

In the article “*Podocnemis expansa* Turtles Hint to a Unifying Explanation for the Evolution of Temperature-Dependent Sex Determination in Long-Lived and Short-Lived Vertebrates” [Sex Dev. 2021;15:23–37, DOI: 10.1159/000515208] by Valenzuela, the female and male lines in Figure 2d should be reversed.

The full Figure 2 (with the corrected panel d) is shown here.

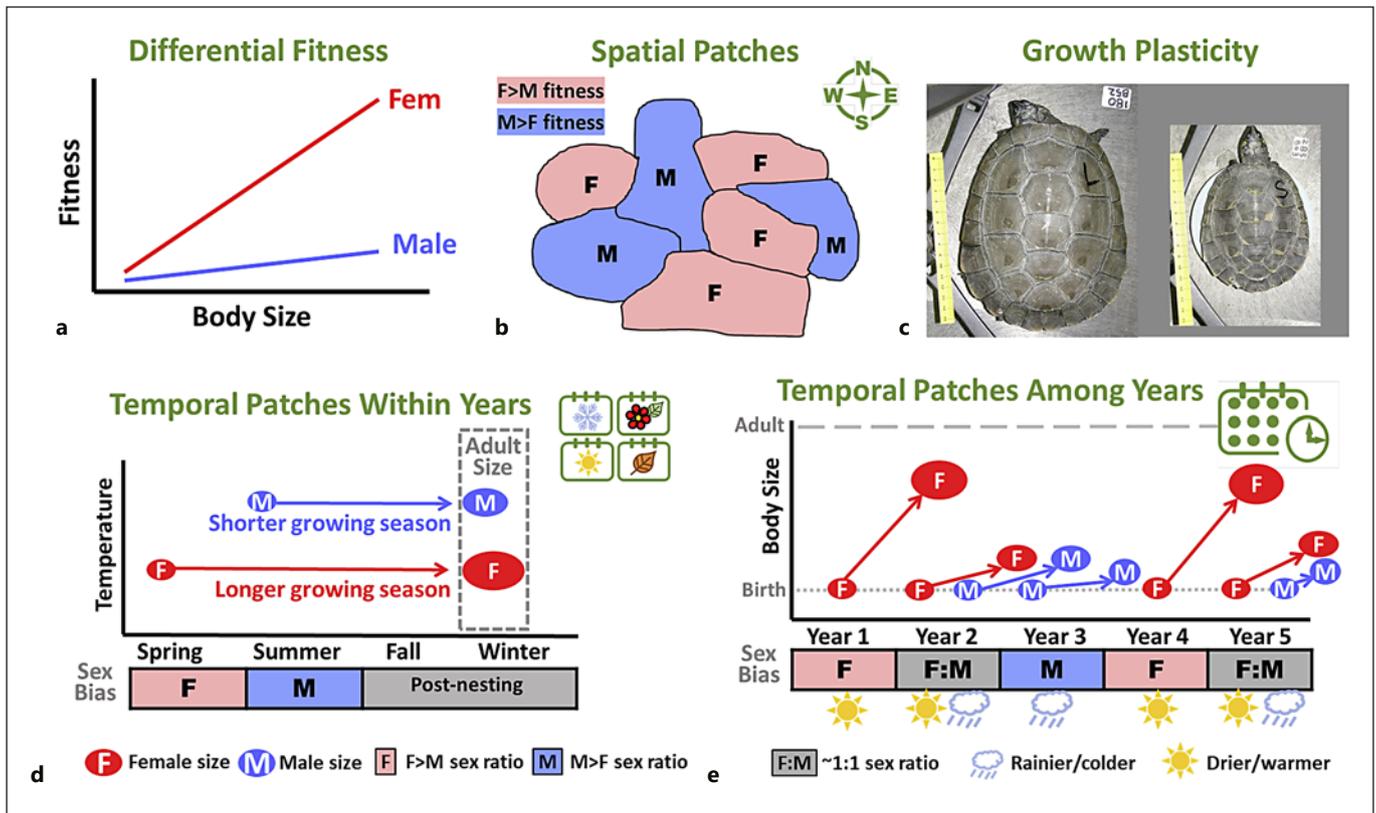


Fig. 2. Fig. 2. Differential fitness hypotheses consistent with the CharnovBull'77 model of adaptive TSD evolution. **a** Females attain greater fitness gains (e.g., via fecundity) than males as a function of body size. **b** Spatially-patchy environments confer greater fitness to one or the other sex in each patch. **c** Largest and smallest 25-month old *P. expansa* clutch mates exhibiting one of the greatest differences in growth rate during a plasticity study [Ceballos et al., 2014]. See text for details. **d** Temporally-patchy environments defined by seasonal temperatures within years confer greater fitness to females (i.e., larger body size – fecundity) when colder nest

temperatures correlate with a longer growing season. **e** Temporally-patchy environments defined by inter/supra-annual temperatures (such as those resulting from El Niño or other cyclical climatic events) confer greater fitness to females when warmer temperatures correlate with optimal resource quality/quantity after hatching (note that TSD would be adaptive under this model when high to full feminization occurs during such years). The panel depicts a hypothetical temporal series where optimal conditions repeat after 4 years; for illustration purposes only.

Podocnemis expansa Turtles Hint to a Unifying Explanation for the Evolution of Temperature-Dependent Sex Determination in Long-Lived and Short-Lived Vertebrates

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Keywords

Adaptive evolution of environmental sex determination · Climate-driven forest phenology of flowering/fruiting · Ecological genetics and life history · Embryonic sexual determination/differentiation/development · Reptilian vertebrates

Abstract

The adaptive significance of temperature-dependent sex determination (TSD) remains elusive for many long-lived reptiles. Various hypotheses proposed potential ecological drivers of TSD. The Charnov-Bull'77 model remains the most robust and explains the maintenance of TSD in short-lived vertebrates, where sex ratios correlate with seasonal temperatures within years that confer sex-specific fitness (colder springs produce females who grow larger and gain in fecundity, whereas warmer summers produce males who mature at smaller size). Yet, evidence of fitness differentials correlated with incubation temperature is scarce for long-lived taxa. Here, it is proposed that the Charnov-Bull'77 model applies similarly to long-lived taxa, but at a longer temporal scale, by revisiting ecological and genetic data from the long-lived turtle *Podocnemis expansa*. After ruling out multiple alternatives, it is hypothesized that warmer-drier years overproduce females and correlate with optimal resource

availability in the flood plains, benefitting daughters more than sons, whereas resources are scarcer (due to reduced flowering/fruiting) during colder-rainier years that overproduce males, whose fitness is less impacted by slower growth rates. New technical advances and collaborative interdisciplinary efforts are delineated that should facilitate testing this hypothesis directly, illuminating the understanding of TSD evolution in *P. expansa* and other long-lived TSD reptiles.

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Sex determination: Any process that establishes and transmits the specification of sexual status of an individual organism (**GO Term 0007530**).

Sex differentiation: The establishment of the sex of an organism by physical differentiation (**GO Term 0007548**).

For millennia, humankind pondered how animals become male or female. Less than a century has elapsed since the discovery of sex chromosomes by Nettie M. Stevens in 1905 [Brush, 1978] and only over half a century since the discovery of temperature-dependent sex determination (TSD) in reptiles by Madeleine Charnier [Charnier, 1966]. Today, the evolutionary lability of sex determination is widely recognized [Bachtrog et al., 2014]. Yet, the causes of this diversity remain a recalcitrant mystery

in biology, particularly for long-lived vertebrates with environmental sex determination (ESD), as is the case for many TSD reptiles that commit to develop as males or females depending on the ambient temperature experienced during embryonic development [Valenzuela and Lance, 2004; Tree of Sex Consortium et al., 2014]. Furthermore, trying to understand why so many reptiles exhibit TSD has led to a variety of potential explanations and to many unsatisfactory empirical tests. Indeed, few examples exist where the adaptive basis of TSD in vertebrates has been empirically validated, all in short-lived taxa, whereas the presence of TSD in long-lived vertebrates has defied conclusive explanations. Here, data from a long-lived reptile, the pleurodiran turtle *Podocnemis expansa*, are revisited, and it is hypothesized that the scenarios that explain TSD in short-lived species may apply also to those with long lifespans.

The Giant Amazonian river turtle, *P. expansa* belongs to the family Podocnemididae in the chelonian suborder Pleurodira. This turtle is widely distributed in the Amazon, Orinoco, and Esequibo river basins in South America where it has cultural and economic importance for diverse human populations inhabiting its distributional range [Hildebrand et al., 1997; Hernandez and Espin, 2006]. Current estimates indicate that Pleurodiran turtles evolved in the late Jurassic about 165-150 Mya, and that by the early Cretaceous, Pan-Pelomedusoides had split from Pan-Chelidae and dispersed from Africa to South America where Podocnemidoidea diversified by the late Cretaceous [Ferreira et al., 2018; Cadena, 2020]. Recent studies date the origin of *Podocnemis* between 90 Mya in the late Cretaceous [Pereira et al., 2017] to 55 Mya in the late Paleocene [Ferreira et al., 2018]. *P. expansa* exhibits the most common TSD pattern seen in turtles where males are produced at low and females are produced at high temperatures (TSDIa or MF TSD) [Alho et al., 1985; Valenzuela, 2001a], yet field and laboratory data suggest that females (albeit few) are induced by even lower temperatures that typically cause high mortality [Valenzuela, 2001a; Lubiana and Ferreira, 2009]. Other aspects of *P. expansa*'s ecology have been well studied [Valenzuela, 2000; Vanzolini, 2003; Chinsamy and Valenzuela, 2008; Mogollones et al., 2010; Ceballos et al., 2014] along with the population genetic structure across its geographic range [Sites et al., 1999; Valenzuela, 2000, 2001b; Bock et al., 2001; Pearse et al., 2006a]. Combined, these data provide insight into why *P. expansa*'s sexual development is under the control of the environmental temperature. Here, salient aspects of turtle sex determination and of *P. expansa*'s ecology and genetics are reviewed, and based

on those data, multiple alternative hypotheses are tested that might explain the maintenance of TSD in this lineage, leading to the proposition that a common adaptive explanation may exist for long-lived taxa as there is for several TSD reptiles with short lifespans.

Turtle Sex Determination

Of the more than 355 turtle species recognized today [The Turtle Conservation Coalition, 2018], 102 have a known system of sex determination [Tree of Sex Consortium et al., 2014; Literman et al., 2017; Rovatsos et al., 2017; Mazzoleni et al., 2019; 2020; Viana et al., 2020], which can be identified by different approaches. On the one hand, incubation experiments across a range of temperatures may be used such that when the resulting sex ratios are always 1:1, TSD is ruled out and a system of genotypic sex determination (GSD) is inferred [Valenzuela et al., 2003]. However, these experiments do not reveal whether these systems are polygenic or if they rely on sex chromosomes. Cytogenetics has been useful for characterizing the type of heterogamety in turtles, either via classic staining techniques that permit visualizing heteromorphic sex chromosomes [Bull et al., 1974; Carr and Bickham, 1981; McBee et al., 1985] or molecular cytogenetics for turtles with homomorphic sex chromosomes [Ezaz et al., 2006; Kawai et al., 2007; Martinez et al., 2008; Badenhorst et al., 2013; Montiel et al., 2017; Lee et al., 2019; Mazzoleni et al., 2019, 2020; Viana et al., 2020]. More recently, PCR or qPCR amplification of sex-linked molecular markers developed in some taxa were applied to identify the presence of sex chromosomes in related taxa [Literman et al., 2014, 2017; Rovatsos et al., 2017]. Importantly, while thermally-induced sex reversals in reptiles are well documented in some lizards with sex chromosomes [Shine et al., 2002; Quinn et al., 2007, 2009; Radder et al., 2008; Holleley et al., 2015; Hill et al., 2018], such cases are unknown in turtles. On the contrary, the co-existence of TSD and GSD proposed to occur in *Pelodiscus sinensis* and *Chrysemys picta* was empirically refuted [Valenzuela et al., 2014; Mu et al., 2015].

The Origins of TSD in *Podocnemis expansa*

Species-level phylogenetic comparative analyses revealed that TSD is the likely ancestral condition in turtles, reptiles, and probably amniotes [Pokorná and Kratochvíl, 2009; Valenzuela and Adams, 2011; Pokorna et al., 2014;

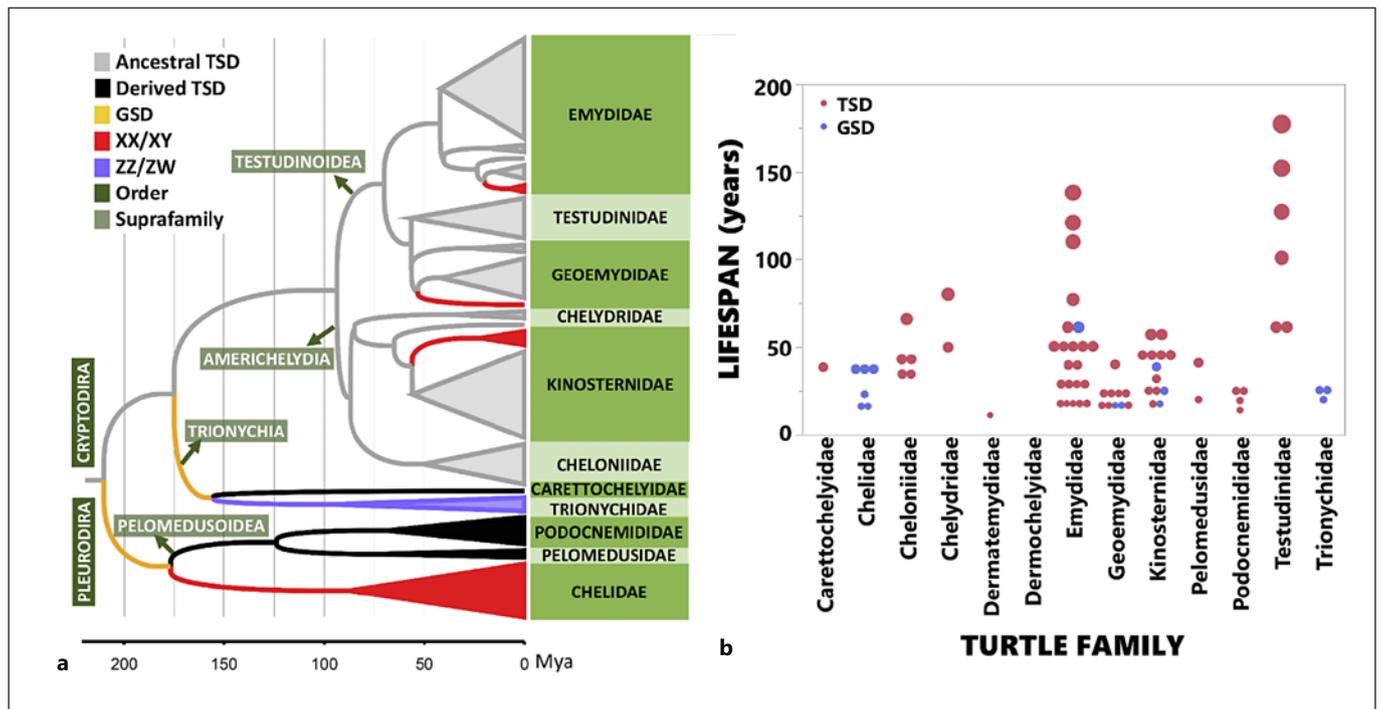


Fig. 1. **a** Phylogenetic relationships among turtle families and their sex-determining mechanism [Bista and Valenzuela, 2020]. **b** Turtle lifespan by taxonomic family [data from Sabath et al., 2016]. Circle size is proportional to lifespan value.

Sabath et al., 2016]. From this ancestral TSD mechanism, sex chromosome systems of GSD evolved at least 5 times in turtles, at least 4 times as male heterogamety (XX/XY), and at least once as female heterogamety (ZZ/ZW) [Bista and Valenzuela, 2020] (Fig. 1a). These sex chromosomes evolved by the co-option of different autosomes in most instances [Montiel et al., 2016], although some lineages reused the same ancestral pair of autosomes, resulting in a case of convergent XX/XY evolution [Montiel et al., 2017].

P. expansa is among those chelonians with TSD, yet it may not have retained the ancestral TSD condition. Instead, *P. expansa* belongs to 1 of 2 turtle lineages where a reversal from GSD back to TSD might have occurred [in Pelomedusoidea (i.e., Podocnemididae + Pelomedusidae), and in Carettochelyidae] [Valenzuela and Adams, 2011; Literman et al., 2018] (Fig. 1a) such that *P. expansa*'s TSD could be an evolutionarily derived trait. Curiously, based on divergence time estimates (www.time-tree.org), these 2 putative evolutionary reversals from GSD to TSD may have occurred at similar times at the split of the 2 lineages in both turtle suborders, namely, 148 Mya in Carettochelyidae (Cryptodira) and 144 Mya

in Pelomedusoidea (Pleurodira) [Valenzuela and Adams, 2011; Bista and Valenzuela, 2020] (Fig. 1a). Although the inference of these reversals is not parsimonious [Pokorná and Kratochvíl, 2014; Sabath et al., 2016], genes involved in sexual development exhibit an accelerated rate of molecular evolution in Carettochelyidae and Podocnemididae, above the uptick experienced by their GSD sister lineages, an observation consistent with the hypothesis that a GSD-to-TSD reversal might indeed have taken place [Literman et al., 2018; Bista and Valenzuela, 2020]. GSD-to-TSD transitions are more common in lizards than in turtles [Sabath et al., 2016], and current climate-induced sex reversals in *Pogona vitticeps* (ZZ/ZW; lifespan = 5–8 years) exemplify how quick such transitions can take place in short-lived reptiles [Holleley et al., 2015].

Why Did TSD Evolve in Pelomedusoidea (Podocnemididae + Pelomedusidae)?

The mechanism by which transitions to and from TSD and GSD occur have been modeled via population genetics [Bull, 1981], quantitative genetics [Grossen et al.,

2010], gene dosage [Quinn et al., 2011], and developmental biology [Uller and Helantera, 2011] frameworks, among others. This review concentrates on potential ecological drivers of the evolution of sex-determining mechanisms (SDM), with special emphasis on some models that may be applicable to *P. expansa*, noting that this is by no means a comprehensive literature review. Further discussion about these and other hypotheses may be found elsewhere [Valenzuela, 2004, 2010a, 2018; Uller and Helantera, 2011; Beukeboom and Perrin, 2014; Bista and Valenzuela, 2020; Schwanz and Georges, 2021].

Explaining the retention of TSD in turtles is easier than explaining a GSD-to-TSD reversal as purported for Pelomedusoidea. For instance, theory predicts that lifespan can render TSD and GSD adaptive, maladaptive, or neutral [Bull and Bulmer, 1989; Valenzuela, 2004; Schwanz and Proulx, 2008; Freedberg and Debenport, 2014]. Consistently, macro-evolutionary variation in lifespan is linked to differences in the rates of evolutionary transitions in sex determination observed between turtles and lizards [Sabath et al., 2016]. Namely, lizards (who are generally shorter-lived than turtles) transitioned from TSD to GSD more often than turtles, likely because skewed sex ratios produced by TSD are detrimental to short-lived taxa, as such biases endangered population survival (a potential cost of TSD) [Sabath et al., 2016]. Moreover, TSD turtles show a tendency to evolve longer lifespans than their GSD relatives, and the same is true within lizards [Sabath et al., 2016]. Thus, TSD would be a more neutral trait in long-lived turtles, where it could have evolved and be retained non-adaptively [Bull, 1980; Girondot and Pieau, 1999; Valenzuela, 2004]. On the other hand, for TSD to be maintained adaptively in short-lived taxa, there must be stronger selective forces that outweigh or neutralize its potential costs; otherwise, a transition to GSD is expected to occur [Bull, 1980; Valenzuela, 2004, 2018; Sabath et al., 2016] as demonstrated in 2 species of *Menidia* fish [Conover and Heins, 1987; Yamahira and Conover, 2003]. Such advantages could also exist in long-lived taxa, but their effect must be large to counter the neutralizing effect of long lifespan so as to permit detection and to lead to adaptive evolution [Valenzuela, 2004]. Indeed, longevity has thus far precluded the collection of lifetime reproductive success needed to make direct inferences about the adaptiveness of TSD in many turtles and other TSD reptiles such as crocodylians or tuatara. Consequently, most studies that examined fitness benefits of incubation temperature in turtles investigated effects immediately post-hatching or in early life, including mortality, morphology (size or shape), growth, energy reserves

(residual yolk mass, body fat, behavior, performance, and physiology) [reviewed in Valenzuela, 2004], but none on lifetime reproductive success.

The average lifespan of Pelomedusoidea is 25.6 years, which is slightly shorter than the average reported for its GSD sister lineage, the family Chelidae (26.7 years) [Sabath et al., 2016] (Fig. 1b), suggesting that if it is true that TSD is a derived trait in Pelomedusoidea, perhaps it arose relatively non-neutrally (though caution should be taken since lifespan estimates may not represent variation within and among populations, and some estimates come from captive individuals and others from wild animals). Importantly, whether TSD evolved neutrally or adaptively in Pelomedusoidea, it would have done so by the accumulation or co-option of a pre-existing thermal sensitivity present in its GSD ancestor [Bull, 1981; Valenzuela, 2004]. Evidence from Cryptodiran turtles demonstrate that such thermal sensitivity does exist in GSD turtles. Indeed, the gene regulatory network underlying gonadal development via environmental, genetic, and epigenetic mechanisms [Capel, 2017] shares a significant number of genes across vertebrates, including turtles, with the notable exception of *Sry* which evolved exclusively in Therian mammals [Wallis et al., 2008]. Significant evolutionary changes have accrued in the transcriptional patterns of these sexual development regulators across vertebrates [Valenzuela et al., 2013; Mizoguchi and Valenzuela, 2020]. Importantly, direct comparisons of gene expression between *C. picta* (TSD) and *Apalone spinifera* (a GSD turtle with ZZ/ZW sex chromosomes) [Badenhorst et al., 2013] revealed that the complete loss of the thermal responses by this network is not a prerequisite for the evolution of GSD. Namely, thermal sensitivity is retained in numerous (but not all) sexual development genes in *Apalone* [Valenzuela et al., 2006, 2013; Valenzuela and Shikano, 2007; Valenzuela, 2008a, b, 2010b; Radhakrishnan et al., 2017, 2018]. Such relic thermal sensitivity could have been co-opted during the reversal from GSD to TSD in Carettochelidae, the sister family to Trionychidae (to which *Apalone* belongs). A similar scenario may be plausible in Pelomedusoidea but gene expression data from its sister family Chelidae is lacking.

Retention of TSD in *Podocnemis expansa*

Irrespective of whether a GSD-to-TSD reversal occurred or not, to explain the presence of TSD in *P. expansa* is to elucidate its retention, either from the ancestor of all turtles or from the ancestor of Pelomedusoidea. One

hypothesis is that TSD might be retained in *P. expansa* via phylogenetic inertia due to lack of genetic variation that precludes this lineage from evolving out of TSD. However, this explanation can be ruled out because variation exists among populations of *P. expansa* in their TSD pattern, as observed also within and among congeneric species (*P. unifilis*, *P. erythrocephala*, and *P. leywana*) [Lance et al., 1992; de Souza and Vogt, 1994; Valenzuela et al., 1997; Valenzuela, 2001a, c; Lubiana and Ferreira, 2009; Paez et al., 2009; Bonach et al., 2011; Gallego-Garcia and Paez, 2016; Gómez-Saldarriaga et al., 2016], as well as in their incubation time [Hildebrand et al., 1997; Valenzuela, 2001c; Lubiana and Ferreira, 2009], clutch size, and other life history traits [Vanzolini, 2003]. Furthermore, variation in the thermal response of the gene network regulating sexual development in turtles and the general lability of sex determination in this clade (TSDIa, TSDII, XX/XY, and ZZ/ZW systems have evolved in turtles) also argue against this hypothesis as an overall explanation for chelonians [Bull, 1980; Ewert et al., 2004; Valenzuela, 2004; Grossen et al., 2010; Uller and Helantera, 2011; Valenzuela et al., 2013; Holleley et al., 2015].

Alternatively, TSD retention in *P. expansa* could be neutral and explained simply because TSD may work equally well as GSD, which on average produces 1:1 sex ratios despite biases at particular locations or years [Bull, 1980; Girondot and Pieau, 1999; Valenzuela, 2004]. But the unescapable reality for species with TSD is that their population sex ratios are tied to an environmental variable, and so are their effective population sizes, rate of loss of genetic variation, and consequently, their extinction probabilities [Bessa-Gomes et al., 2004; Girondot et al., 2004; Valenzuela and Lance, 2004; Bachtrog et al., 2014]. Directional climate change could skew sex ratios drastically [Neuwald and Valenzuela, 2011; Jensen et al., 2018; Valenzuela et al., 2019] to the point that the evolution of a GSD system would be favored to restore the investment into male and female production closer to balance [Bull, 1980]. Consistent with this scenario, evolutionary turnover of sex determination (mostly TSD-to-GSD transitions) occurred in turtle lineages that split from their sister clades around geological times associated with climate change events [Valenzuela and Adams, 2011], yet other lineages preserved their TSD and survived these climatic changes. *P. expansa*'s sex ratios at birth vary among years and populations. For instance, reports range from a highly feminized sex ratio (11:1 female:male) detected in a longer-term study of a population in the Orinoco river [Mogollones et al., 2010] to slighter female-bias or male-bias recorded with lesser sampling in single years in oth-

er populations [Valenzuela, 2001a; Bonach et al., 2011]. Further, no systematic female-biased sex ratios at a population-wide scale have been reported for *P. expansa* that are tied to contemporary global warming. In fact, although the number of females varies among current populations, males are more abundant in some localities [Portelinha et al., 2014; Forero-Medina et al., 2021] (but the effect of overexploitation of nesting females which might mask feminization by climate change remains unclear). Additionally, the lifespan of *P. expansa* is estimated from captivity as 25.4 years [Tacutu et al., 2013], which is high for Podocnemididae (Fig. 1b) (although it is still lower than the average and maximum lifespan reported for turtles overall, i.e., 41.3 and 177 years, respectively) [Sabath et al., 2016]. However, reported estimates of lifespan vary substantially such that the actual lifespan of *P. expansa* remains uncorroborated. For instance, the lifespan estimate of 25.4 years [Sabath et al., 2016] reported in Tacutu et al. [2013] derives from captivity [Snider and Bowler, 1992] and differs from other estimates based on extrapolation without empirical evidence ranging from >30 years [Ojasti, 1971] to about 80 years [Hernandez and Espin, 2006]. Furthermore, it is unclear whether anthropogenic overexploitation of *P. expansa*, especially if targeted against the largest (perhaps older) individuals, might result in underestimates of lifespan. Nonetheless, *P. expansa*'s lifespan and the presence of overlapping generations could buffer against the detrimental effects of skewed sex ratios produced over multiple years.

Adaptive TSD in *Podocnemis expansa*?

Again, the extended lifespan of *P. expansa* also means that if TSD is to be adaptive, its benefits must be much greater than any costs and strong enough to be detectable. But could TSD be adaptive in long-lived turtles at all? What are the potential costs and benefits of TSD in *P. expansa*? Some adaptive hypotheses for the retention of TSD predict that sex ratio biases produced by individual females via TSD are advantageous, correlated tightly with temperature, and impossible to achieve under GSD.

One such hypothesis proposes that TSD is adaptive because it reduces inbreeding by producing unisexual clutches [Ewert and Nelson, 1991; Burke, 1993]. *P. expansa*'s time to maturity is calculated as 5–10 years [Ramírez, 1956; Ojasti, 1971; Mittermeier, 1978], consistent with 5–6 years estimated by a preliminary skeletochronology analysis [Chinsamy and Valenzuela, 2008], but as 15 years using carapace annuli [Pritchard and

Trebbau, 1984] or 17 years based on a very small sample size which renders this estimate unreliable [Hernandez and Espin, 2006]. Variation in these estimates is expected considering that in *P. expansa*, as in turtles in general, female onset of maturity depends more on the attainment of a minimum size rather than on age [Alho and Pádua, 1982 and references therein], a point that will become important later on. Nonetheless, any of these values combined with *P. expansa*'s lifespan would allow interbreeding of numerous cohorts, diluting the probability that siblings will reproduce among themselves. Furthermore, *P. expansa* exhibits high levels of multiple paternity, with 2-10 minimum number of sires per clutch and up to 13 groups of siblings detected in natural nests [Valenzuela, 2000; Fantin et al., 2017a, b], which reduces the relatedness of clutch-mates and consequently the potential for inbreeding. Multiple paternity is less detectable in highly decimated populations of *P. expansa* with depauperate genetic variability [Pearse et al., 2006b]. Finally, within-nest temperature gradients [Valenzuela, 2001a] reduce the occurrence of unisexual clutches. Therefore, TSD is not a crucial or unique inbreeding avoidance mechanism in *P. expansa* and is probably not maintained for its role as such.

Another hypothesis states that TSD is adaptive because it produces biased sex ratios which are favored by natural selection in small breeding kin groups [Hamilton, 1967], as overproduction of females would bolster population growth [Bull and Charnov, 1988]. When extreme, however, drastically small demes or drastically skewed sex ratios could push demes to extinction instead [Berec et al., 2001]. This hypothesis assumes that breeding groups in TSD species are smaller and more isolated than in GSD species, a phenomenon that should result in lower heterozygosity in TSD than in GSD taxa, for which no genetic evidence has been reported [Burke, 1993; Shine, 1999]. Furthermore, heterozygote deficiency was detected in some but not all populations of *P. expansa* throughout its range [Sites et al., 1999; Valenzuela, 2001b; Pearse et al., 2006a] and is attributable to recent bottlenecks [Pearse et al., 2006a]. Consistently, populations have declined due to centuries of human overexploitation which have decimated this species in many regions to the point that it is now listed in the Appendix II of CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora; www.cites.org). This hypothesis is therefore inconsistent with data from reptiles in general [Burke, 1993; Shine, 1999] and for *P. expansa* in particular.

Other adaptive models predict that temperatures frequently encountered in nature by individuals living in

spatially or temporally patchy habitats have a lifetime differential fitness effect between the sexes (in terms of survival and/or reproductive output) (Fig. 2a, b) [Charnov and Bull, 1977]. These fitness effects can be exerted directly by the incubation temperature or exerted by a variable tightly correlated with the environmental temperature. Under this scenario, TSD enhances the fitness of reproductive females by enabling their offspring to develop into the sex that attains maximal fitness under incubation conditions that cannot be predicted by the parents. This differential fitness hypothesis is the most robust of the models proposed thus far, and several posed alternatives vary in the mechanism linking incubation temperature to fitness [Shine, 1999; Valenzuela, 2004].

One variant states that natal homing favors ESD by providing the fitness differential needed in the Charnov-Bull'77 model [Reinhold, 1998]. This hypothesis proposes that daughters are produced at nesting sites that confer higher survival, to which they return to nest as adults, thus attaining higher fitness than sons whose reproductive output is unaffected by their natal site if they survive to maturity. *P. expansa* is a gregarious nester, and females reutilize nesting beaches with high fidelity when available [Ojasti, 1971; Alho et al., 1979], perhaps following a social facilitation behavior akin to that reported for its congener *P. unifilis* [Escalona et al., 2009, 2019]. However, although initial studies detected genetic structure among nesting beaches in *P. expansa* using microsatellite DNA consistent with natal homing [Valenzuela, 2001b], later meta-population analyses using mitochondrial DNA demonstrated a lack of natal homing in both sexes [Pearse et al., 2006a]. Thus, this hypothesis can be refuted in *P. expansa* and has been empirically refuted in other freshwater turtles [Valenzuela and Janzen, 2001].

A second variant states that temperature affects offspring mortality during development differentially for males and females, such that TSD is adaptive because it produces the best-fit sex at temperature extremes [Burger and Zappalorti, 1988]. In *P. expansa*, mortality rates during development and sex ratio are independent [Valenzuela, 2001a]. It should be noted that differential mortality cannot be the sole cause for biased primary sex ratios produced under TSD [Bull and Vogt, 1979; Valenzuela et al., 2003]. Instead, TSD is a mechanism that affects the commitment and differentiation of the developing gonad into a testicular or ovarian fate directly [Valenzuela, 2008c; Weber et al., 2020]. Gathering evidence to test this hypothesis requires artificially producing the rare sex at temperatures that normally produce the opposite sex (via e.g., hormonal manipulation) [Rhen and Lang, 1995].

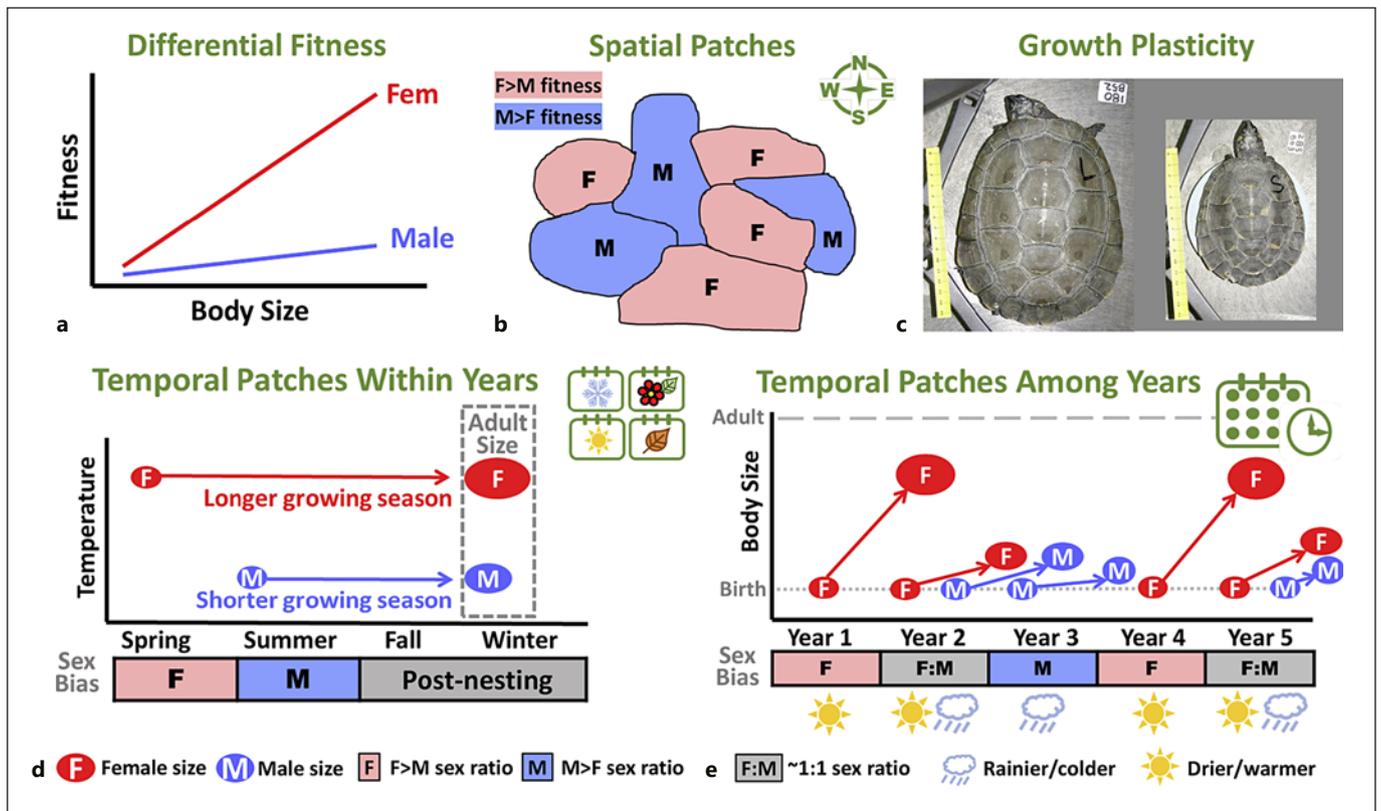


Fig. 2. Differential fitness hypotheses consistent with the Charnov-Bull'77 model of adaptive TSD evolution. **a** Females attain greater fitness gains (e.g., via fecundity) than males as a function of body size. **b** Spatially-patchy environments confer greater fitness to one or the other sex in each patch. **c** Largest and smallest 25-month old *P. expansa* clutch mates exhibiting one of the greatest differences in growth rate during a plasticity study [Ceballos et al., 2014]. See text for details. **d** Temporally-patchy environments defined by seasonal temperatures within years confer greater fitness to females (i.e., larger body size – fecundity) when colder nest temperatures

correlate with a longer growing season. **e** Temporally-patchy environments defined by inter/supra-annual temperatures (such as those resulting from El Niño or other cyclical climatic events) confer greater fitness to females when warmer temperatures correlate with optimal resource quality/quantity after hatching (note that TSD would be adaptive under this model when high to full feminization occurs during such years). The panel depicts a hypothetical temporal series where optimal conditions repeat after 4 years; for illustration purposes only.

Under such conditions, evidence in favor of this model would be found if individuals of the rare sex suffer higher mortality than the common sex or higher than individuals of the same sex from temperatures that normally produced mixed sex ratios. While no such data have been collected for *P. expansa*, mortality was not differential in snapping turtles when using hormonal manipulations [Rhen and Lang, 1995], and the reptiles that match this hypothesis (i.e., snakes) have GSD [Burger and Zappalorti, 1988], indicating that differential mortality is not a determinant for TSD evolution.

Another variant [Roosenburg, 1996] proposes that females choose nest sites according to the size of their eggs in ways that bias offspring sex ratio, assuming that larger eggs produce larger offspring, and because daughters

benefit more than males from a larger initial size through growth post-hatching. This notion can be ruled out in *P. expansa*, because larger females produce more and larger eggs which are buried deeper [Valenzuela, 2001c] where temperatures are cooler overall (masculinizing) [Valenzuela, 2001a] instead of warmer, as predicted by this model. Furthermore, larger clutches of *P. expansa* occupy larger egg chambers which experience wider thermal clines than smaller clutches, resulting in mixed sex ratios [Valenzuela, 2001a] and reducing the potential for nesting females to control sex ratios.

In a similar vein, other adaptive hypotheses consider the differential fitness that males and females may gain as a function of increased body size (Fig. 2a) and the potential for temperature to influence those sizes. One of these

models was originally proposed based on scant data at the time and states that TSD is favored in species with sexual size dimorphism (SSD) if the larger sex is produced by temperatures that enhance growth, whereas GSD would occur in taxa that are less dimorphic [Head et al., 1987; Deeming and Ferguson, 1988; Ewert and Nelson, 1991; Valenzuela, 2004]. *P. expansa* exhibits female-larger SSD, and common garden experiments revealed that growth during the first 25 months of age is affected positively by egg size, and it is temperature- and resource-dependent [Ceballos et al., 2014]. Namely, size at hatching and growth over the first 2 years of life is plastic (Fig. 2c), enhanced not only by incubation temperature but also by other environmental factors, such as water temperature post-hatching, food quality, and food quantity [Ceballos et al., 2014]. But notably, both sexes are equally plastic, and plasticity can change through time [Ceballos et al., 2014]. Also, larger offspring at hatching (from laboratory experiments and in the field) experienced higher survival [Valenzuela, 2001c]. Nonetheless, this model predicts the co-evolution of SDM and SSD in turtles (or reptiles) in a way that to falsify it, it must be tested at the macroevolutionary level and not in a single species. However, species-level phylogenetic analysis based on larger datasets showed that female-larger SSD is ancestral in turtles and that monomorphic or male-larger SSD taxa evolved from this ancestral state, yet sex determination and SSD do not co-evolve [Ceballos et al., 2012] as predicted by this model. Thus, this macroevolutionary hypothesis has been ruled out as a general explanation for the evolution of TSD across turtles. A recent theoretical variant proposing that TSD is favored when males and females follow different maturation schedules if temperature influences juvenile survival (even if it does so equally for both sexes) was not supported in turtles [Schwanz et al., 2016].

A Novel Look at an Old Hypothesis – Temporal Patches

This leads us to a hypothesis that has been neglected for long-lived reptiles, namely, that TSD is adaptive because incubation temperatures predict future environmental conditions experienced by offspring after birth that affect male and female fitness differentially [Conover, 1984]. In the known cases where this model applies, the environmental temperature experienced by the developing offspring is a reliable cue of the length of the ensuing growing season during the first year of life before sexual maturity is attained (Fig. 2d). Thus, offspring born at

colder temperatures earlier in the spring become females and are larger at maturity (accruing greater fitness via fecundity gains) than males who are born at warmer temperatures later in the season and consequently grow less but whose fitness is less dependent on body size (Fig. 2a). This hypothesis has been demonstrated by elegant studies in a few short-lived vertebrates: the silverside fish *Menidia menidia* [Conover, 1984; Duffy et al., 2015] and *M. peninsulae* [Yamahira and Conover, 2003], and in *Ambiolurus muricatus* lizards [Warner and Shine, 2008]. Some data suggest that the same may be true in *Gnathopogon caeruleus* [Fujioka et al., 2015] and *Micrometrus minimus* [Schultz, 2008] fish and in *Agama picticauda* [Steele and Warner, 2020] and other short-lived lizards [Warner et al., 2009]. The short life span of these species makes this adaptive scenario plausible, while the protracted growing season of long-lived taxa such as turtles would preclude its applicability in this exact manner.

However, based on data from *P. expansa*, it is hypothesized here that seasonal shifts in sex ratios may also occur in long-lived taxa in ways that confer an adaptive advantage to TSD. The difference, however, is that the time-scale of the seasonal shifts in sex ratios that matters for these long-lived vertebrates may be across years instead of within years (Fig. 2e). This hypothesis is based on the observations (detailed below) that

1. Sex ratios change among years within populations of *P. expansa* [e.g., Lance et al., 1992; Valenzuela et al., 1997; Valenzuela, 2001a];
2. Nesting females may be as small as 50 cm [Alho and Pádua, 1982] or 52 cm carapace length [Hildebrand et al., 1997];
3. Offspring exhibit an impressive plasticity in growth and are able to attain remarkably large sizes in their first year of life under optimal resource availability (quality and quantity) [Ceballos et al., 2014];
4. Natural climatic oscillations affect temperature, irradiance, and precipitation during the nesting season, influencing sex ratios as well as resource availability in the flood plains where *P. expansa* migrate to feed after hatching [Wright and Calderon, 2006; Zanin and Satyamurty, 2020].

Seasonal flood pulses are important for the life cycle of *P. expansa*. Males and females migrate in groups from backwaters, lakes, and small tributaries toward the nesting beaches, sometimes for hundreds of kilometers (sometimes >420 km) as the water levels drop and sandbanks emerge for females to oviposit [Hildebrand et al., 1997; Ferrara et al., 2014]. The exact timing of this migration and oviposition varies by basin and sub-basins

throughout *P. expansa*'s distributional range in South America, because hydrological cycles are not uniform, as some depend on local precipitation whereas others depend on regional events that occur a long distance away from the nesting areas [Hildebrand et al., 1997; Vogt, 2008; Zanin and Satyamurty, 2020]. But importantly, the onset of rains and rising water levels in the nesting areas induce hatching, and neonates are guided by vocalizations from adults to join in a mass migration into the flood plains where they feed for an extended period [Hildebrand et al., 1997; Vogt, 2008; Ferrara et al., 2014]. Likewise, these flood plains attract other aquatic animals such as numerous fish that exploit food resources during the high water pulses [Ropke et al., 2016].

Importantly, resource availability in the flood plains and the rest of the home range of *P. expansa* is affected by climatic cycles and extreme anomalies that vary over time in their frequency and amplitude. Indeed, natural climatic oscillations, such as supra-annual El Niño events that reduce average rainfall and increase average temperature and irradiance (including in *P. expansa*'s distributional range in South America) [Wright and Calderon, 2006; Zanin and Satyamurty, 2020], enhance fruit production, whereas mild dry seasons (when dry season rains occur locally, i.e., “wetter” dry seasons) discourage flowering [Wright and Calderon, 2006]. On the other hand, neotropical forests are capable of masting [Norden et al., 2007], including those in the Amazon basin [Mendoza et al., 2015], and these massive fruiting events at supra-annual intervals provide ad libitum feeding conditions for frugivores [Mendoza et al., 2015]. These cycles affect the trophic networks that depend on the forest phenology, as evidenced by extreme anomalies in these climatic oscillations that result in famine for frugivores and granivores, as observed in some communities during mild dry seasons following El Niño [Wright et al., 1999]. This happened because the high production induced by El Niño over-utilized stored plant reserves, thus limiting the next reproductive event, an effect not countered by the mild dry season that followed that particular El Niño event, since rainier dry seasons reduce fruit production [Wright et al., 1999].

P. expansa is mostly herbivorous but opportunistically omnivorous, a trait tied to the seasonality of the flood plain habitat [Eisemberg et al., 2017]. Information about *P. expansa* feeding habits of juveniles and adults include stomach contents of mostly fruits and seeds, along with leaves, flowers, stems, plus vertebrate and invertebrate parts [Hildebrand et al., 1997; Vogt, 2008; Eisemberg et al., 2017; Cunha et al., 2020]. Scavenging dead fish and

rare predation of live fish by *P. expansa* is also reported by indigenous people [Hildebrand et al., 1997], while in captivity, sub-adults prefer fish and beef [Ojasti, 1971; Eisemberg et al., 2017]. While reports concern mainly juvenile to adult individuals, less is known about the natural diet of hatchlings, although in *P. unifilis* (the congener closest in adult size to *P. expansa*) fish consumption is high in juveniles [Eisemberg et al., 2017].

Thus, the following scenario is hypothesized here. Warmer (feminizing) drier years, when more female *P. expansa* are produced by incubation at higher temperatures, would be correlated with optimal resource availability in the flood plains, which would benefit daughters more than sons. On the other hand, during rainier/colder years, when temperatures during the nesting season are more masculinizing and resources are more scarce (due to reduced flowering/fruitletting), more sons would be born who will not be as negatively affected by the depressed growth rates (Fig. 2a, d). The longevity and overlapping generations of *P. expansa* would buffer populations against extreme biased sex ratios produced among years. Consistently, existing data show that sex ratios in *P. expansa* vary considerably among years due to changes in climatic conditions. For instance, in the Caquetá river in Colombia, 0–60% females were produced in 1997 [Valenzuela, 2001a], 70–100% females were produced in 1993 [Valenzuela et al., 1997], and hatchlings gathered at random from nesting beaches in 1991 were all females [Lance et al., 1992].

Females will particularly benefit from years of extreme bonanza, which plasticity studies show they are capable of, as higher protein and high abundance diets accelerate growth rates dramatically in *P. expansa* [Ceballos et al., 2014]. Indeed, in captivity, female and male *P. expansa* grew from an average of 5.3 cm of straight carapace length at birth to >15 cm in the first year and to >20 cm in the second year [Ceballos et al., 2014], nearing 50% of the 52 cm of straight carapace length of the smallest nesting female observed in the wild [Hildebrand et al., 1997]. Such growth rate (100 mm in the first year and 150 mm over 2 years) is much greater than the estimate from the same population of 15 mm/year for juvenile females [Ojasti, 1971] and the 34–43 mm/year recorded for head-started juveniles released into the wild [Hernandez and Espin, 2006] (Fig. 2c). Even more telling, centroid size (cs, a measure of size akin to general area obtained from geometric morphometric 2D data, which is more relevant to fecundity components of female fitness than straight-line carapace length), shows that *P. expansa* grew from 96 cs at birth, to >314 cs at 1 year and to >410

cs at 2 years of age under optimal resources [Ceballos et al., 2014]. Such remarkably enhanced growth rates could allow females to reach maturity much earlier at similar or larger body sizes, thus accruing higher fitness via fecundity gains from earlier reproduction and from larger clutches, compared to females growing under less optimal resources. This scenario is consistent with life history theory [Bernardo, 1993] as well as turtle life history, where the lifetime fecundity benefits of higher juvenile growth rates that result in earlier maturation of female turtles at larger size could not be erased by growth after first reproduction [Congdon et al., 2018]. These observations in turtles also agree with recent empirical evidence that clutch size at first reproduction is a reliable indicator of lifetime reproductive success in animals [Powers et al., 2020].

The frequency of resource-bonanza years with feminizing temperatures proposed here will be affected by the periodicity of climatic events such as El Niño (average of 3–5 years, range of 2 to 7–8 years) [Grelaud et al., 2009] and other supra-annual climate cycles that affect conditions in the distributional range of *P. expansa* [Marengo and Espinoza, 2016]. These cycles do not need to be perfectly predictable, because TSD confers the adaptive plasticity necessary for females to take advantage of the differential fitness afforded by optimal conditions when they are available [Charnov and Bull, 1977; Valenzuela, 2018]. The model does not require complete feminization during these bonanza years for TSD to be adaptive, but the stronger the female bias, the greater the fitness advantage accrued by reproductive females. It should be noted that anthropogenic disturbances from contemporary climate change, habitat disturbance, and overexploitation could alter the climatic cycles, disrupt migration patterns, and decrease genetic variation needed for adaptive evolution on which the proposed scenario relies, potentially adding to the factors endangering the survival of these populations.

Conclusion and Future Directions to Test This Novel Hypothesis for *Podocnemis expansa* (and Other Long-Lived Taxa)

In this commentary, it is speculated that the temporal patches hypothesis, which successfully explains the adaptiveness of TSD in short-lived fish and lizards, might also operate in longer-lived TSD turtles but at an inter-annual and supra-annual scale. Indeed, some existing ecological information is consistent with this notion, but much

data are still needed to properly corroborate or refute this hypothesis. Clearly, lifetime reproductive success for females would represent the “smoking gun” in terms of critical observational data. However, in the absence of lifetime reproductive success for long-lived turtles such as *P. expansa*, other indirect data could be used to test the novel hypothesis proposed here. For instance, while we are gaining valuable insights into the migratory behavior of *P. expansa* newborns and adults into the flooded forest after hatching as described earlier, it is imperative to collect: (i) climatic data at nesting areas and flooded plains simultaneously to test whether incubation conditions and temperature/precipitation at feeding areas are correlated; (ii) data on plant phenology and other resource abundance at the feeding areas used by hatchlings to test whether resource availability varies at annual and supra-annual scales in ways that may affect hatchling fitness; (iii) dietary information from individuals in their first year or life in consecutive cohorts that captures climatic variability at annual and supra-annual scales to determine whether feeding habits of newborns vary across years, and whether diet correlates with climatic conditions during nesting.

While dietary information remains scarcer for *P. expansa* than *P. unifilis*, indirect approaches such as stable isotope analysis, which has been applied successfully to these taxa in the past [Lara et al., 2012], could be used to illuminate broad dietary patterns. Such approach would be particularly useful if calibrated experimentally with captive specimens fed a variety of diets [Ceballos et al., 2014] so as to identify annual or supra-annual trends in feeding habits.

This could also be complemented with skeletochronology analysis of digits collected non-lethally to assess individual age and growth rates in early life, also calibrated using the same captive specimens from the stable isotope experiments described above. It would be expected that the largest animals in the wild would show evidence of enhanced growth during the first year post-hatching (as determined by skeletochronology) compared with smaller individuals of the same age, particularly among adults, and especially among adult females. Thus, these data will enable testing the prediction that there are fluctuations in growth rates that correspond with boom-bust years, with the largest adult females showing evidence of enhanced growth rates in the first year of life.

Additionally, experiments are needed to assess heritability *sensu stricto* of the thermal responses underlying TSD, a necessary component for adaptive evolution, but one which currently remains undocumented at levels that

allow selection to operate meaningfully. Clutch effects, which estimate heritability *sensu lato*, have been reported for sex ratios at the pivotal temperature (which produces 1:1 sex ratios under TSD). But heritability values calculated from clutch effects are inflated by non-genetic components such as maternal yolk allocation of hormones [Bowden et al., 2000; reviewed in Valenzuela et al., 2014] and underestimated by the presence of multiple paternity which exists in *P. expansa* [Valenzuela, 2000; Pearse et al., 2006b; Fantin et al., 2017a]. Clutch effects on gene expression of regulators of sexual development were detected in other turtles [Valenzuela et al., 2013], and a similar analysis can be conducted easily in *P. expansa* and other turtles using an experimental design specific to detect heritability *sensu stricto*.

Combined, these data will illuminate whether temperature during embryonic development is a good predictor of resource quality and quantity available after hatching, and whether such variation affects offspring fitness by fueling differential growth rates in nature in ways that benefit female growth during female-producing boom years, resulting in larger adult females that would accrue fecundity gains, thus rendering TSD adaptive in this long-lived taxon. Importantly, whether a similar scenario as that put forth here for *P. expansa* might be at play for other TSD congeners, Pelomedusoid relatives, or more distant long-lived reptiles, sympatric or allopatric, remain open questions worthy of empirical examination to test the generality of the proposed hypothesis. For instance, this hypothesis may apply to sympatric Podocnemididae relatives whose female nests multiply in each reproductive season, since not all clutches laid by an individual female within a year need to benefit from optimal conditions for TSD to be adaptive (indeed, environmental conditions during many years may not be optimal), or because the warm

and dry conditions that would give rise to the optimal resources proposed by the model may last through the entire nesting season and affect all clutches laid by a given female. The same is true for sympatric species whose sex ratios in typical years are influenced by variation in the nest conditions at a micro- and macro-geographic scale, because the optimal conditions proposed by the model are pseudo-periodic climatic anomalies whose magnitude would override micro-geographic variation in nest locations to a great degree (including differences in soil type, elevation, etc.), resulting in feminized sex ratios (though complete feminization may not be needed for TSD to be adaptive under the model). Thus, we are at an exciting crossroads when new technical advances and collaborative interdisciplinary efforts should facilitate our understanding of TSD evolution not only in *P. expansa* but in other long-lived TSD reptiles whose sexual development has been placed by nature in the hands of environmental whims.

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Conflict of Interest Statement

The author has no conflicts of interest to declare.

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