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The selective basis for increased egg retention: early incubation temperature determines hatchling phenotype in wall lizards (*Podarcis muralis*)

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The thermal environment during development influences many aspects of the phenotype of hatchling reptiles. We hypothesized that temperature should differentially affect early incubation stages, in which differentiation dominates over growth, and late incubation stages, characterized by high growth rates. To test this idea, we incubated eggs of wall lizard (*Podarcis muralis*) under three regimes with the same mean temperature (29 °C), one constant and two variable with opposite sequences: first cold (25 °C) and then hot (32 °C), and vice versa. Hatchlings incubated at high temperature during the initial period had shorter hindlimbs and tails than those incubated under the other two temperature regimes and shorter heads than those incubated initially at low temperature. Thus, temperature experienced by embryos during the early external incubation period produced similar phenotypic responses compared to those reported in previous studies for the same constant temperature applied over the whole incubation period. Because female wall lizards select lower body temperatures during pregnancy, an increase of intrauterine retention would extend the time of exposure of developing embryos to suitable temperatures. Diminution of body temperature during pregnancy is contrary to the expected pattern under the hypothesis that egg retention has evolved to accelerate development, as proposed by the cold-climate model for evolution of viviparity in squamates, and the results of the present study support the alternative hypothesis of developmental optimization as a special case of the broader maternal manipulation view. © 2007 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2007, **92**, 441–447.

ADDITIONAL KEYWORDS: developmental optimization – developmental plasticity – evolution of viviparity – maternal manipulation – thermal variation.

INTRODUCTION

Developmental plasticity in morphological, physiological and life history traits is widespread in ectothermic animals and can explain a substantial amount of within- and among-population phenotypic variation (Shine, Elphick & Harlow, 1997; Deeming, 2004). A sizeable literature developed in the last decade indicates that temperature is likely to be the main single environmental factor influencing early development in ectothermic vertebrates (Johnston, Vieira & Hill, 1996; Deeming, 2004). The thermal environment during the incubation period varies both among and within populations, among years or among successive clutches (Packard, Tracy & Roth, 1977; Marco & Pérez-Mellado, 1998; Qualls & Shine, 1998; Shine, 2004). Even for a single clutch, the incubation temperature can experience dramatic diel variations, as well as directional or stochastic variation of daily mean temperatures along the time from oviposition to hatching (Castilla & Swallow, 1996; Shine & Elphick, 2001). Therefore, the effects of temperature on hatchling phenotype that have been extensively demonstrated in laboratory experiments are also likely to be operative in nature.

By contrast to birds, reptile embryos are viable under a wide range of incubation temperatures (Birchard, 2004; Booth, 2004) but the thermal conditions experienced during development can affect

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*Corresponding author. E-mail: fbrana@uniovi.es hatchling morphology, physiology and behaviour (Burger, 1989; Elphick & Shine, 1998). Important thermal effects on embryogenesis have been demonstrated in a number of squamate reptiles and specifically in the wall lizard, *Podarcis muralis* (Van Damme *et al*., 1992; Ji & Braña, 1999; Braña & Ji, 2000). Rather than a pattern of continuous variation, most of these studies have demonstrated the existence of thresholds over which the effects of temperature were important and affected several aspects of the hatchling phenotype. In wall lizards, there is an apparent threshold situated between 29 °C and 32 °C, so that hatchlings from eggs incubated at or above the later temperature exhibit morphological differences and perform poorly in functions such as locomotion and growth (Van Damme *et al*., 1992; Braña & Ji, 2000).

Growth and development are processes that to some extent are dissociated during embryogenesis. Early development is characterized by tissue differentiation and organogenesis with rapid changes in external embryonic morphology, whereas late development is characterized by growth in size and physiological changes (Deeming & Ferguson, 1991). Embryonic differentiation and formation of extra-embryonic membranes are well advanced before any substantial growth of the embryo has occurred (Andrews, 2004). Female wall lizard laid eggs containing embryos at stages 26–28 in Dufaure & Hubert's (1961) developmental series (Braña, Bea & Arrayago, 1991), which represents approximately two-thirds of morphological development (hatching occurs at D&H stage 40), but less than 1% of pre-hatching growth in mass. Therefore, most differentiation occurs early in development, whereas growth is much more intense in later stages. Consequently, we hypothesized that thermal variation should affect hatchling phenotype in a different way depending upon the phase of development in which it was applied; in particular, hatchling morphology should be more affected by the thermal environment experienced in the early stages of development after egg-laying, whereas hatchling size should be more affected by thermal variation in comparatively later stages. To test this hypothesis, we experimentally incubated lizard eggs under variable thermal regimes with similar mean temperature and examined size, morphology, and locomotor performance of hatchlings that had experienced different temperatures during the supposedly significant phases.

MATERIAL AND METHODS

The wall lizard (*P. muralis*) is a small [female snout–vent length (SVL) in our sample was in the range 51.50–65.08 mm] oviparous lacertid lizard that is widely distributed throughout south Europe. Female wall lizards with advanced oviductal eggs ($N = 26$) were caught by the use of a noose in Noreña (Asturias, Northern Spain) during the reproductive season in 2002, housed in terraria, and allowed to oviposit in moist potting ground. Terraria were checked for clutches several times daily, and freshly laid eggs were weighed to the nearest 0.0001 g soon after oviposition and half-buried in moistened vermiculite (mass ratio of distilled water to dry vermiculite 2 : 1; approximately −12 kPa water potential). Eggs were placed individually in 50-mL containers and assigned to one of three experimental incubation treatments with similar mean temperature: one constant (29 °C) and two sequentially variable regimes (cold–hot: 25–32–29 °C; hot–cold: 32–25–29 °C). Variation around the selected temperature was controlled in each incubator based on recordings from one probe placed in one container with moistened vermiculite similar to those containing eggs. In the variable regimes, the eggs were first set at the initial temperature (25 °C or 32 °C) and then at the other temperature, each for 13 days, and finally at 29 °C during the last period until hatching (6–9 days in both variable regimes); this sequence was arranged to equalize the time spent at the two 'extreme' temperatures and to obtain the same mean temperature in the three treatments because the total duration of the incubation period is slightly variable. Previous data for eggs of the same population of *P. muralis* indicated that the average incubation time at 29 °C and a 2 : 1 water to vermiculite ratio was 33.8 ± 0.1 days (range: 32.5–35.5 days; Ji & Braña, 1999; present study: 32.9 ± 0.1 days; range: 31.7–35.5 days). In the present study, mean ± SE temperatures were 28.9 ± 0.03 °C (range: 28.3–29.4 °C) for the constant regime incubator, 25.2 ± 0.08 °C (range: 24.6–26.0 °C) for the cold incubator, and 32.0 ± 0.03 °C (range: 31.6–33.0 °C) for the hot incubator; overall mean temperature was 28.6 °C for both variable regimes.

For clutches of four or more viable eggs, one randomly selected egg was dissected to determine the stage of embryonic development at oviposition, according to the stages described by Dufaure & Hubert (1961). The remaining eggs were equally distributed among incubation treatments. Splitting eggs between treatments ensured that possible variation among families was not confounded with thermal effects. Within a few hours of hatching, lizards were weighed to the nearest 0.001 g and sexed by applying gentle pressure on the tail base, which causes the eversion of the hemipenes in males (Harlow, 1996). Morphological data (see below) were obtained from ventral images of every hatchling measured with image analysis software. Within 1 day of hatching, lizards were tested for running performance by chasing them along a 1.2-m racetrack with one transparent side that allowed filming at constant 25 frames s^{-1}. Videotapes were later examined for sprint speed for the fastest 20-cm interval and for maximal distance travelled without stopping. Two trials were conducted for each lizard at a 30-min interval and the best performance was selected for analysis. Prior to each trial, lizards were maintained for 30 min in an incubator to set their body temperature at 35 °C, close to the optimum for maximum speed; for methodological details, see Braña & Ji (2000).

In addition to initial mass, we separately analysed two aspects of hatchling phenotypes: morphology (measurements of SVL, head length and width, tail length, and forelimb and hindlimb length) and locomotor performance (burst speed and maximal distance steadily run). Hatchling mass was analysed using analysis of covariance (ANCOVA), with egg mass as covariate, to test the effects of temperature treatment. Because of the presumable intercorrelations among the variables considered to describe hatchling morphology, we utilized a multivariate analysis of variance (MANOVA) to test the effects of the same factors and their interaction on hatchling morphology. Prior to this analysis, the assumption of homogeneity of variances was tested at the univariate (Cochran's test) and multivariate (Box's *M*-test) levels. Tail length was analysed with a separate ANCOVA (with SVL as covariate) because tip mutilation of several individuals should have diminished the sample size for general morphological analysis. Wall lizards exhibit strong sexual dimorphism and, accordingly, sex was considered as a second factor in all analyses in addition to incubation treatment.

RESULTS

Except for one dead embryo at stage 24 in Dufaure & Hubert's (1961) developmental series, freshly laid eggs from 13 different clutches contained embryos at stages 25 ($N = 2$), 26 ($N = 2$), 27 ($N = 8$), and 28 ($N = 1$). The yolk-free mass of the initial embryos averaged 0.717 ± 0.163 mg, and represented approximately 0.25% of the average mass of hatchlings incubated from the same clutches. The incubation time differed among temperature treatments (ANCOVA, with egg mass as covariate; $F_{2,52} = 51.618$, $P < 0.0001$; shorter for constant temperature regime, Scheffé test; Fig. 1) and also between sexes ($F_{1,52} = 6.494$, $P = 0.014$; longer for males, Scheffé test). Hatchling mass did not differ among incubation treatments (ANCOVA, with egg mass as covariate; $F_{2,52} = 0.358$; $P = 0.701$) or between sexes ($F_{1,52} = 0.194$, $P = 0.661$).

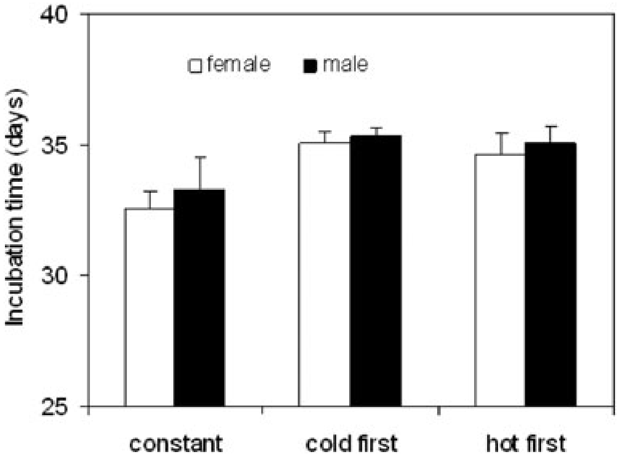

Figure 1. Variation of incubation time (mean ± standard deviation) for eggs incubated under three thermal regimes having the same mean temperature and according to the sex of the hatchlings.

Hatchling morphology was affected both by incubation treatment (Wilks' lambda = 0.696; d.f. = 10, 98; $P = 0.048$) and by sex (Wilks' lambda = 0.636; d.f. = 5, 49; $P = 0.0003$). Hatchlings incubated at high temperature (32 °C) during the initial period had shorter heads than those incubated initially at low temperature (25 °C), and shorter hindlimbs than those incubated under the other two temperature regimes (Scheffé tests a posteriori; $P < 0.05$; Fig. 2). Males had larger heads, longer hindlimbs, and fewer ventral scale rows than females. Tails of hatchlings maintained at 32 °C in the earlier phase of incubation were shorter than those of hatchlings incubated at other temperatures in that phase (ANCOVA, with SVL as covariate; $F_{2,43} = 5.616$, $P = 0.006$; Scheffé test a posteriori; Fig. 2), and were longer overall in males than in females ($F_{1,43} = 5.906$, $P = 0.019$); there was also a marginally nonsignificant interaction effect of sex and thermal treatment affecting tail length ($F_{2,43} = 3.061$; $P = 0.057$).

Locomotor performance was not affected by incubation treatment in our experiment. Neither incubation treatment, nor sex had significant effects on burst speed (ANOVA; $F_{2,51} = 0.783$, $P = 0.463$, for temperature treatment; $F_{1,51} = 0.116$, $P = 0.735$, for sex), or on maximal distance of continuous running (ANOVA; $F_{2,51} = 0.641$, $P = 0.531$, for temperature treatment; $F_{1,51} = 0.0.075$, $P = 0.785$, for sex) of hatchling wall lizards.

DISCUSSION

Despite the fact that early events of the embryogenesis are highly canalized to ensure coordinated development and production of viable phenotypes

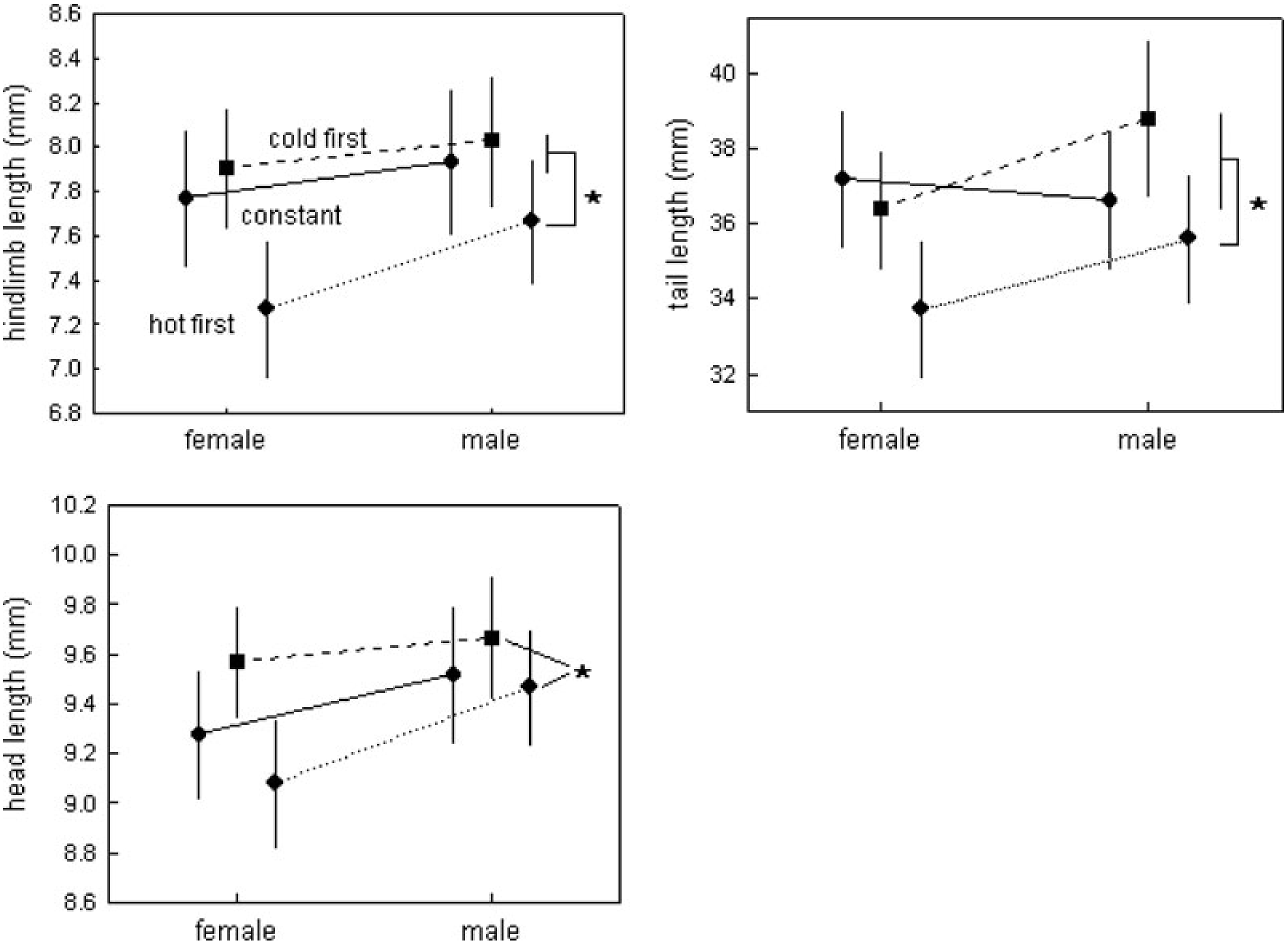

Figure 2. Effects of temperature regimes and sex on several aspects of hatchling morphology (mean and 95% confidence intervals were represented for each measurement). There are significant sex (male > female) and temperature regime effects for all traits. Particular differences between thermal regimes, as revealed by Scheffé's tests a posteriori, are denoted by asterisks.

over wide temperature ranges (Johnston *et al*., 1996; Mabee, Olmstead & Cubbage, 2000), the present study demonstrated that temperature variation during the external incubation period affected some morphological features (head, limb and tail proportions) of hatchling wall lizards. These morphological modifications were highly consistent with previous results on the postoviposition development of wall lizards at three constant temperatures, which resulted in shorter heads, tails, and hindlimbs of hatchlings incubated at 32 °C compared to those incubated at 26 °C and 29 °C (Braña & Ji, 2000). These three constant temperatures almost exactly match the initial treatment temperatures in the present study and therefore, taken together, these results support the hypothesis that temperature during the initial phase of incubation has the main influence in determining hatchling morphology. That is, embryos experiencing high temperatures (32 °C in the present study) only during the early phase of external incubation, exhibited the same kind of morphological modifications as those incubated at high temperatures during the entire incubation period. Our results agree with the developmental tenet that variations in environmental conditions are likely to produce the strongest effects when they occur early in ontogeny (Lindström, 1999; Mabee *et al*., 2000). However, because development is faster at 32 °C than at 25 °C and the time to switching temperature was set constant (13 days), embryos in the hot-first treatment would have been exposed to high temperatures over a wider range of developmental stages than embryos in the cold-first treatment were exposed to cold temperatures. Consequently, because we do not know how far development will have progressed in either treatment during the first time interval, we can only state with certainty that the reported effects have been determined during the early incubation period (the first 13 days), but we are unable to assess which precise developmental stages are critical to produce plastic responses.

Most oviparous squamates retain eggs for an important fraction of embryogenesis (Shine, 1983, 1985; Blackburn, 1995) and we have also documented that wall lizards and some closely-related species retain eggs until quite advanced developmental stages (Braña *et al*., 1991). Egg retention was generally interpreted as a trait evolved either to provide faster development or to enhance incubation conditions within a buffered environment during a sensitive phase of development (Shine, 1985; Guillette, 1993). Thus, the fact that temperature experienced during the early incubation period had important effects on hatchling phenotype can help to explain the evolution of egg retention because maternal thermoregulation should provide optimal thermal conditions for development. Because high incubation temperatures produce detrimental effects on hatchling morphology and performance in wall lizards (Van Damme *et al*., 1992; Braña & Ji, 2000), even if applied only during the earliest phase of external incubation (present study), gravid females should select lower temperatures to provide optimal conditions to developing embryos. Gravid wall lizards had lower field body temperatures than similarly-sized males and nonpregnant females (Braña, 1993), and such a difference is likely to be a consequence of the decrease of thermoregulatory set points experienced during pregnancy (Tosini & Avery, 1996). This decrease seems to be contrary to the expected pattern under the cold-climate hypothesis for the evolution of viviparity, which posits that egg retention has evolved to accelerate development in cold environments in which low nest temperatures would slow or even preclude developmental progress (Packard *et al*., 1977; Shine, 1995). However, according to the function shown in Figure 3, there is only a slight decrease of incubation time above the average temperature selected by pregnant females (32.62 °C versus 34.04 °C for nonpregnant females; Braña, 1993) and this temperature is likely to be much higher than the average temperatures experienced in natural nests (Braña & Ji, 2000; for *Podarcis atrata*, see also Castilla & Swallow, 1996). Therefore, avoidance of even brief exposure of developing embryos to critically high temperatures could have been the selective basis for the evolution of lower thermoregulatory set points by pregnant females of *P. muralis*, as previously suggested for some viviparous lizards (for *Sceloporus jarrovi*, see Beuchat, 1986; Mathies & Andrews, 1997). Thus, assuming that high temperatures would also produce detrimental effects on preoviposition embryonic stages, now protected by intrauterine development, we have a plausible evolutionary scenario for the increase of egg retention until the developmental stage at oviposition exhibited by wall lizards because any advance in retention would extend the time of exposition to suitable temperatures that can yet produce developmental acceleration. This is consistent with the 'maternal manipulation' view on the evolution of viviparity (Shine, 1995; Webb, Shine & Christian, 2006) because females modify thermal conditions during retention to optimize hatchling phenotypes.

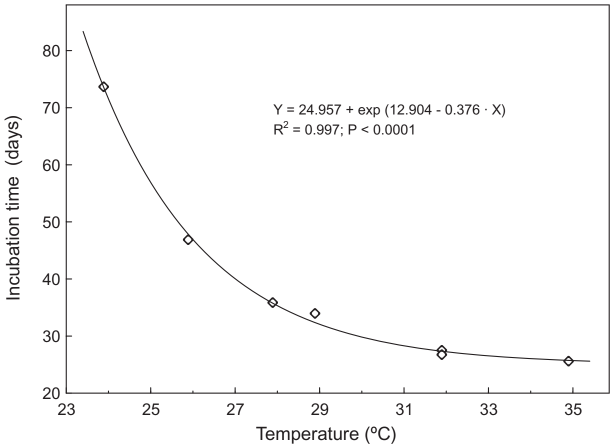

Figure 3. Relationship between incubation temperature (constant) and duration of the incubation of wall lizard eggs, according to data from Van Damme *et al*. (1992) and Ji & Braña (1999).

The incubation time in *P. muralis* was longer under variable temperature regimes, irrespective of the temperature sequence, compared to under the constant regime. The average temperature in the constant treatment was slightly higher than in both variable regimes but the differences were so small (0.2–0.3 °C) that we consider them unlikely to have caused important differences in developmental rates, considering the low decrease in incubation time at increasing temperatures above 29 °C (Fig. 3). Because the relationship between temperature and development time is not linear in reptiles (Gutzke & Packard, 1987; Deeming & Ferguson, 1991), variable thermal regimes can produce either earlier (Shine, 2004) or later (present study) hatching with respect to constant regimes, with the outcome depending upon the shape of the response function and the position of the selected temperatures in the viable range interval. The incubation time in wall lizards steeply shortens as the temperature increases from 24 °C to 29 °C but, from 29 °C to the upper critical threshold for viability (near 35 °C; Van Damme *et al.*, 1992), there is only a smooth decrease (Fig. 3). Within our experimental range, the shape of the function relating incubation time to temperature results in longer incubation times in variable regimes because these include one period at a comparatively low temperature (25 °C) during which incubation progresses much more slowly.

The prediction that thermal variation in the late stages of incubation should affect hatchling size was not supported by the results of our study. In many reptiles and other ectothermic animals, there is a negative relationship between rearing temperature and size at a given stage of development (Atkinson, 1994). Previous studies conducted on the same population analysed here consistently reported a definite negative effect of incubation temperature on hatchling size of wall lizards when applied over the whole incubation period (range 24–35 °C; Van Damme *et al.*, 1992; Ji & Braña, 1999; Braña & Ji, 2000). By contrast, the present study shows that variable thermal regimes with similar average temperatures do not produce significant differences in either hatchling SVL or body mass. One possible explanation for this discrepancy could be that, in our experimental design, the time spent at different temperatures during the more intense growth phase was too short to produce important differences because the very final phase of development was set at a constant temperature to equalize mean temperatures among treatments.

The time from oviposition to hatching was significantly longer for males across incubation treatments. This is likely to be a genuine sex-specific response to incubation treatment because there were no differences in egg size or in hatchling size between sexes that could explain this difference, and we do not have any reason to propose that the developmental stage at oviposition is different for male and female embryos. A number of incubation-induced between-sex differences have been documented in squamate reptiles (Burger & Zappalorti, 1988; Elphick & Shine, 1999; Flatt *et al.*, 2001). Although the interaction between sex and incubation regime has been suggested as a plausible basis for the evolution of temperature-dependent sex determination (Shine, Elphick & Harlow, 1995; Elphick & Shine, 1999), it remains unclear in most cases whether between-sex differences have some specific evolutionary significance or are mere by-products of constitutive physiological differences. Between-sex differences in the early developmental rate occur in many taxa and are likely to be influenced by the hormonal environment during development independently of thermal variation, as suggested by documentation of this phenomenon in homoeothermic animals (birds, Cook & Monaghan, 2004; humans, McLachlan & Storey, 2003).

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