

# The physiology of hibernation among painted turtles: the midland painted turtle (*Chrysemys picta marginata*)

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## Abstract

Midland painted turtles from Michigan were submerged at 3°C in normoxic and anoxic water. In prediver, and in turtles submerged for up to 150 days, plasma PO<sub>2</sub>, PCO<sub>2</sub>, pH, [Cl<sup>-</sup>], [Na<sup>+</sup>], [K<sup>+</sup>], total Mg, total Ca, lactate, glucose, and osmolality were measured; hematocrit and mass were determined, and plasma [HCO<sub>3</sub><sup>-</sup>] was calculated. Anoxic turtles developed a severe metabolic acidosis, accumulating lactate from a prediver value of 4.4 mmol/L to a 150-day value of 185 mmol/L, associated with a fall in pH from 7.983 to 7.189. To buffer lactate increase, total calcium and magnesium rose from 3.7 and 2.6 to 58.9 and 11.8 mmol/L, respectively. Plasma [HCO<sub>3</sub><sup>-</sup>] was titrated from 39.2 to 4.8 mmol/L in anoxic turtles. Turtles in normoxic water had only minor disturbances of their acid–base and ionic statuses, associated with a much smaller increase of lactate to 23 mmol/L; there was a marked increase in hematocrit from 29.1% to 42.1%. We suggest that it is ecologic, rather than phylogenetic, relationships that determine the responses of painted turtles to prolonged submergence associated with hibernation. © 2000 Elsevier Science B.V. All rights reserved.

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## 1. Introduction

Painted turtles (*Chrysemys picta*) range from the east to the west coasts of the USA, and from the Gulf coast to southern Canada. There are

four well-marked subspecies. Their approximate ranges (Conant and Collins, 1998) are *C. p. dorsalis* (southern painted turtle) in the south-central USA, *C. p. bellii* (western painted turtle) in the northern plains states west of the Mississippi River, *C. p. marginata* (midland painted turtle) from Tennessee northward between the Mississippi River and the Appalachian Mountains and into southern Quebec and Ontario, and *C. p. picta* (eastern painted turtle) between the Appalachians and the Atlantic Ocean from Georgia to Nova

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Scotia. (Subspecies are hereafter referred to by only their subspecific epithet.)

Bishop and Schmidt (1931) suggested that *marginata* was the parent form of the other three subspecies. In contrast, Bleakney (1958) suggested that *marginata* was derived as a hybrid of *bellii* and *dorsalis*, resulting from intergradation of the latter two as they spread northward from southern refugia after the Wisconsinan glaciation (about 18,000–20,000 yr ago). He based his supposition on the intermediate plastral pattern of *marginata* (*dorsalis* has no pattern and *bellii* has an extensive pattern) and the similarity in dorsal scute alignment (anterior edges staggered rather than aligned transversely) of the two. He suggested that the Wisconsinan refugium for *bellii* was in or near New Mexico, and that as this form spread north and east, it encountered *dorsalis*, which was moving up the Mississippi River drainage from a refugium in the lower portions of the drainage (e.g., Louisiana). Bleakney hypothesized that the two advancing fronts met near the confluence of the Missouri, Mississippi, and Ohio rivers, and that the hybrid form (now *marginata*) then moved north and east to occupy its present range. Bleakney envisioned a third (perhaps incipient) subspecies, *picta*, to have had a refugium on the southeastern coastal plain, from which it moved northward along a corridor bordered by the Appalachian Mountains and the Atlantic Ocean.

Bishop and Schmidt's hypothesis has received no support, but it also has not been disproven. Several subsequent morphological studies of intergradation among painted turtle subspecies, however, have led to conclusions that support Bleakney's hypothesis (Ernst, 1970; Ernst and Ernst, 1971), and his theory seems to be generally accepted (reviewed by Ernst et al. (1994) and by Rhodin and Butler (1997)). However, since the present ranges of *bellii* and *dorsalis* do not meet, these and other studies of natural intergradation among subspecies have not included this pairing (Hartman, 1958; Waters, 1964, 1969; Pough and Pough, 1968; Ernst and Fowler, 1977). Thus, the support for Bleakney's theory on the origin of *marginata* rests solely on the observation that certain morphological characters are intermediate between those of *bellii* and *dorsalis*.

If one assumes that an intermediate morphology is evidence of a hybrid origin of *marginata*, then one might suspect that other characters, such as genetic or physiologic traits, might also be intermediate, although not necessarily to the same degree. Here, we assess the physiologic responses of *marginata* to simulated hibernation, and find that its responses are not intermediate between those of *bellii* and *dorsalis*, instead being similar to those of *bellii* from Wisconsin and *picta* from Connecticut, suggesting that all northern subspecies of painted turtles have relatively similar physiologic adaptations to the prolonged cold submergence associated with hibernation.

## 2. Materials and methods

### 2.1. Animals

Adult midland painted turtles (*Chrysemys picta marginata*) were collected during the summer of 1998 in Shiawassee County, Michigan under permit from the Michigan Department of Natural Resources. Turtles were maintained in an AAALAC-approved aquatic facility at the University of Alabama, where they were kept in well water, provided basking platforms under full-spectrum light on a 12D-12L photoperiod, and fed catfish food pellets supplemented with whole fish.

### 2.2. Blood sampling and analyses

The methodologies for simulated overwintering and for blood sampling are similar to those of Ultsch et al. (1985). Briefly, in late September, we stopped feeding the turtles and in early October moved them into a cold room at 15°C. They were separated into two equally sized groups and placed in shallow ( $\approx 13$  cm) water with access to air, where they were cooled at 1 °C/day to 3°C. They were maintained at 3°C for three days whereupon control (pre-dive) blood samples were taken from five turtles. Samples were taken after clamping the neck, followed by decapitation anterior to the clamp and pithing of the severed head. A 1 cm hole was then trephined in the plastron

over the heart. A heparinized (100,000 units/L ammonium heparin in turtle ringer's solution) tuberculin syringe was used to withdraw an anaerobic aliquot of blood ( $\approx 0.4$  ml), which was immediately analyzed for pH,  $PO_2$ , and  $PCO_2$  (Radiometer BMS 3 MK2 Blood Micro System and PHM 73 pH/blood-gas monitor, thermostated at  $3^\circ C$ ). The  $CO_2$  electrode output was read as pH (Radiometer PHM 240 pH/ion meter) and converted to  $PCO_2$  using the linear relationship between  $\log PCO_2$  and pH. Blood-gas electrodes were calibrated with known gas mixtures (Wösthoff M301/a-F gas-mixing pump); the pH electrode was calibrated with precision buffers (Radiometer).

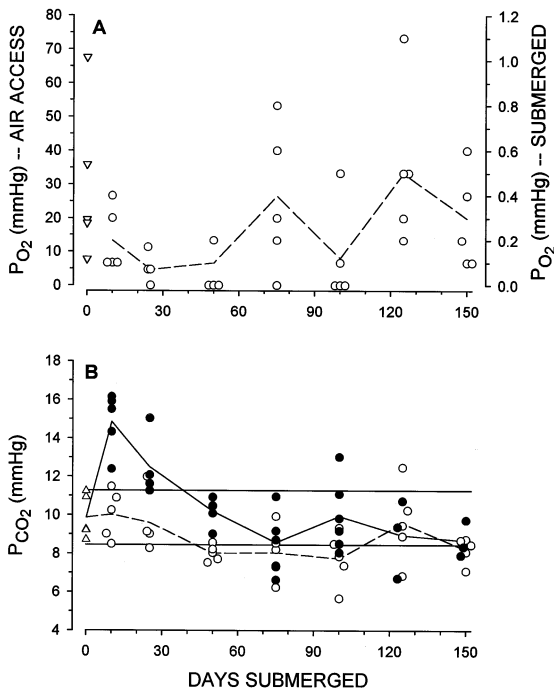


Fig. 1. A: plasma  $PO_2$  of *C. p. marginata* controls (predive, open triangles and left y-axis) and submerged in normoxic water (open circles and right y-axis) at  $3^\circ C$ . B:  $PCO_2$  of control (open triangles) and submerged turtles; anoxic submergence, filled circles, normoxic submergence, open circles. The 95% confidence interval for the predive turtles is shown as parallel lines. A dashed line connects mean values for the sampling periods for the turtles submerged in normoxic water, and a solid line connects mean values for the turtles submerged in anoxic water.

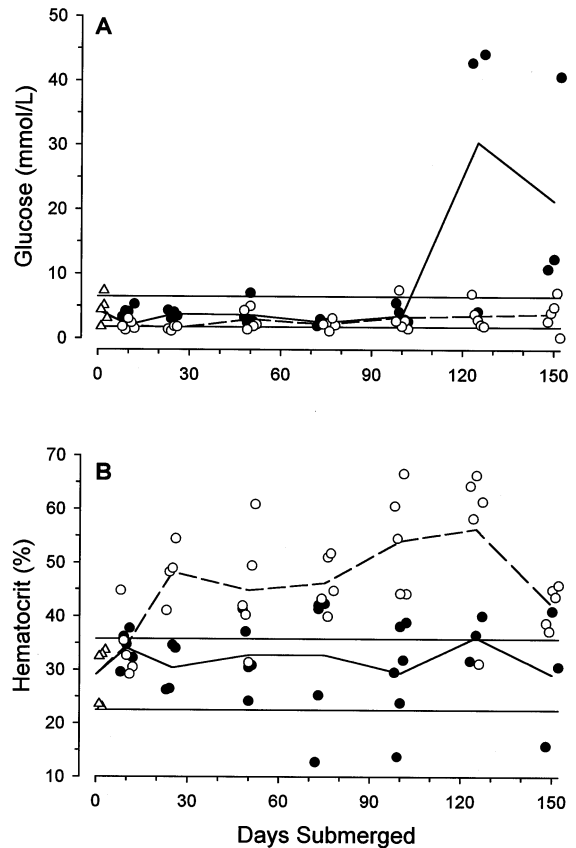


Fig. 2. As in Fig. 1B, for glucose concentration and hematocrit.

The turtle was then inverted and elevated, and a 20-gauge needle with attached catheter tubing (PE 90) was inserted into the ventricle. Blood was collected as it drained from the catheter and centrifuged (3 min at 10,000 g) and the decanted plasma was stored at  $-80^\circ C$  for later analysis. Duplicate microhematocrit tubes were filled from the catheter and hematocrit determined by centrifugation for 4 min at 13,000 g (Adams Microhematocrit II centrifuge). The plasma was later used for determinations of  $[Na^+]$  and  $[K^+]$  (Radiometer FLM 3 flame photometer),  $[Cl^-]$  (Radiometer CMT10 chloride titrator), lactate and glucose (YSI 2300 Stat-Plus Analyzer), total Mg and Ca (Perkin-Elmer 280 atomic absorption spectrophotometer), and osmolality (Precision Systems  $\mu$ Osmette 5004). Plasma  $[HCO_3^-]$  was calculated using the Henderson-Hasselbalch equa-

tion with  $\alpha_{\text{CO}_2} = 0.0812$  (Reeves, 1976) and a  $\text{pK}'$  that depended on the blood pH. Jackson and Heisler (1983) found the  $\text{pK}'$  of prediver (air-breathing) painted turtles at 3°C to be 6.293 and of submerged anoxic turtles to be 6.350. We found the lowest pH of control (prediver) turtles to be 7.967, so when any subsequent samples had a  $\text{pH} \geq 7.967$  we used  $\text{pK}' = 6.293$ , and when samples had a  $\text{pH} < 7.967$  we used  $\text{pK}' = 6.350$ .

The remaining turtles were divided into two groups. Each group was maintained submerged by placing a plastic grate in the water column above the turtles. Aerators, drawing air from outside the cold room, were placed above the grating of one group, maintaining the water  $\text{PO}_2$  at or near 158 mmHg. The tank containing the other group was made nearly airtight by attaching closed-cell insulation along the edges and covering it with a plastic lid. Nitrogen was bubbled through airstones above the grate, which maintained the  $\text{PO}_2 < 5$  mmHg. The water in both tanks was flushed occasionally, without allowing the turtles air access, with pre-equilibrated water at 3°C.

Turtles were removed for sampling ( $n = 3-5$ ) as described above, except that the necks were clamped underwater to prevent breathing. Sam-

ples were taken from both groups on 0 (prediver controls), 10, 25, 50, 75, 100, 125, and 150 days of submergence.

### 2.3. Statistics

Statistical analyses were performed with SigmaStat 1.0 for Windows and with Statistica; we accepted the 0.05 level of significance. Comparisons were done with MANOVA, one-way ANOVA, and a Kruskal–Wallis test when data were non-parametric. Student–Newman–Keuls, Bonferonni tests, or Tukey's HSD were used for multiple comparisons. Data are given as mean  $\pm$  S.E.

## 3. Results

In a tank not used for sampling turtles, eight turtles submerged in anoxic water survived  $132 \pm 6$  (range 104 to 153) days. Five others submerged in normoxic water were still alive after 217 days; at which time one was removed for blood sampling; two of the remaining four recovered when allowed access to air, warmed to room temperature overnight, and placed in a tank with shallow

Table 1

Prediver values for body mass, blood gasses, acid–base variables, Hct, ions, total Ca and Mg, osmolality, and glucose compared to the values for the same variables after 150 days of submergence of *Chrysemys picta marginata* in normoxic and in anoxic water at 3°C. Values are mean  $\pm$  S.E

	Prediver ( $n = 5$ )	Normoxic water (150 days) ( $n = 5$ )	Anoxic water (150 days) ( $n = 3$ )
$\text{PO}_2$ (mmHg)	$29.88 \pm 10.40$	$0.30 \pm 0.10^{\text{a,b}}$	0
$\text{PCO}_2$ (mmHg)	$9.86 \pm 0.51$	$8.21 \pm 0.30$	$8.66 \pm 0.55$
pH	$7.983 \pm 0.006$	$7.868 \pm 0.046^{\text{b}}$	$7.189 \pm 0.031^{\text{a}}$
$[\text{HCO}_3^-]$ (mmol/l)	$39.23 \pm 1.85$	$22.90 \pm 2.25^{\text{a,b}}$	$4.84 \pm 0.21^{\text{a}}$
$[\text{Na}^+]$ (mmol/l)	$117.2 \pm 2.5$	$104.7 \pm 7.4$	$99.2 \pm 8.1$
$[\text{K}^+]$ (mmol/l)	$2.13 \pm 0.13$	$3.00 \pm 0.19^{\text{b}}$	$10.57 \pm 1.58^{\text{a}}$
$[\text{Cl}^-]$ (mEq/l)	$72.6 \pm 2.4$	$54.4 \pm 4.9^{\text{a}}$	$44.5 \pm 0.5^{\text{a}}$
Total [Mg] (mmol/l)	$2.60 \pm 0.13$	$5.00 \pm 0.80^{\text{a,b}}$	$11.70 \pm 0.10^{\text{a}}$
Total [Ca] (mmol/l)	$3.74 \pm 0.34$	$6.60 \pm 1.70^{\text{b}}$	$58.90 \pm 3.20^{\text{a}}$
Lactate (mmol/l)	$4.42 \pm 1.25$	$22.90 \pm 4.70^{\text{a,b}}$	$185.00 \pm 7.00^{\text{a}}$
Glucose (mmol/l)	$4.35 \pm 0.94$	$3.82 \pm 1.15$	$21.30 \pm 9.70^{\text{a}}$
Osmolality (mOsm/kg plasma)	$250.0 \pm 5.0$	$224.0 \pm 14.0^{\text{b}}$	$397.0 \pm 21.0$
Hematocrit (%)	$29.1 \pm 2.4$	$42.1 \pm 1.7$	$29.1 \pm 7.3$

<sup>a</sup> Significantly different from prediver values (MANOVA).

<sup>b</sup> Significantly different from anoxic values (MANOVA).



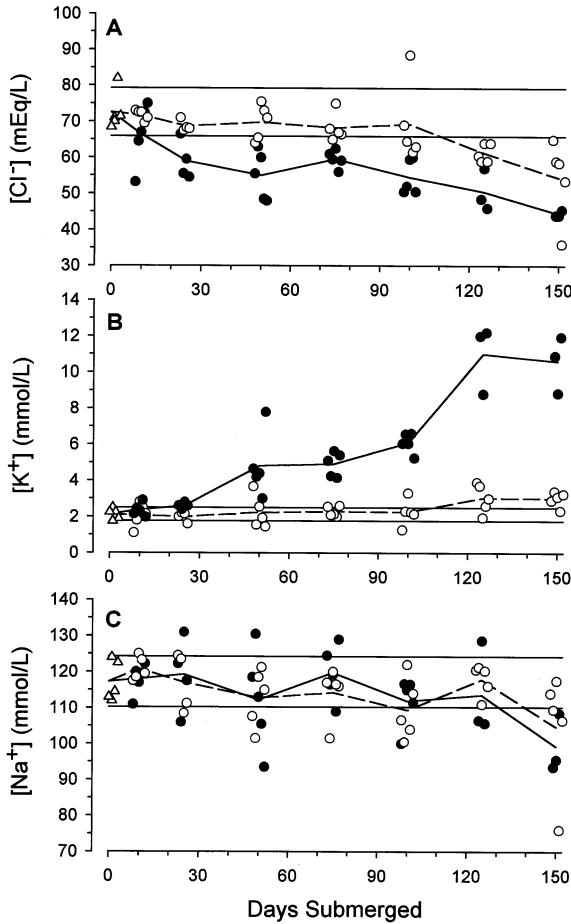


Fig. 5. As in Fig. 1B, for  $[\text{Cl}^-]$ ,  $[\text{K}^+]$ , and  $[\text{Na}^+]$ .

Final  $[\text{Cl}^-]$  decreased 25% in turtles in normoxic water and 39% in anoxic turtles (Fig. 5A). Final  $[\text{K}^+]$  increased to 10.6 mmol by 150 days in anoxic turtles, but remained unchanged in turtles submerged in normoxic water (Fig. 5B).  $[\text{Na}^+]$  was maintained at or near prediver levels in both groups (Fig. 5C). Total [calcium + magnesium] increased 64.3 mmol in anoxic turtles while remaining unchanged in turtles submerged in normoxic water (Fig. 6A and B). Osmolality increased 58.8% to 397 mOsm by 150 days of submergence in anoxic turtles while remaining unchanged in turtles submerged in normoxic water (Fig. 6C).

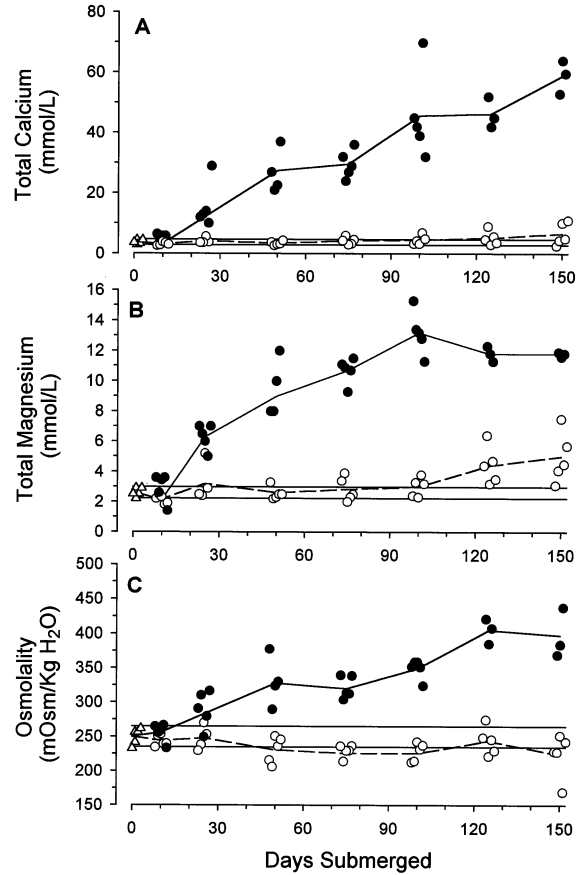


Fig. 6. As in Fig. 1B, for total Ca, total Mg, and osmolality.

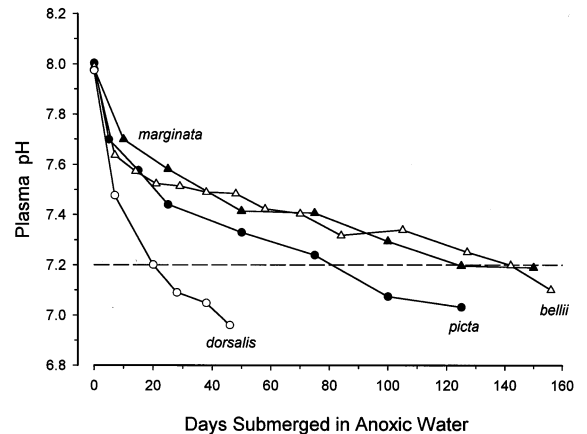


Fig. 7. Decline in plasma pH of the four subspecies of *Chrysemys picta* submerged in 3°C anoxic water. Data for *C. p. picta* from Utsch et al. (1999), for *C. p. bellii* from Utsch and Jackson (1982), and for *C. p. dorsalis* from Utsch et al. (1985).

#### 4. Discussion

The acid–base and ionic responses of *marginata* are qualitatively similar to those found in previous studies of *dorsalis*, *bellii*, and *picta* (Ultsch and Jackson, 1982; Jackson and Ultsch, 1982; Ultsch et al., 1985, 1999), but quantitatively most closely resemble those of northern *bellii* and northern *picta*. Lactate accumulated in submerged turtles due to a decreased or absent O<sub>2</sub> supply. Anoxic turtles accumulated a profound lactate load, while those in normoxic water had a much lower lactate load due to their ability to extract enough O<sub>2</sub> from the water sufficient to accommodate a significant aerobiosis. The accumulation of lactate in both groups caused an associated fall in pH and titrated the [HCO<sub>3</sub><sup>-</sup>] to low levels in anoxic animals, but not to zero, which indicates that bicarbonate was being exported to the blood from an outside source. That source was likely the skeleton, in particular the shell, from MgCO<sub>3</sub> and CaCO<sub>3</sub> reservoirs, as indicated by the large increases in total calcium and magnesium in the plasma (Jackson, 1999; Jackson et al., 2000). The rise in [K<sup>+</sup>] and the decrease in [Cl<sup>-</sup>] aid by electrically compensating for the increase in the strong ion, lactate. If just [K<sup>+</sup>] and [Cl<sup>-</sup>] are considered, however, the resulting SID (strong ion difference) would suggest a pH far below measured values. The Ca and Mg levels, when taken into account, move the SID in a direction that would compensate for the observed acidosis (Jackson et al., 1984).

The rate at which turtles accumulate lactate seems to be the major factor in determining their survival during anoxic submergence (Ultsch and Jackson, 1995). Based on Bleakney's hypothesis that *marginata* arose as a hybrid of *bellii* and *dorsalis*, we would expect the response to anoxic submergence of *marginata* to be intermediate between that of *bellii* and *dorsalis*. However, using the rate of fall in pH during submergence in anoxic water as a basis for comparison, we find that the response of *marginata* is much more similar to that of northern (Wisconsin) *bellii* and northern (Connecticut) *picta*. Using a fall of 0.8 pH units (to 7.2) as a basis for comparison, all

the northern subspecies reach pH 7.2 after > 80 days of submergence, while the southern *dorsalis* attain this pH after only 20 days of submergence (Fig. 7). The differences cannot necessarily be attributed to a north–south physiologic cline, as the musk turtle (*Sternotherus odoratus*), which has a fairly wide latitudinal range, showed no physiologic cline between northern (Michigan) and southern (Alabama) populations with respect to their responses to submergence in anoxic water at 3°C (Ultsch and Cochran, 1994). Although these data do not definitively negate Bleakney's hypothesis of a *bellii* and *dorsalis* hybrid origin of *marginata*, they do cast doubt upon it. One possibility for explaining our results is that *marginata* is a hybrid of *picta* and *bellii*, two northern forms already adapted to extended overwintering periods. Another is that *marginata* is not a hybrid, but was already present during the Wisconsinan glaciation, being harbored in an as yet undiscovered refugium somewhere in the southern portion of its present range. A third is that Bleakney was correct, and that *marginata* has developed, through natural selection, an ability to tolerate long-term hibernation that is similar to that of *bellii* and *picta*; however it should be noted that musk, map, and softshell turtles have not developed this ability over a similar time period. We conclude that while our data do not point definitively to any one evolutionary origin of *marginata*, they do not seem to support Bleakney's hypothesis for subspeciation and suggest a need for further scrutiny into the evolutionary history of the painted turtle complex.

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