CULEX TRITAENIORHYNCHUS GILES: SOME EFFECTS OF TEMPERATURE AND PHOTOPERIOD ON LARVAL DEVELOPMENT AND SELECTED ADULT ATTRIBUTES

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Abstract: A Pakistan strain of *Culex tritaeniorhynchus* was exposed to conditions of 18, 22, 28 and 34°C at 16L: 8D and 18 and 22°C at 9L: 15D in an incubator. Cooler temperatures reduced immature survival from eclosion to emergence and slowed the rate of development resulting in larger and heavier adults at emergence with increased energy reserves in the form of triglycerides. The rate of mating, blood feeding and the synthesis of triglycerides from sucrose increased as a function of temperature. Short day length further slowed the rate of development increasing the size and weight, but not triglyceride content, of adults at emergence. The insemination rate also decreased under shorter day length. These results suggested that cold-induced quiescence, but not necessarily diapause, was induced by the present experimental regimens.

The climate of Punjab Province, Pakistan, presents an extremely varied temporal series of ecological regimens to its mosquito fauna. To persist, species must withstand temperatures ranging from 45°C in midsummer to 0°C in mid-winter, seek more ameliorated microhabitats or evolve physiological mechanisms to alter metabolism during periods of stress. Although aestivation has yet to be reported, several *Culex* species, including *Culex tritaeniorhynchus* Giles, appear to undergo a cessation of reproductive activity in response to shorter days and/or cooler temperatures and overwinter as inseminated, unfed and nulliparous females; males and larvae are not collected during mid-winter (Reisen, 1978 and unpubl.). However, on warm days during winter, *Cx. tritaeniorhynchus* females occasionally are collected biting bovid baits (Reisen, 1978), suggesting that if a diapausing state is indeed realized, it must not be obligatorily governed by photoperiod or require an elongated period of cold exposure prior to reactivation. Under slightly colder winter conditions in Japan, overwintering diapause is induced during autumn by a combination of cooler temperatures and shorter day lengths (e.g., Omori, *et al.*, 1965a, b; Wada,

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et al., 1973) and persists through the first warm days of spring. Some gonotrophic dissociation occurs in autumn (Oda and Wada, 1973); however, the accrual of fat reserves prior to diapause seems to be accomplished mainly through sugar feeding.

The purpose of the present study was to examine the effects of different temperatures and long and short day photoperiods on immature development and adult characteristics under controlled laboratory conditions. Emphasis was placed on studying those attributes related to overwintering behavior to determine if diapause could be induced in strains of *Cx. tritaeniorhynchus* collected from Punjab Province, Pakistan.

METHODS AND MATERIALS

Strain: The Balloki strain from Punjab Province, Pakistan, was used throughout, and was well-adapted to mating in 3.8 liter carton cages (Reisen, *et al.*, 1979) and feeding on restrained laboratory mice. The females used to produce the cohorts for experimentation were held under normal insectary conditions of 28°C, 60% R. H. and 16L: 8D photoperiod with 1.5 hr crepuscular periods simulated by gradually changing the illumination produced by 60 watt incandescent bulbs from 18 to 0 Lux.

Biological methods: Egg rafts, within 12 hrs of oviposition, were placed in an incubator to hatch under the test temperature and photoperiod regimens. On the morning of eclosion, 216 1st instar larvae were counted into each of 4 replicate, 16 × 27 cm enameled pans (density = 0.5 larvae/cm² of pan surface area) filled with well-water to a depth of 1.5 cm. and were fed a total of 1.075 gm of finely sifted liver powder (Siddiqui, *et al.*, 1976). The addition rate of the food was adjusted for the larval development rate at each temperature. Pupae were counted daily into plastic jars and the numbers of males and females successfully emerging recorded. Adults unable to leave the water surface were considered dead. The length of the wing, from its insertion at the mesothorax to its most distal margin excluding wing scales, and dry weight were determined for the first and last 5 females and 5 males emerging from each of the four replicate pans. The triglyceride content of the weighed females was determined using the colorimetric method described by Shahid, *et al.* (1980).

Ten females per pan were released into plastic jars (vol. = 1200 cm³) where they were continuously offered a 10% sucrose solution in vials with sponge wicks. After four days, the females were killed by freezing, dried for 2 days at room temperature in a dessicator, weighted and the triglyceride content determined. The remaining males and females were pooled by date of emergence and released into 3.8 liter carton cages, in which they were continuously offered 3% sucrose in vials with sponge wicks, and, at night, a restrained laboratory mouse as a blood source. Each morning all blood engorged females were isolated individually in vials with dilute straw-infusion water as an oviposition substrate (Reisen and Siddiqui, 1978). Dying females as well as some unfed females were dissected to determine if they were inseminated, and during the short-day photoperiod, if oögenesis had progressed beyond Stage I (Clements, 1963). Females ovipositing hatching egg rafts were released into a separate cage and were offered a mouse as a blood source. Those

refeeding were re-isolated for a second oviposition. The number of eggs in each raft was counted under a microscope to determine fecundity.

The entire experiment was done in an incubator set at 18, 22, 28 and 34°C, with a 16L:8D photoperiod and at 18 and 22°C with a 9L:15D photoperiod. The lowest temperature at which restrained mice survived was 18°C. The photoperiod was established by turning the incubator fluorescent lights off and on, with a 1 to 2 hr dusk simulated at light-off by allowing the used cells of a flashlight to dim gradually from about 100 to 0 lux. *Cx. tritaeniorhynchus* had been found to mate readily at 16L:8D and 28°C in 3.8 liter cages under these illumination regimes (Reisen, *et al.*, 1979).

Statistical methods: Median developmental time (E_{50}) was calculated by regressing the cumulative proportion of adults emerging, transformed to probits, as a function of time in days from eclosion, transformed to $\log_e$. Temperatures within the 16L:8D photoperiod were compared by model I analysis of variance (ANOVA) with individual means compared by *posteriori* least significant range tests (Sokal and Rohlf, 1969). Measurements of the adults subsampled from each pan were considered to be nested within pans, and the among-pan mean square was used to test the main effects and first and second order interaction terms. The effect of shorter photoperiod was tested individually at each temperature using Student's t-tests or ANOVA. Observed sex ratios were compared using 2×2 contingency Chi Square adjusted by Yates' correction factor (Sokal and Rohlf, 1969). Non-replicated observations were tested over temperature using a Model I regression analysis with the regression coefficients tested for significant departure from 0 by ANOVA (Sokal and Rohlf, 1969).

RESULTS

Effects of temperature at 16L:8D Survival from eclosion to pupation did not significantly vary among temperatures, but pupal to adult survival was significantly lower at 18°C when many of the adults, especially the males, did not successfully shed the pupal integument and/or leave the water surface (Table 1). Overall survival from eclosion to emergence was highest at 28°C. The sex ratio at emergence (males/total) was lowest at 18°C and significantly departed from 1:1 at 18 and 28°C ($p < 0.05$).

Median developmental time (E_{50}) varied inversely with water temperature (Table 1). Males developed significantly faster than females, and this disparity varied disproportionately over temperature, since the sex by temperature interaction term in the ANOVA was significant ($p < 0.01$). The relation of temperature (t) to the velocity of immature development ($V = 1/E_{50}$) was expressed by the law of heat summation, $V = (t - t_0)/K$, where t_0 is the developmental point and $k = a$ a thermal summation constant (Mogi, 1978). V varied linearly as a function of temperature for both males and females, with $t_0 = 8.3$ and 9.2°C for males and females, respectively (Fig. 1). The thermal summation constant of 158.7 for females and 164.2 for males implied the number of degree days above t_0 required for the completion of immature development.

Table 1 The effects of temperature and photoperiod on selected immature attributes.

Attribute	Photoperiod: Temperature (°C):	16L: 8D ¹					9L: 15D ²	
		18	22	28	34	group $\bar{x}$	18	22
Survivorship ³								
L to P		.813 ^a	.767 ^a	.866 ^a	.647 ^a		.598*	.899 ^{ns}
P to A		.600 ^b	.831 ^a	.913 ^a	.806 ^a		.629 ^{ns}	.886 ^{ns}
L to A		.488 ^b	.646	.791 ^a	.521 ^b		.380 ^{ns}	.796 ^{ns}
Sex ratio (♂ /total)		.356 ^b	.520	.448 ^a	.486 ^a		.442*	.493 ^{ns}
E ₅₀ (days) ⁴ ♀ ♀		17.67	13.08	7.59	6.85	11.30 ^a	18.62*	13.82*
	♂ ♂	17.51	11.97	7.55	6.85	10.97 ^b	18.03 ^{ns}	13.25*
	group $\bar{x}$	17.59 ^a	12.53 ^b	7.57 ^c	6.85 ^d			

¹ Means compared within 16L: 8D; means in each row or group means followed by the same least significant range test ($P > 0.05$).

² Means compared between photoperiods at the same temperature, ns: $P > 0.05$; *: $P < 0.05$; **: $P < 0.01$.

³ L=Larval stages I to IV; P=pupae; A=adult.

⁴ E₅₀=median developmental time to adult emergence in days.

Figure 1 The velocity of immature development (velocity, $v = E_{50}^{-1}$, where E_{50} is median emergence in days) for males (squares) and females (circles) plotted as a function of water temperature, T , in $^{\circ}\text{C}$. r^2 = the coefficient of determination indicating the goodness of fit.

Figure 2 The percentage of emerging females imbibing a blood meal (B) and mating (I) plotted as a function of holding temperature, t , in $^{\circ}\text{C}$ for long day (16L: 8D=filled squares and circles) and short day (9L: 15D=open squares and circles) photoperiods. Regression functions calculated from long day photoperiod data; r^2 =coefficient of determination indicating the goodness of fit.

Table 2. The effects of temperature and photoperiod on selected adult attributes.

Attribute	Photoperiod:		16L: 8D ¹				9L: 15D ²			
	Temperature (°C):		18	22	28	34	group $\bar{x}$	18	22	
♀ ♀ inseminated (%)			2.08	20.0	34.41	41.73		0.0 ^{ns}	5.40**	
♀ ♀ blood fed (%)			23.81	40.47	58.80	79.68		6.58**	62.57**	
Fecundity (eggs/♀) ³ : GC1			62.3(14)	101.6(9)	125.3(57)	124.0(84)	117.94(164) ^a	65.0(3) ^{ns}	112.8(9) ^{ns}	
	GC2		0(0)	45.0(2)	125.9(28)	111.7(53)	115.29(83) ^a	0(0)	98.3(4) ^{ns}	
Length gc (days) ³ : GC1	group $\bar{x}$		62.3(14) ^b	91.3(11) ^b	125.5(85) ^a	119.3(137) ^a		65.0(3)	108.3(13)	
	GC2		17.5(18) ^a	14.6(22) ^b	7.6(115) ^c	6.8(153) ^d		16.8(3) ^{ns}	12.2(45) [*]	
Wing length (mm) T1 ⁴ : ♀ ♀			NE	4.3(3) ^a	3.0(29) ^a	2.9(101) ^a		NE	3.3(4) [*]	
	♂ ♂		3.25	3.13	2.80	2.74	2.85 ^a	3.49	3.26	
T2 ⁴ : ♀ ♀			2.95	2.85	2.62	2.50		3.22	3.03	
	♂ ♂		3.22	3.20	2.81	2.62	2.82 ^a	3.39	3.32	
group $\bar{x}$			2.90	2.90	2.47	2.46		3.16	2.91	
			3.08 ^a	3.02 ^a	2.67 ^b	2.58 ^b		3.32**	3.13**	
Dry weight (mg) T1 ⁴ : ♀ ♀			.383	.435	.349	.410	.351 ^a	.464	.360	
	♂ ♂		.321	.352	.286	.275		.407	.319	
T2 ⁴ : ♀ ♀			.391	.365	.363	.316	.325 ^b	.433	.408	
	♂ ♂		.320	.322	.266	.259		.405	.329	
group $\bar{x}$			.353 ^a	.368 ^a	.316 ^a	.315 ^b		.427**	.354 ^{ns}	
			.522 ^c	.582 ^{bc}	.664 ^b	.782 ^a		.529 ^{ns}	.590 ^{ns}	
Dry weight after sucrose gain ⁵ (%)			135.3 ^b	145.8 ^b	188.3 ^a	216.0 ^a		118.0 ^{ns}	153.7 ^{ns}	
Triglyceride (mg/♀) T1 ⁴			.131	.062	.086	.072	.088 ^a	.070	.040	
	T2 ⁴		.154	.029	.029	.029	.060 ^b	.075	.072	
$\bar{x}$			.142 ^a	.046 ^b	.057 ^b	.050 ^b		.072**	.056 ^{ns}	
			.206 ^b	.133 ^c	.218 ^b	.251 ^a		.107**	.169**	
Triglyceride after sucrose gain ⁵ (%)			146.2 ^c	306.4 ^b	398.5 ^{ab}	504.2 ^a		143.8 ^{ns}	323.9 ^{ns}	

Footnotes 1-2 see Table 1.

³ GC1 and GC2 refer to the first and second gonotrophic cycles, respectively; (No. hatching egg rafts and ovipositing females in parentheses).⁴ T1 and T2 refer to the first and last five adults of each sex emerging, respectively.⁵ gain (%) = $(T_4 - T_0)/T_0 \times 100$, where T₀ is the estimate at emergence and T₄ is the estimate after feeding for 4 days on 10% sucrose solution.

The proportion of females imbibing a blood meal and mating (i.e., the number of dissected females that were inseminated or that oviposited hatching egg rafts) decreased as a linear function of decreasing temperature (Table 2 and Fig. 2). The extrapolated temperatures at which no blood feeding or mating would occur were 10.7 and 15.3°C, respectively.

There was no significant difference ($p > 0.05$) between female fecundity (egg/female) in the first and second gonotrophic cycles (Table 2). Fecundity at 18 and 22°C was significantly lower than fecundity at 28 and 34°C. The mean duration from emergence to oviposition (GC 1) significantly decreased with increasing temperature; however, the length of the second gonotrophic cycle, the night of re-feeding to oviposition 16 (2), did not differ significantly among temperatures. At 18°C, no female laid more than 1 egg raft, and none of the rafts hatched or contained embryonated eggs.

Mosquitoes reared at 18 and 22°C had longer wings than those reared at 28 and 34°C (Table 2). Females had longer wings than males and this difference varied disproportionately over temperature, since the sex-by-temperature interaction term in the ANOVA was significant ($p < 0.05$). Adults emerging first did not have significantly longer wings than those emerging last ($p > 0.05$). Similarly, the dry weight of adults reared at 18 and 22°C was heavier at emergence than the dry weight of adults reared at 28 and 34°C (Table 2). Females were heavier than males, and the difference remained proportionate, since the sex-by-temperature interaction term was not significant ($p > 0.05$). The mean weight of adults emerging first was significantly greater than adults emerging last, suggesting some form of larval intra-specific competition for food as described previously by Siddiqui, et al. (1976). The significantly greater weight of females emerging first was attributed to fat reserves at emergence, since the triglyceride content was also significantly greater (Table 2). Adults reared at 18°C had significantly more triglycerides at emergence than did those reared at 22, 28 and 34°C.

The dry weight of females offered 10% sucrose solution for four days increased markedly from estimates made at emergence and the percent gain ranged from 135.3 to 216.0% (Table 2). The mg of dry weight (dw) and the percent gain was significantly higher at 28 and 34°C than at 18 and 22°C and increased as a linear function of temperature (t), ($dw\% = 35.75 + 5.32 t$, $r^2 = 0.982$). Weight increases were attributed to triglyceride synthesis from the imbibed sucrose, since the greatest percent triglyceride increases were also realized by females held at 34 and 28°C. The rate of triglyceride synthesis (ts) was temperature dependent, i.e., the percent of triglyceride gain increased as a linear function of temperature (t): $\%ts = -201.19 + 21.18t$, $r^2 = 0.955$.

Effects of photoperiod at 18 and 22°C: Under a "short day" illumination, there was no difference in survival from larval eclosion to adult emergence; however, the median developmental time was generally slower (Table 1). The sex ratio of the immatures reared at 18°C increased significantly, but the proportion of males remained lower than the expected 1:1 ratio when tested by Chi square ($p < 0.05$).

None of the females held at 18° and significantly fewer females held at 22° mated under the shorter photoperiod (Table 2). In addition, significantly less

and more females at 18 and 22°C, respectively, imbibed blood meals. Females taking blood meals matured eggs at all photoperiods and temperatures studied, although under 9L: 15D, 1♀/16♀♀ dissected (3.2%) at 22° exhibited gonotrophic disassociation, i.e., took a blood meal, but after death 2 or more days later, ovarian development had not progressed beyond Stage Ib.

Fecundity at 9L: 14D did not differ significantly at either 18 or 22°C (Table 2). The duration of the first gonotrophic cycle at 18° did not differ significantly, although at 22°C the duration of both the first and second gonotrophic cycles were significantly shorter.

At 9L: 14D the wing lengths of adults reared at 18° and 22°C and the dry weight at 18°C, but not 22°C, were significantly longer and heavier than adults reared at 16L: 8D, respectively (Table 2). Females were again larger and heavier than males. At 18°, but not 22°C, the wing lengths of the adults emerging first were significantly longer; no significant differences in the dry weights of the adults emerging first and last was found. At 22°C, there was significant among-rearing pan variability ($P < 0.05$) in both the wing length and dry weight ANOVAs which contributed to the inability to detect significant differences in the time of emergence effects. The triglyceride content of the emerging females was significantly less at the 9L: 15D photoperiod and 18°C, while no significant difference was detected at 22°C. This was unexpected, since at 18°C the dry weights were significantly greater at emergence and usually the triglyceride content was significantly correlated with dry weight (Shahid, *et al.* 1980).

The dry weight of females after being offered 10% sucrose for 4 days increased markedly from the values estimated at emergence, but the weight attained and the percent weight gain did not vary between photoperiods (Table 2). Unexpectedly, the mg of triglyceride accrued at 9L: 15D was significantly less and greater than the mg of triglyceride accrued at 16L: 8D and 18 and 22°C, respectively; however, there were no significant differences in the percentage of triglyceride gained. Thus, although the actual values of triglyceride at emergence and after 4 days of being offered 10% sucrose were significantly lower at 18° under the short day photoperiod, the relative quantity of triglycerides synthesized was comparable. Discrepancies in the absolute values were attributed to variability among the groups of mosquitoes measured.

DISCUSSION

As defined by Clements (1963), "Quiescence is a state of inactivity induced by an unfavorable environment and it ceases on amelioration of the environment. Diapause is terminated only after reactivation, i.e., exposure to a factor such as cold for a period of time." Faculative diapause in the adults of multivoltine species is characterized by the accrual of fat reserves usually in response to an exogenous cue such as photoperiod. The influence of photoperiod in some mosquitoes is mediated by temperature which reduces the rate of development, so that a critical number of short-day photoperiods are experienced and diapause is induced (e.g. Beach, 1978).

In Punjab, differences between the parity rate of biting and resting *Cx. tritaen-*

niorhynchus females during autumn suggested that the population bifurcates into gonotrophically inactive overwintering and active non-overwintering segments (Baker, *et al.* 1979; Reisen, *et al.* 1980). Parallel observations have been recorded in Japan by Fujimiya and Yajima (1976). In Punjab, the rate of insemination typically decreases in November, as indicated by an accrual of uninseminated resting females; however, by January all females were inseminated (Reisen, *et al.*, 1977), suggesting that the uninseminated females either mated or did not overwinter. Females collected during December and January are typically without blood in the gut, nulliparous and have Stage I ovaries, although blood fed and gravid females may be collected during every month of the year. These data suggest that an overwintering state is perhaps achieved, but loosely maintained due to mild winter temperature regimens.

In the present study, cooler temperatures and shorter day length elongated larval development and resulted in the emergence of larger adults with more energy reserves. These results agreed with studies in Japan by Yoshida, *et al.* (1974) who found that cooler temperatures and shorter photoperiods elongated immature development, and by Nakamura, *et al.* (1968) and Fujimiya and Yajima (1976) who found that the diapausing generation was always significantly larger in size than females that remained gonotrophically active during autumn.

The mating and blood feeding rates were slower and the time from emergence to initial oviposition was greatly elongated at cooler temperatures, which, through time, could result in an accrual of uninseminated, unfed nullipars under field conditions. At 18° the shorter photoperiod decreased the proportion of females feeding, while at 22° the proportion feeding actually increased. These observations contrasted with the findings of Eldridge (1962) who reported that a Japanese strain of *Cx. tritaeniorhynchus* was reluctant to feed on a shaved chick at 28° and 8L: 16D. In addition, in the present study, little gonotrophic dissociation was observed at 9L: 15D and 22°C, whereas this phenomenon was commonly reported under similar experimental conditions with strains of *Cx. tritaeniorhynchus* from Japan, although rarely observed under field conditions (e.g. Harada, *et al.*, 1968, Takahashi, 1970, and Oda and Wada, 1973). The rate of triglyceride synthesis from sucrose did not increase under the shorter photoperiod, but rather decreased with temperature, as was expected from similar experiments with other species of mosquitoes (Van Handel, 1966a and b).

Survivorship and longevity did not appear to be greatly enhanced, and adults at all temperatures and photoperiods were dead 6 weeks after emergence. In contrast, Omori *et al.* (1965a), using semi-natural conditions in Japan, induced *Cx. tritaeniorhynchus* females to hibernate in October and more than 20% of females fed with sugar survived for more than 20 weeks, November through March. In Pakistan, females would have to survive at least 15 weeks to overwinter from November through late February or early March, when the first overwintering females can be collected at bovid baits. These data suggest that a true diapause state was probably not induced during the present study.

The results of the present study indicated that some of the attributes characteristic of diapausing *Cx. tritaeniorhynchus* were induced, although these attributes were

not separable from quiescence, or cold-induced physiological changes, and did not impart marked longevity. Speculative explanations included:

1. Incubator conditions were not conducive for diapause; i.e., metabolic processes were not slowed sufficiently.
2. The photoperiod regimens were too extreme and "not recognized" as the diapause induction cue.
3. The exposure of the immature stages to a critical number of "short day" photoperiods does not induce diapause.

In Japan, Omori, *et al.* (1965a) were able to induce diapause experimentally and keep females alive throughout the winter. They found that survival was enhanced in hibernacula that were continuously dark, had little air movement and were thermally stable at about 11°C. Conditions in the present study retained a diel photoperiod, were warmer (min. temp. = 18°C), but had reduced air movement in paper carton cages with a wet cotton pad on the gauze top to increase humidity. However, the habitats of diapausing females appear to differ considerably. In Japan, *Cx. tritaeniorhynchus* females have been collected from brush piles and caves (Bullock, 1959), but crevices and rodent burrows in the stone walls supporting permanent terraced rice fields seemed to be the preferred microhabitat (e.g. Omori, *et al.* 1965a, Wada, *et al.* 1973). In Punjab overwintering females can be recovered throughout the winter in closely spaced agricultural crops such as clover (*Trifolium* spp.) and in dense clumps of elephant grass (*Imperata* spp.). Hibernacula in the Lahore area of Punjab are thus more subject to diel temperature changes, which ranged in 1978, for example, from a minimum of 2°C in January to a maximum of 25°C in February, and are exposed to diel illumination changes. Perhaps, the constant incubator temperatures of 18°C may have been too warm.

Natural photoperiods in Lahore area range from 14:17L:9:43D on 21 June to 10:09L:13:51D on 21 December, with 11:00L:13:00D occurring on 1 November. Photoperiods in the incubator were markedly longer and shorter than the natural condition. Some "long day" insects such as *Acronycta* enter diapause only after exposure to critical photoperiods; days which are either too long or too short fail to induce diapause (Lees, 1955). Possibly, the 9L:15D photoperiod presented in the present study was not recognized as diapause induction stimulus by *Cx. tritaeniorhynchus*.

Bifurcation of overwintering and non-overwintering populations may begin as early as October in Punjab. At this time, afternoon water temperatures at breeding sites were 25°C, dropping to 15 or 16°C by early morning; however, developmental time in nature remained at 6 to 7 days (Reisen and Siddiqui, 1979). In the present study at 9L:15D and 22°C, E_{50} exceeded 13 days, and thus if the immature stages were receptive to the induction cue, they should have experienced a sufficient number of "short-day" photoperiods to induce diapause. Our results suggest that the exposure of the immature stages to a given number of short-day photoperiods was not the triggering stimulus responsible for diapause induction. In agreement, Omori, *et al.* (1965b) used adults reared from their laboratory colony (no details given) for their diapause experiments, and Eldridge (1962) found that

blood feeding activity of females reared at 14L:11D decreased after exposure to 2 to 4 "short-day" (8L:16D) photoperiods.

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