness advantage does not have to be substantial to favour TSD over genotypic sex determination [6], quantifying lifetime fitness in organisms that can live many decades is challenging. As a proxy, because mortality rates are highest among hatchlings (e.g. [28]), most studies of the evolutionary significance of CSD in reptiles have perhaps justifiably focused on hatchlings. Surprisingly, however, the overwintering aspect of early life in many species has not received attention from this standpoint, although it has been well studied from various physiological perspectives [19], including the remarkable cold tolerance abilities of many species that may be the most advanced of any amniote vertebrate [19,22,29].

Although we did not observe statistically significant treatment-related mortality, sex- and treatment-specific differences in overwintering physiology and body size of our neonatal painted turtles occurred. Because incubation temperature and hatching sex generally are confounded in reptiles with TSD, relatively few studies have isolated the phenotypic effects of these two factors. Where this has been done, sexual dimorphism in early-life traits in reptiles with TSD has been unmistakably documented, including for physiologically related aspects [30–32]. Although additional work is required to further identify sex- and treatment-specific effects on physiological traits in reptiles with TSD and their influence on individual fitness, even the seemingly small size differences documented here may have considerable fitness effects. Painted turtle hatchlings only 1% longer than their siblings were more likely to survive the necessary terrestrial migration from nest to water [33].

The mechanism underpinning sexual dimorphism in early-life metabolism is unclear, but may involve the endocrine system. Sex steroid hormones circulate at different levels in males and females in vertebrates, including in hatchlings of turtles with TSD (e.g. [34]). Moreover, androgens can inhibit lipid storage and enhance glucose levels, thereby elevating metabolism (e.g. [35]), whereas oestrogens have the opposite effect (e.g. [36]). The extent to which the activities of these sex hormones or aromatase (the enzyme that converts androgens to oestrogens) are temperature sensitive could explain the sexual dimorphism in oxygen consumption and yolk catalysis that we detected. Male C. picta are generally exposed to cooler incubation and overwintering temperatures because their nests experience less solar radiation [22], thus their physiologies may be conditioned to prepare for longer periods of sub-zero temperatures. By contrast, female turtles may employ alternative strategies, because their nests are more likely to enter shallow and irregular periods of sub-zero temperatures. Hence, the sex-specific differences in metabolic rates and yolk use may reflect two strategies for winter survival. Such effects are not limited to the turtles in this study or even to vertebrates. For example, the supercooling temperature for male beetle larvae (Alphitobius diaperinus) is 6°C lower (−17°C) than that of females (−11°C) [37], and this difference relates to the ability to produce antifreeze proteins that inhibit the growth of ice crystals [38].

Our results may be more general than supposed. Hatchlings of many, if not most, freshwater turtle species with TSD in the Northern Hemisphere typically remain in the natal nest or in other terrestrial locations over the winter. Similarly, the tuatara overwinters in the nest in the Southern Hemisphere and has evolved a cold-adapted form of TSD [6]. Few, if any, turtle species with genotypic sex determination do so [18,19]. Thus, we propose that sex-specific overwintering physiology of hatchlings could serve as a proximate mechanism for the adaptive maintenance of TSD in turtles. Of course, exceptions to this hypothesis may arise (sea turtles, snapping turtles, etc.), but our experimental results combined with the underappreciated frequency of terrestrial overwintering by hatchlings of many turtle taxa with TSD is intriguing. Indeed, residual yolk and other sources of stored energy are critical to meeting basal metabolic needs after hatching and during aestivation [19,21]. These maternally sourced energy provisions also provide fuel for essential and demanding post-hatching activities, such as nest emergence and migration. In our study, all hatchlings had significant yolk reserves in autumn before the overwintering period, and these yolk reserves appeared depleted in 50% of males and 40% of females in the warm and cool overwintering treatments, respectively. Thus, atypical aestivation temperatures may induce sex-specific physiological stress in territorially aestivating hatchling turtles [20].

TSD may offer no selective advantage and be neutral, or quasi-neutral, in populations that do not overwinter territorially. Our study nonetheless raises the tantalizing possibility that thermal regimes outside of the incubation environment may confer sex-specific benefits in species with TSD that overwinter in their incubation sites. If generalizable to other species with TSD, our results could help explain the otherwise puzzling persistence of this enigmatic sex-determining mechanism in turtles.

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