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Incubation Temperature Influences Sex-Steroid Levels in Juvenile Red-Eared Slider Turtles, *Trachemys scripta,* a Species with Temperature-Dependent Sex Determination[1]

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ABSTRACT

Incubation temperature determines gonadal sex in the red-eared slider turtle, *Trachemys scripta.* However, little is known about the long-term effects of incubation temperature on traits other than gonadal sex in this species. To investigate the hypothesis that incubation temperature (independent of gonadal sex) influences sex steroid levels after hatching, we incubated eggs of the red-eared slider turtle at three temperatures (26, 28.6, and 31°C). We then measured plasma levels of dihydrotestosterone, estradiol, progesterone, and testosterone in 6-wk-old males from 26°C and 28.6°C eggs, and in 6-wk-old females from 28.6°C and 31°C eggs. We found that dihydrotestosterone levels were not influenced by incubation temperature or gonadal sex. However, progesterone levels were significantly higher in males from 26°C eggs than in males from 28.6°C eggs. In contrast, testosterone levels did not differ between males from 26°C versus males from 28.6°C eggs, but they were significantly higher in females from 28.6°C than in females from 31°C eggs. Progesterone and testosterone levels did not differ between males and females from 28.6°C eggs. Temperature also influenced estradiol levels in both sexes, but the effects were enigmatic. We conclude that incubation temperature has lasting effects on sex steroid levels even after hatching.

INTRODUCTION

Temperature during embryonic development determines gonadal sex in some lizards, many turtles, and all crocodilians studied to date (reviewed in [1–3]). Although advances have been made toward understanding the developmental mechanism of temperature-dependent sex determination (TSD; reviewed in [4–6]), most in-depth research on this topic has been conducted on a small number of species. Likewise, incubation temperature effects on traits other than gonadal sex have been examined in just a few TSD species. Unfortunately, the vast majority of studies on any given TSD species have focused on either 1) the mechanism of sex determination during embryogenesis or 2) the effects of incubation temperature on other traits after embryonic development is completed. As a result, it is difficult to integrate these studies of different species in order to obtain a coherent picture of how incubation temperature simultaneously determines gonadal sex and influences post-hatching traits like body size, energy reserves, metabolism and growth, behavioral thermoregulation, and adult sociosexual behaviors [7–14].

For example, there is a relatively large data base on TSD in the red-eared slider turtle, *Trachemys scripta*, but virtually no information concerning incubation temperature effects after hatching (but see [15]). In this species, low temperatures (i.e., < 28.4°C) produce all males, intermediate temperatures (i.e., 28.4–29.4°C) produce mixed sex ratios, while high temperatures (i.e., > 29.4°C) produce all females [16]. Various experiments suggest that temperature determines gonadal sex by influencing sex steroid metabolism during embryonic development. First, exogenous natural and man-made estrogens induce ovarian differentiation at normally male-producing temperatures and only have their effect during the temperature-sensitive period of gonadal development [17–19]. Second, these estrogens act synergistically with increasing incubation temperatures to produce more females than would be expected if estrogens and temperature act via separate developmental pathways [20, 21]. Finally, aromatase inhibitors can block ovarian differentiation at temperatures that normally produce females [22, 23]. Consequently, it is thought that temperature directly (or indirectly) regulates the expression of aromatase enzyme, which in turn affects estrogen production and ovarian differentiation [4].

Because sex determination is a threshold trait (i.e., sex ratio varies, but no hermaphrodites are produced), individuals with estrogen levels below a certain threshold develop as males and individuals with estrogen levels above the threshold develop as females. This model predicts that individuals of the same sex from different incubation temperatures are exposed to different hormonal milieus during embryonic development. Consequently, temperature-induced variation in hormone production could affect numerous physiological and behavioral traits later in life. To investigate the hypothesis that embryonic incubation temperature (independent of gonadal sex) influences sex steroid levels after hatching, we incubated eggs of the red-eared slider turtle at three different temperatures (26, 28.6, and 31°C). Whereas 26°C produces only males and 31°C produces only females, a temperature of 28.6°C produces ~4:1 ratio of males to females. We then raised turtles to 6 wk of age and determined basal levels of sex steroids and steroid levels in response to FSH. In another turtle species, FSH treatment stimulates male-specific production of testosterone [24], and we were anticipating a similar sex-specific (and possibly temperature-influenced) steroid response in the red-eared slider turtle. By choosing these incubation temperatures, we were able to compare temperature effects within each sex and test for sex differences in turtles from the same temperature.

MATERIALS AND METHODS

Egg Incubation and Turtle Husbandry

Animals were treated according to a research protocol approved by the University of Texas institutional animal care and use committee. Turtle eggs were purchased from a commercial supplier (Robert Kliebert, Hammond, LA) and transported to the lab for candling, used to establish

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embryo viability. The eggs used in this experiment were collected and brought to the lab within 24 h of oviposition. To avoid clutch effects, eggs were randomized among treatment groups before incubation. Viable eggs were placed in groups of 35 into trays containing 1:1 vermiculite:water and were incubated at 26°C, 28.6°C, or 31°C in computer-controlled incubators. Temperature was also monitored by HOBO temperature loggers (Onset Computer Corporation, Pocassett, MA), and by daily readings of in-incubator thermometers. After hatching, turtles were maintained at room temperature (~25°C) in plastic shoeboxes filled with tap water approximately 3 cm deep and were fed lettuce and commercial turtle food on a daily basis.

Gonadotropin Challenge and Blood Sampling

A blood sample was drawn from 112 turtles (37 males from 26°C, 43 males and 16 females from 28.6°C, and 16 females from 31°C) at 6 wk of age: red-eared slider turtles are sexually immature at this age. Turtles from each temperature were randomly assigned to one of three groups: 1) a nontreated control, 2) a vehicle-treated control (0.5 ml of nanopure water), and 3) an FSH group (50 μg ovine FSH dissolved in 0.5 ml of nanopure water; 1 U/mg; Sigma, St. Louis, MO). Nontreated turtles were anesthetized on ice and then pithed while vehicle-treated and FSH-treated turtles were first injected i.p. with either water vehicle (0.5 ml H_2O) or FSH, maintained at room temperature for 3 h, anesthetized on ice, and then pithed. Blood was then collected directly from the heart with a heparinized 1-cc syringe, transferred to 0.5-ml microfuge tubes, and centrifuged at 3000 rpm for 10 min at 4°C. Plasma was drawn off and frozen at −80°C. When killed, each turtle was diagnosed for gonadal sex by examination of the structure of the gonads with a dissecting microscope. This procedure is very accurate and has been validated a number of times using histological methods [17–23].

RIA

Plasma was pooled from 2–3 individuals to make 600-μl samples for RIA because a pilot study indicated that hormone levels were very low. Samples were then assayed for dihydrotestosterone (DHT), estradiol-17β (E_2), progesterone (P_4), and testosterone (T) using the methods of Tousignant and Crews [10]. The antibodies used for RIA were DT3–351 for DHT, E26–47 for E_2, P11–192 for P_4, and T3–125 for T (Endocrine Sciences, Calabasas Hills, CA). Briefly, plasma samples were mixed with approximately 500 cpm each of tritiated DHT, E_2, and T. After a 1-h equilibration time at 25°C, steroids were extracted with 3 ml of ether. Extracts were then dried under a stream of nitrogen in a dry bath at 37°C. Steroids were resuspended in 500 μl of isooctane saturated with ethylene glycol and separated on celite columns. The eluted steroids were dried under a stream of nitrogen in a dry bath at 37°C and resuspended overnight at 4°C in 330 μl of PBS with gelatin.

A single 100-μl aliquot of each resuspended sample was used to determine individual recoveries. Duplicate aliquots of each resuspended sample (100 μl) were used for RIA with approximately 2000 cpm (50 μl) of the appropriate labeled steroid and the corresponding antibody (100 μl). Assay sensitivity was 13 pg DHT/ml plasma, 13 pg E_2/ml plasma, 106 pg P_4/ml plasma, and 10 pg T/ml plasma. Intraassay coefficients of variation for a pooled sample of leopard gecko plasma were 16% for DHT, 18% for E_2, 12% for P_4, and 17% for T. Interassay coefficients of variation for the same sample were 18% for DHT, 17% for E_2, 20% for P_4, and 13% for T. We also ran quality control standards of known concentration in the low, medium, and high ranges of the standard curve for each steroid, with the following results, respectively. For DHT, intraassay coefficients of variation were 12%, 6%, and 6%, and interassay coefficients of variation were 18%, 9%, and 11%. For E_2, intraassay coefficients of variation were 11%, 4%, and 6%, and interassay coefficients of variation were 10%, 8%, and 9%. For P_4, intraassay coefficients of variation were 17%, 7%, and 8%, and interassay coefficients of variation were 14%, 1%, and 5%. For T, intraassay coefficients of variation were 9%, 4%, and 5%, and interassay coefficients of variation were 14%, 9%, and 10%.

Statistical Analysis

Plasma steroid levels were log-transformed to eliminate heteroscedasticity. Transformed steroid levels were compared using separate two-way ANOVAs for males and females for each steroid. We used two-way analyses with incubation temperature and FSH treatment as main effects because a three-way, fully factorial analysis was not possible (no females are produced at 26°C and no males are produced at 31°C). Since untreated and vehicle-treated groups did not differ within any of the experimental groups, they were combined into a single control group for the following analyses. Thus, independent variables in the two-way ANOVAs were incubation temperature, juvenile FSH administration 3 h before turtles were killed at 6 wk of age (combined controls vs. FSH treated), and their interaction. The following hierarchical set of comparisons among treatment groups was planned a priori. We first determined whether the interaction between incubation temperature and juvenile FSH administration was significant. Since this interaction was not significant for any sex steroid for males or for females, we examined the main effects of incubation temperature and FSH treatment. Because there were only two temperature and two gonadotropin groups, there was no need to make any correction for multiple comparisons. An analogous procedure was used to analyze sex effects, FSH effects, and sex-by-FSH effects for males and females from 28.6°C eggs. Recoveries for a number of P_4 samples were low, which prevented their inclusion in our statistical analysis. An outlier for T in the 26°C male group was also thrown out. All statistics were done using Version 3.1 of JMP [25] for Apple Macintosh (Cupertino, CA).

RESULTS

Incubation Temperature Effects in Males

Levels of DHT in 6-wk-old males were not influenced by incubation temperature, FSH challenge, or the interaction between incubation temperature and FSH challenge ($P > 0.05$ in all cases; 26°C males = 40 ± 6 pg/ml plasma; 28.6°C males = 33 ± 5 pg/ml plasma).

P_4 levels in 6-wk-old males were influenced by incubation temperature during embryonic development: males from 26°C eggs had significantly higher P_4 levels than males from 28.6°C (ANOVA; F ratio = 5.45; $df = 1$; $P = 0.04$; Fig. 1). However, there was no significant effect of FSH challenge nor a significant interaction between incubation temperature and FSH challenge ($P > 0.05$).

Like DHT, T levels in 6-wk-old males were not influenced by incubation temperature (Fig. 2), FSH challenge, or the interaction between incubation temperature and FSH challenge ($P > 0.05$ in all cases).

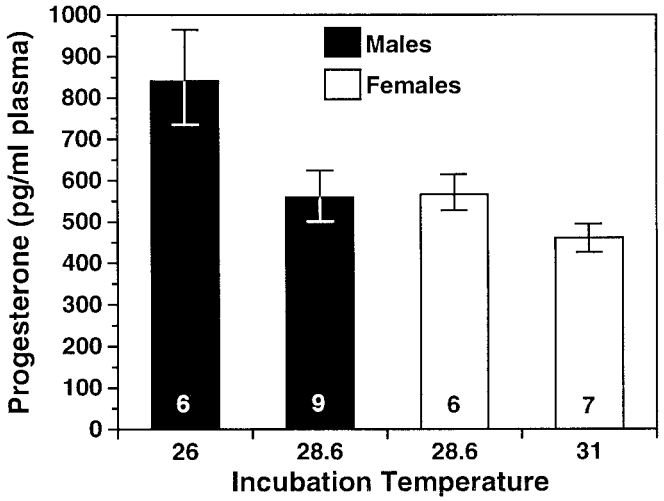

FIG. 1. Plasma P_4 levels of male and female juvenile red-eared slider turtles as a function of incubation temperature. P_4 levels are least-squares means (± 1 SE) from the ANOVA described in the text. These means represent the means for each group while controlling for all other independent variables in the experiment. Sample sizes for each group are indicated at the base of the histogram bars.

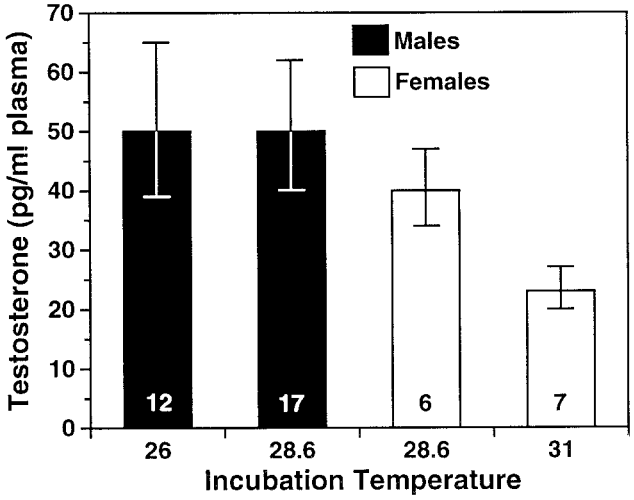

FIG. 2. Plasma T levels of male and female juvenile red-eared slider turtles as a function of incubation temperature. T levels are least-squares means (± 1 SE) as noted in Figure 1. Sample sizes for each group are indicated at the base of the histogram bars.

Six-wk-old male turtles from 26°C eggs had E_2 levels well above the level of assay sensitivity (t-test = 8.088, $P < 0.0001$), while males from 28.6°C had undetectable levels of E_2 (i.e., < 13 pg E_2/ml plasma), indicating a highly significant incubation temperature effect (Fig. 3). There was no effect of FSH challenge on E_2 levels in males from 26°C (t-test = 1.13, $P = 0.28$). Since E_2 levels were undetectable in males from 28.6°C, we were not able to test for FSH effects.

Incubation Temperature Effects in Females

Levels of DHT in 6-wk-old females were not influenced by incubation temperature, FSH challenge, or the interaction between incubation temperature and FSH challenge ($P > 0.05$ in all cases; 28.6°C females = 39 ± 5 pg/ml plasma; 31°C females = 40 ± 4 pg/ml plasma).

Six-wk-old females from 28.6°C eggs tended to have higher P_4 levels than females from 31°C (Fig. 1). However, P_4 levels did not differ significantly between incubation temperatures (ANOVA; F ratio = 3.63; $df = 1$; $P = 0.09$). There was no significant effect of FSH challenge nor a significant interaction between incubation temperature and FSH challenge for P_4 levels ($P > 0.05$).

Incubation temperature influenced the level of T in females: 6-wk-old females from 28.6°C eggs had significantly higher T levels than females from 31°C (ANOVA; F ratio = 5.32; $df = 1$; $P = 0.047$; Fig. 2). There was no significant effect of FSH challenge nor a significant interaction between incubation temperature and FSH challenge for T levels ($P > 0.05$).

Incubation temperature also significantly influenced plasma E_2 levels in females (Fig. 3). Six-wk-old female turtles from 31°C eggs had E_2 levels above the level of assay sensitivity (t-test = 8.69, $P < 0.0001$), while E_2 levels were undetectable in females from 28.6°C (i.e., < 13 pg E_2/ml plasma). FSH challenge did not influence E_2 levels in females from 31°C (t-test = 0.97, $P = 0.38$). Since E_2 levels were undetectable in females from 28.6°C, we were not able to test for FSH effects at this temperature.

Sex Differences

There was no detectable sex difference in the levels of DHT, P_4, T, or E_2 in 6-wk-old turtles from an incubation temperature of 28.6°C ($P > 0.05$ in all cases; Figs. 1, 2, and 3). In addition, there were no FSH effects nor an interaction between gonadal sex and FSH challenge in turtles from this temperature ($P > 0.05$ in all cases).

DISCUSSION

In this study, we found that embryonic incubation temperature had lasting effects on post-embryonic sex steroid levels in both male and female red-eared slider turtles, a species with TSD. Levels of P_4 were inversely related to incubation temperature in both sexes whereas T levels only varied with temperature in females. However, there were no detectable differences in P_4 or T levels between males

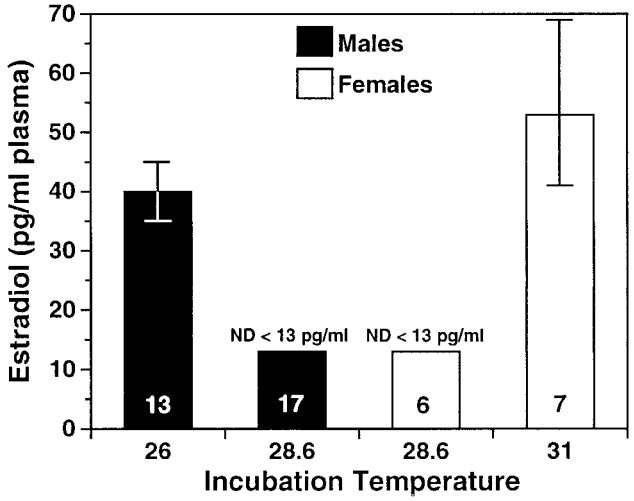

FIG. 3. Plasma E_2 levels of male and female juvenile red-eared slider turtles as a function of incubation temperature. E_2 levels in males and females from 28.6 were below assay sensitivity (i.e., < 13 pg E_2/ml plasma). E_2 levels are least-squares means (± 1 SE) as noted in Figure 1. Sample sizes for each group are indicated at the base of the histogram bars.

and females from the same incubation temperature. The latter result indicates that differences in hormone levels between males from 26°C eggs (i.e., higher P_4 and T) and females from 31°C eggs (i.e., lower P_4 and T) are primarily due to incubation temperature and not gonadal sex. Interestingly, the pattern of incubation temperature effects (i.e., 26 vs. 31°C) on 6-wk-old turtles is the same as previously described for whole-body P_4 and T concentrations during the temperature-sensitive period of embryonic development [26]. Conferring additional veracity to the current data, both the concentrations of hormones and the magnitude of the temperature differences were very close to those observed in the earlier study of embryos. In addition to these in vivo effects, incubation temperature similarly influenced in vitro steroidogenesis in adrenal-kidney-gonad complexes taken from embryos incubated at 26 and 31°C [27, 28]. In our study, incubation temperature also influenced E_2 levels in both sexes. Although it is unclear why E_2 levels were significantly higher in males from 26°C and females from 31°C and undetectable in both sexes from 28.6°C, similar U-shaped responses have been reported for various physiological traits. Despite this enigma, plasma E_2 levels in males from 26°C and females from 31°C at 6 wk of age were generally similar to those reported during the temperature-sensitive period of embryonic development [26]. In contrast to P_4, T, and E_2 levels, we did not detect any incubation temperature or gonadal sex effects on DHT levels. However, we cannot judge the validity of these results, as DHT levels have not been measured previously in embryos or young red-eared slider turtles. Finally, we did not detect any change in sex steroid levels in response to FSH challenge. Considering results from in vitro studies, it is possible that the ambient temperature of the turtles in our experiment (i.e., ~25°C) reduced their responsiveness to gonadotropin treatment. Cool ambient temperatures (i.e., 10 and 20°C), relative to a warm ambient temperature (i.e., 28°C), eliminated or blunted T secretion in response to ovine FSH treatment of testes taken from sexually mature males [29, 30].

However, the lack of response for any steroid in either sex in the juvenile turtles in our experiment could also be due to developmental differences in sensitivity to mammalian gonadotropins. In fact, in vitro studies of testicular steroid secretion reveal that ovine FSH stimulated testosterone production about 10-fold in testes from adults but did not increase testosterone production in adrenal-kidney-gonad complexes from putative male (or female) embryos [28–30]. Although adrenal-kidney-gonad complexes from embryos did respond to FSH treatment with significant increases in P_4 and E_2, the responses were dependent upon developmental stage and incubation temperature and were relatively weak [28]. Unfortunately, we cannot make many other comparisons among developmental stages for the hormones that we measured because there are few published studies on sex steroid levels in sexually mature red-eared sliders. Nevertheless, plasma E_2 levels in our 6-wk-old turtles were generally similar to those reported in ovariectomized adult females [31]. While this suggests that the gonads may be quiescent with respect to E_2 production in immature turtles, we do not know what the normal circulating levels of E_2 are like in intact, cycling females. In another study of female red-eared sliders [32], plasma levels of P_4 were similar to, but slightly lower than, those we found in juvenile females from 31°C eggs. Yet samples in that study were taken after females were administered P_4: plasma P_4 levels were presumably at basal levels immediately after hormone administration and increased thereafter. Another caveat is that the authors did not report the age, the size, nor the reproductive status of the females used in their experiment so it is difficult to make any inferences about developmental (or any other) changes in P_4 levels. In the only study that has reported circulating levels of sex steroids in mature males, P_4 levels were one to two orders of magnitude higher than those we found for immature males, whereas T and DHT levels were generally undetectable [33]. Although the level of detectability for each hormone was not explicitly reported, it is interesting that P_4 levels are substantially higher than both T and DHT levels in both immature and mature male red-eared sliders. Another fascinating finding from this study was that melanistic and nonmelanistic males differed with respect to their serum and testicular levels of progesterone. Consequently, it will be important to determine whether incubation temperature effects on pigmentation in hatchlings [15] are mediated by temperature-induced differences in sex steroid levels and whether such differences are correlated with color polymorphism in adulthood.

Overall, the results from our experiment and these earlier studies suggest that embryonic incubation temperature may have lasting effects on steroid-dependent physiological and behavioral traits much later in life. Indeed, in many vertebrates, variation in early sex steroid exposure of this kind has permanent developmental effects on adult sexual and aggressive behavior. This finding is significant because one would have to wait years to examine incubation temperature effects in adult red-eared sliders: males attain sexual maturity at 3–5 yr of age while females reach maturity at approximately 8 yr of age [34]. In fact, it would be difficult to study the persistence of incubation temperature effects in the majority of TSD reptiles because of their longevity. Nevertheless, previous work on an early-maturing TSD lizard, the leopard gecko, *Eublepharis macularius*, indicates that such temperature-induced variation in sex steroid levels, and consequently behavior, persist into adulthood (reviewed in [12]).

Perhaps the most intriguing question is how, at a developmental level, incubation temperature maintains its persistent effects on sex steroid levels in TSD reptiles. Although we have no clear answer, lessons may be drawn from intrauterine position effects in mammals. The intrauterine position of a mammalian fetus relative to same- or opposite-sex siblings affects its exposure to estradiol and testosterone [35, 36], thus influencing subsequent morphology, physiology, and behavior [37–40]. For example, adult male rats that were located between male siblings in utero, and thereby exposed to higher testosterone levels, have significantly higher testosterone and lower estradiol levels than adult male rats that were located between female siblings in utero [38]. In rats and mice, the effects of this early steroid exposure on adult steroid physiology are mediated by changes in steroidogenic enzyme activities in the reproductive tract [37, 41]. Thus, it would be interesting to determine whether similar changes in the activity (or expression) of steroidogenic enzymes occur in the reproductive system of TSD reptiles.

Considering recent evidence that much of the molecular machinery for gonadogenesis is evolutionarily conserved, an analogy between incubation temperature and intrauterine position effects on subsequent gonadal physiology may reflect common developmental mechanisms. Indeed, the gonadal anlagen is initially bipotential and consists of a cortical region that gives rise to the ovary and a medullary

region that gives rise to the testis in all amniotic vertebrates [42]. Moreover, genes clearly involved in mammalian sex determination (e.g., *AMH, SF-1, WT1,* and *SOX9*) [43–45] have also been identified and implicated in avian sex determination [43, 46–48] and temperature-dependent sex determination (TSD) in the red-eared slider turtle [43, 49, 50] (A. Fleming and D. Crews, personal communication). Some of these genes (i.e., *AMH* and *SF-1*) are known to regulate the expression of steroidogenic enzymes during gonadal differentiation in mammals and may also do so in reptiles with TSD. In conclusion, our results with the red-eared slider turtle show that incubation temperature has lasting effects on sex steroid levels even 6 wk after embryonic development has been completed. It remains to be determined, however, whether this variation in steroid levels is related to temperature-induced differences in metabolism, growth, and behavior that have been demonstrated in other TSD reptiles [7–14] or to variation in pigmentation in the red-eared slider turtle [15, 33].

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