Reproductive biology of a cave-associated population of the frog *Rana palustris*

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Aspects of the reproductive biology of a trogloxene population of the pickerel frog, *Rana palustris* (Anura: Ranidae), were studied at a cave on the edge of the Ozark plateau in Missouri. Sexual dimorphism in body size was marked; there was almost no overlap in adult body size ranges, and the ratio of mean adult female snout–vent length (SVL) to adult male SVL was 1.26. All males with SVL of >45 mm were sexually mature. Males showed a clear cycle of spermatogenesis, with a peak in mid-summer and a decline in spermatogenic activity through autumn and winter. Minimum size at maturity for females was 59 mm SVL. Females completed vitellogenesis during the summer, before their arrival at the study site. Of 28 females above the minimum size at maturity, 27 contained egg clutches. Clutch size, clutch mass, and egg size (mass) show significant positive correlation with SVL. Mean (±SD) clutch size was 1759 ± 548. Fat bodies were present in both males and females in autumn, but were totally depleted before the animals emerged from hibernation. Trogloxene *R. palustris* do not diverge significantly from patterns of reproductive biology typical of other temperate zone *Rana*. Current or past patterns of cave use have had no detectable effect on reproductive characteristics of trogloxene *R. palustris*.

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Certains aspects de la biologie de la reproduction ont fait l'object d'une étude chez une population trogloxène de la Grenouille des marais *Rana palustris* (Anura : Ranidae) dans une grotte située aux abords du plateau Ozark dans le Missouri. Le dimorphisme sexuel de la taille était très marqué; il n'y avait presque pas de chevauchement entre les étendues de taille des adultes mâles et femelles, et le rapport longueur moyenne museau – anus (SVL) des femelles adultes à la SVL des mâles adultes était de 1,26. Tous les mâles de SVL de 45 mm avaient atteint leur maturité sexuelle. Chez les mâles, le cycle de la spermatogénèse était bien marqué : l'activité de la spermatogénèse était maximale au milieu de l'été, puis diminuait durant l'automne et l'hiver. La taille minimale à la maturité était de 59 mm (SVL) chez les femelles. Les femelles avaient terminé leur vitellogénèse au cours de l'été, avant leur arrivée dans la grotte. Vingt-sept des 28 femelles plus grandes que la taille minimale à la maturité sexuelle contenaient des masses d'oeufs. La taille des masses d'oeufs, leur masse et la taille des oeufs (masse) se sont avérées en corrélation positive avec la SVL. La taille moyenne d'une masse d'oeufs était de 1759 ± 548 oeufs. Les corps gras étaient présents chez les mâles et les femelles à l'automne, mais avaient complètement disparu avant la fin de l'hibernation. La biologie de la reproduction des *Rana palustris* trogloxènes ne diffère donc pas significativement de celle des autres *Rana* de la zone tempérée. L'utilisation de la grotte dans le passé et maintenant ne semble pas avoir d'effet sur les caractéristiques de la reproduction des *Rana palustris* trogloxènes.

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Introduction

Certain populations of the pickerel frog, *Rana palustris*, in the limestone karst areas of the interior highlands and adjacent regions of the United States exhibit an ecology unique among temperate zone anurans in that they use caves and springs during a considerable portion of the year (Resetarits 1986). This species is the only North American frog commonly found in caves. Schaaf and Smith (1970) and Resetarits (1986) suggested that caves play an important and perhaps critical role in the distribution and ecology of *R. palustris* in these regions. The unique nature of this species' habitat use raises questions regarding origin and history of cave use and its importance in these populations.

Cave-dwelling organisms, in general, have a suite of reproductive characters related to the energetic constraints and environmental stability of the cave environment; these include small clutch size, large ovum size, and infrequent reproduction, often coupled with an extended life-span (Poulson 1963; Barr 1968; Culver 1982). Reproductive traits that are suggestive of cave adaptation may be observed in troglophilic and trogloxene members of generally troglobitic genera, as is the case in the amblyopsid cavefish (Poulson 1963) and beetles of the genus *Ptomaphagus* (Peck 1986), in which gradients of reproductive adaptation to cave (or cave-like) conditions exist.

The presence of such reproductive characteristics in cave-associated populations of a species not known to breed in subterranean waters or springs would suggest adaptation for facultative (troglophilic) use of such sites for breeding purposes, perhaps during periods of climatic change (Resetarits 1986).

Cave use in *R. palustris* is of particular interest, both in relation to other trogloxenes and in the fact that it may be the most "cave adapted" of temperate zone Anura, an order which contains no true troglobites or troglophiles.

The ecology of cave use in a population of *R. palustris* is treated elsewhere (Resetarits 1986). The purpose of this paper is to describe the reproductive biology of this population and to determine if its unique habitat use is paralleled by features of its reproductive biology.

Materials and methods

Specimens were obtained from Ralph's Ford Bluff Shelter (RFBS), a cave in southeastern Boone County, Missouri, described elsewhere (Resetarits 1986).

Seventy-five *Rana palustris* were collected at RFBS (33 females and 42 males). Females were sampled on 11 dates from September through April. Males were sampled on 13 dates from July through April. Samples of each sex cover their respective periods of cave use (see Resetarits 1986). Frogs could not be tracked to breeding sites or summer habitat, therefore specimens are lacking for these periods. Specimens were preserved in 10% formalin within 24 h of capture.

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Animals were then weighed to the nearest 0.01 g and snout–vent length (SVL) was measured to the nearest millimetre. Coelomic fat bodies were removed and mass (wet) determined for all specimens. Sex was confirmed by dissection.

Reproductive biology of females

Ovaries and oviducts were removed from each female (N = 33) and weighed to the nearest 0.1 mg. Average individual ovum mass was determined by weighing a cluster with a minimum of 40 ova from each female, counting the ova in the cluster, and dividing the cluster mass (in grams) by the number of ova in the cluster. This ratio and the total clutch mass were then used to calculate the clutch size for each female. This estimate was necessary because of large clutch size and the difficulty of separating and directly counting individual ova. All females had fully yolked ova exhibiting little size variation within clutches.

Reproductive biology of males

The left testis was removed from each male (N = 42) and weighed to the nearest 0.1 mg; length was measured to the nearest 0.1 mm. The right testis was used in cases where the left was damaged during dissection. Testes were placed in individual vials of 10% formalin, dehydrated, and cleared in isopropyl alcohol and toluene. Tissue was then embedded in paraffin, sectioned at 7–8 μm, and stained with hematoxylin, Biebrich Scarlet – Orange II, and Fast Green.

Spermatogenic activity was determined using Sluiter et al. (1950). An average of 20 cross-sectioned tubules from each male was used for estimating overall spermatogenic activity.

Statistical analysis

Female reproductive parameters were analysed by standard regression techniques: Pearson's product–moment correlation coefficient (r) and multiple regression. Fat body data for both sexes and testis data were analysed using analysis of covariance (ANCOVA) with SVL as the covariate (Sokal and Rohlf 1981).

Results

Body size and sexual dimorphism

Mean ($\pm$ SD) SVL and total body mass for adult females were 68 $\pm$ 6.3 mm (limits 59–86 mm) and 35.08 $\pm$ 11.57 g (limits 19.88–62.60 g), respectively. Adult males averaged 54 $\pm$ 3.7 mm (limits 45–61 mm) and 16.56 $\pm$ 3.91 g (limits 9.15–25.23 g), respectively. Male and female differences were significant for both SVL (p < 0.001) and body mass (p < 0.001).

Reproductive biology of females

Individuals larger than 60 mm were mature, based on the presence of fully developed ova. The largest immature and the smallest mature female each measured 59 mm SVL, suggesting that RFBS females mature near that size.

Clutch size and SVL were positively correlated (r = 0.82, p < 0.001; Fig. 1). Mean clutch size was 1759 $\pm$ 548 (SD) (limits 704–2896). Mean clutch mass was 4.66 $\pm$ 1.97 g (limits 1.86–9.68 g). Clutch mass also positively correlated with SVL (r = 0.85, p < 0.001). Mass of an average ovum (within clutch) ranged from 1.6 to 3.3 mg (mean 2.6 $\pm$ 0.4 mg). Average ovum mass and SVL were positively correlated (r = 0.56, p < 0.01). Clutch size and ovum mass showed a suggestive positive correlation (r = 0.37, df = 26, p < 0.06). An average 13.0 $\pm$ 1.95% (SD) (range 7.0–16.9%) of total female body mass (including clutch and fat body mass) was ova. A slight tendency existed for larger females to produce a greater relative clutch mass, but this correlation was not significant (r = 0.32, p > 0.10).

Multiple regression was used to examine the relative contribution of clutch size and ovum size (mass) to increase in clutch mass associated with increasing body size (SVL). Because of
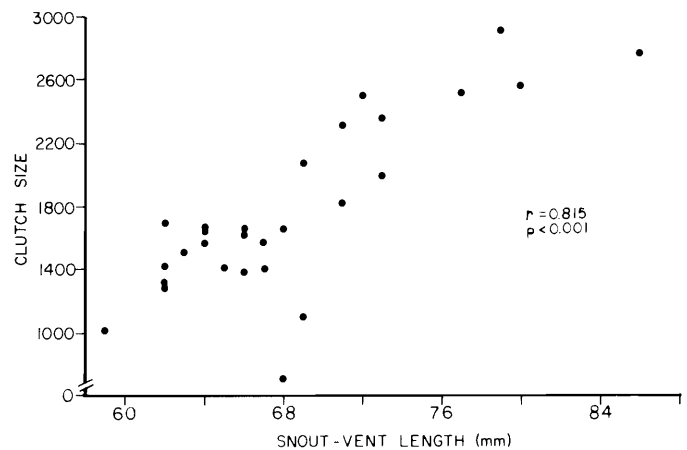

FIG. 1. Clutch size vs. body size in *Rana palustris* at RFBS.

the correlation between clutch size and ovum size, strict partitioning of variance was not possible (Draper and Smith 1981). Using multiple regression, we varied order of entry of the two variables into the model to determine their importance. The two variables explained 98.8% of the variation in total clutch mass. Both variables contributed significantly to the explanation of variation in total clutch mass, with clutch size contributing a greater amount of variation regardless of order of entry of variables into the model.

Production of ova was completed during summer, before the autumn arrival of females at RFBS. Females collected in September at RFBS contained a full complement of mature ova. No discernable change occurred in ova or oviducts during the overwintering period. All females above minimum body size at maturity (59 mm SVL) contained a full complement of mature ova.

Reproductive biology of males

All males in this study were sexually mature. Males <45 mm SVL were not represented. Testis mass (Fig. 2a) was lowest in late winter. Males were absent from the cave during April–June. In July, males showed highest testis mass for the study period (July–March), suggesting an increase in testis mass between April and July. This increase was followed by a steady decrease in percent testis mass from July through March ($F_{1,12}$ = 2.46, p < 0.05).

Throughout the sampling period, tubules in nearly all stages of spermatogenesis were present within individual testes. Relative abundance of tubules in each stage varied among months, resulting in a definite cycle of spermatogenic activity. Spermatogenic activity was at its highest level in July and decreased steadily from this time through the overwintering period, reaching lowest levels in late winter (Fig. 2b).

Mature spermatozoa were present in all testes examined, regardless of time of year. The quantity of mature spermatozoa in individual tubules and the proportion of tubules containing mature spermatozoa increased from July to March, corresponding with the decrease in other maturation stages. Spermatozoa were abundant through winter and early spring. Most spermatozoa were associated with Sertoli cells, but some free spermatozoa were present in the lumen of all tubules containing mature spermatozoa. This situation predominated even in specimens taken just before emergence (late March); spermatozoa were still found in bundles associated with Sertoli cells, with relatively few free within the lumen. Spermiation was apparently initiated after emergence from hibernation.
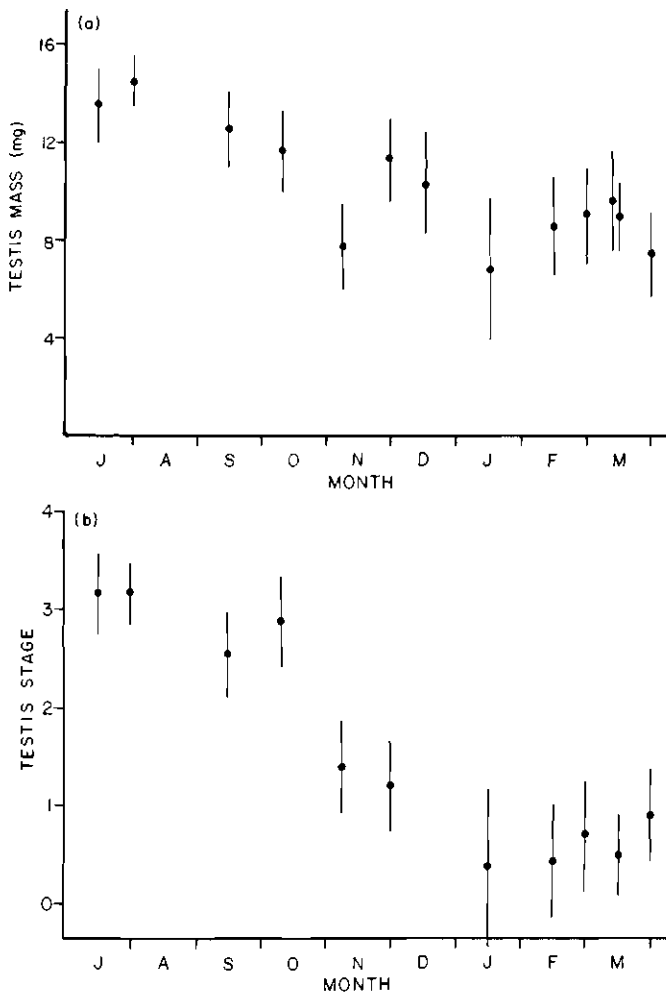
FIG. 2. Reproductive cycle in RFBS males between July and March. All data are plotted as mean ± 1 SE. Data are corrected for body size using analysis of covariance (ANCOVA). (a) Testis mass versus date; (b) spermatogenic stage versus date. Stage 0, testis tubule with a few primary spermatogonia present; stage 1, testis tubule with primary spermatogonia and one small cell nest; stage 2, testis tubule with primary spermatogonia and several small cell nests; stage 3, testis tubule with primary spermatogonia, several small cell nests, and at least one large cell nest; stage 4, testis tubule with closed row of cell nests on its periphery; stage 5, testis tubule almost completely filled with cell nests.

Coelomic fat bodies

Males and females began the overwintering period at RFBS with large fat bodies (Figs. 3a and 3b). Mean percent fat body in September was greater in males (1.74%) than in females (1.23%). Depletion of reserves was apparent from the earliest collection date for both males (July) ($F_{1,12} = 11.19$, $p < 0.0001$) and females (September) ($F_{1,10} = 3.95$, $p < 0.005$), and continued until reserves were totally absorbed. Though reduction proceeded at a slower rate, fat stores in females had been totally absorbed earlier (January) than those in males (February), owing to initially smaller relative reserves. Males and females were completely devoid of fat reserves well before the end of the overwintering period.

Discussion

Body size and sexual dimorphism

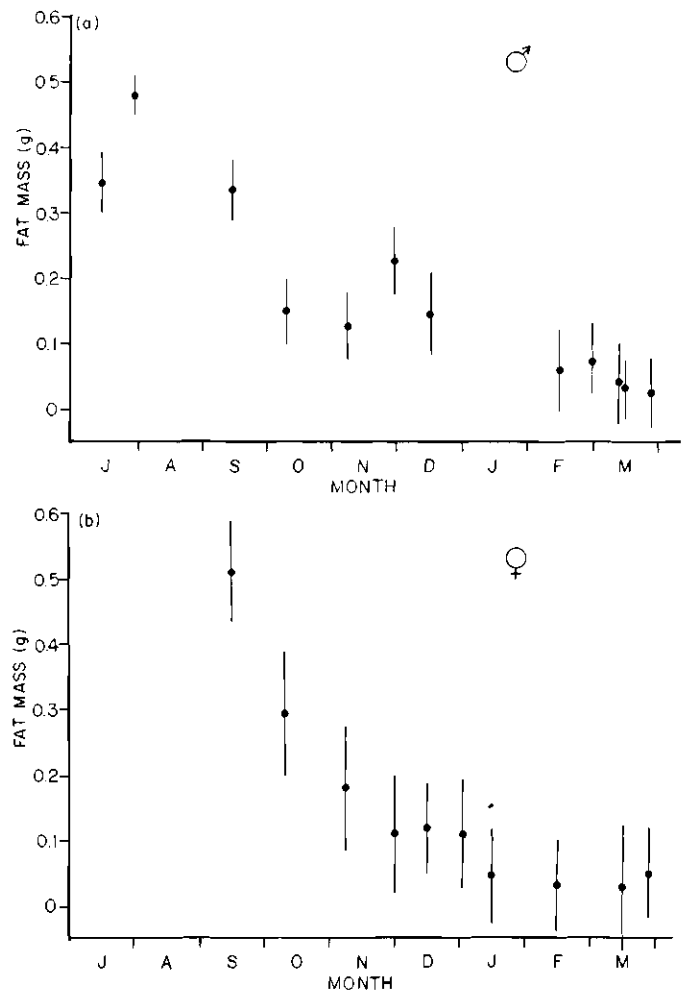Rana palustris at RFBS show a high degree of dimorphism in adult body size, with little overlap between mature males

FIG. 3. Coelomic fat mass (mean ± 1 SE) plotted against date (July–March). Data are corrected for body size using ANCOVA. (a) Males; (b) females.

and females. Pope (1944), Walker (1946), Wright and Wright (1949), and Smith (1961) reported similar sexual dimorphism in size in other parts of the species' range.

Woolbright (1983) collected data on sexual dimorphism in size from the recent literature on 18 species from the genera Bufo, Eleutherodactylus, Hyla, and Rana. He compared the ratios of mean adult female SVL to mean adult male SVL. The range of ratios was 0.92–1.34. The ratio in the RFBS population, 1.26, exceeds all except those of three species of Eleutherodactylus from Puerto Rico and Jamaica. The significance of this high degree of sexual dimorphism in size in the RFBS population is unknown.

Reproductive biology of males

Similar male reproductive cycles occur in other species of temperate zone ranids: Rana pipiens (Glass and Rugh 1944), Rana esculenta (Lofts 1964), and Rana catesbeiana (Schroeder 1975). The cycle in Rana palustris is most similar to that described for R. pipiens by Glass and Rugh (1944). Compared with that of R. pipiens, the spermatogenic cycle of trogloxene R. palustris is less seasonal, and peak spermatogenesis is not as active, nor the quiescent period as inactive. Similarity to the R. pipiens cycle suggests that the spermatogenic cycle in R. palustris is initiated postbreeding, in late May or early June. The absence of specimens from early April through mid-July precludes determining if the increased testis

mass and activity evident in July began in preparation for breeding, or began after cessation of breeding activity. A slight upward trend in testis mass and testis activity in February and March may indicate prebreeding initiation of spermatogenesis.

Reproductive biology of females

As all but one of the females at or above the minimum size at maturity produced clutches in a sample taken from a hibernating population, mature females in this population probably produce at least one clutch per year. The alternate explanation, that only gravid females overwinter in the cave, seems unlikely. One or more clutches per season is generally the case in temperate zone *Rana*; however, data are usually from animals captured at or near breeding sites, which may bias the sample in favor of gravid females.

Clutch size is lower than the 2000–3000 reported by Wright and Wright (1949). Mean and limits of clutch sizes at RFBS lie near the lower end of the limits when compared with those of members of the *R. pipiens* complex, though data from ovarian counts in these other species are lacking. Clutch size in *Rana palustris* lies close to that predicted using Terentjev's (1960) formula, which is based primarily on temperate zone *Rana*. Predicted clutch size is 1915, and the mean for the RFBS population is 1759 ± 548 (SD).

Ovum size (mass) in trogloxene *R. palustris* is not notably different from that reported for other temperate zone *Rana* (McAlister 1962; Kuramoto 1978; Berven 1982), and shows no trend toward small clutch size and large ovum size as observed in the reproductively divergent *Rana tagoi*, a species that breeds in subterranean springheads (Kuramoto 1978). *Rana tagoi* represents one extreme of reproductive strategies within the genus and displays the adaptations one would expect to see in a hypothetical troglobitic or troglophilic *Rana*.

Ovum size (mass) often shows no correlation with body size in anurans. Kuramoto (1978) found significant positive correlation in four of nine anuran species (three of six *Rana*). At RFBS, ovum size shows a significant positive correlation with body size, and explains a significant amount of the variation in total clutch mass. *Pseudacris triseriata* (Pettus and Angleton 1967), *Rana pipiens* (McAlister 1962), *Rana temporaria* (Honig 1966), *Rana sylvatica* (Berven 1982), *Rana japonica*, *Rana tsushimensis*, and *Rana nigromaculata* (Kuramoto 1978) also show significant positive correlation between ovum size and body size.

Kuramoto (1978) found no significant positive correlation between clutch size and ovum mass in nine species examined. A positive correlation between clutch size and ovum mass is uncommon; a suggestive positive correlation exists between these parameters at RFBS. At RFBS, both increased egg number and increased ovum mass contribute to the increase in total clutch mass that occurs with increasing body size. Larger clutch size and larger ovum size contribute positively to potential female fitness in different ways: larger clutch size by increasing the total number of offspring produced and larger ova by potentially increasing the survival chances of individual offspring by allowing larger size at hatching (Salthe and Duellman 1973) and faster growth (Kaplan 1980). Partitioning the increase in total clutch mass between these two factors has implications for the specific reproductive strategy of trogloxene *R. palustris*. Increasing ovum mass at the expense of clutch size is a major feature of troglobitic animals (Poulson 1963; Barr 1968; Culver 1982; Peck 1986), and appears in *R. palustris* in rudimentary form. Whether this is related to cave use is unknown. Pettus and Angleton (1967) observed a

similar reproductive resource allocation strategy in a Colorado population of *Pseudacris triseriata* in response to high elevation.

Fat utilization

Males and females had considerable fat reserves (fat bodies) at the start of the overwintering period. Males contained initially greater relative reserves than females. Fat reserves were rapidly reduced in both sexes, and were totally depleted before the end of overwintering. Reduction of these reserves appeared to proceed more rapidly in males, but because of greater initial (relative) reserves, males maintained measurable fat bodies until February whereas females were depleted in January. More rapid use of coelomic fat reserves by males has no obvious explanation, as presumably they are used primarily for maintenance, because both testis mass and testis activity are low over the winter. A potential explanation is that males may be mobilizing some of the fat into a more accessible form in preparation for initiation of spermatogenesis and the demands of the breeding season. One might still expect greater maintenance requirements for reproductive females. What is most apparent in both is that fat bodies are entirely absorbed over the winter.

Winter stress results in considerable overwintering mortality, especially in juveniles (Resetarits 1986). An interesting aspect of this situation is that although effects of coelomic fat depletion per se on mortality, growth, and reproduction are unknown, the frogs do indeed choose the highest (also the most stable) ambient temperature available within the cave during winter (Resetarits 1986). The effect over the overwintering period would be increased metabolic costs. The magnitude of these costs and the presumed advantage of choosing the highest available temperature are unknown, though temperature stability may be important in reducing overwintering mortality due to freezing.

Aside from slight differences in degree of seasonality in the spermatogenic cycle, the reproductive biology of *R. palustris* differs little from that of closely related species. Resetarits (1986) has discussed the possible role of climatic change associated with Pleistocene glaciations in the origin of cave use in *R. palustris*. Cave use presently centers around the cave as a seasonal or temporary refugium, and may suggest a more intensive use of caves in the past (Resetarits 1986). Evidence of more intensive use of caves, particularly the use of epigean portions of cave streams as breeding sites, might be expected in aspects of the reproductive biology of the species. Such adaptations (especially increased egg size and decreased egg number) can be seen in *R. tagoi* breeding in similar habitat in the mountains of Japan (Kuramoto 1978). This study suggests that whatever the past pattern of cave use may have been in *R. palustris*, it has failed to leave a definitive mark on the reproductive biology of the species. Although their use of caves appears unique among temperate anuran amphibians, trogloxene *R. palustris* populations are not distinctly different from their wholly epigean congeners in terms of reproductive biology.

mended RFBS as a study site. N. Aspinwall, O. J. Sexton, H. M. Wilbur, R. A. Alford, and R. N. Harris reviewed this or earlier versions of the manuscript. This project was partially supported by a grant from the Cave Research Foundation to W.J.R.

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