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Effects of environmental factors on the regulation of egg diapause in the walking-stick insect, *Ramulus irregulariterdentatus* (Phasmatodea: Phasmatidae)

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Abstract. Environmental factors that regulate egg diapause in the walking-stick insect, *Ramulus irregulariterdentatus*, were studied. Insects collected in fields at Okayama, Japan soon after hatching in spring were reared under constant laboratory conditions. After adult emergence, their eggs were placed outdoors or incubated under constant laboratory conditions. Most eggs kept at 15 or 20°C hatched more than 100 days after oviposition. At 25°C, however, many eggs remained in diapause throughout the experimental period although a small number hatched slightly earlier than those kept at the lower temperature. The maternal photoperiod affected the egg period at 20 and 25°C; eggs from females reared under short day conditions hatched significantly earlier. A low temperature of 10°C shortens diapause development, whereas 5°C did not have a clear effect on time to hatching. When eggs of females reared under long day conditions were placed outdoors from June to early July, most hatched the next spring. On the other hand, eggs of females reared under short day conditions hatched before winter if they were laid before mid-July. The short day eggs laid in August and September hatched successfully the following April, and there was a positive correlation between the date of oviposition and time of hatching. It is concluded that low temperatures in mid-winter do not terminate diapause development. The results revealed that both maternal regulation of diapause intensity and the rate of diapause development play an important role in maintaining a monovoltine life cycle in *Ramulus irregulariterdentatus*.

INTRODUCTION

Diapause is a dynamic process, which consists of various phases, each with different prerequisites. Diapause induction and intensification are generally followed by a period of diapause maintenance, during which the intensity of diapause gradually decreases (Tauber et al., 1986). It is known that low temperatures in winter are effective in terminating diapause (Andrewartha, 1952; Lees, 1955; Tauber et al., 1986; Danks, 1987; Hodek, 1996, 2002; Košťál, 2006). However, the environmental regulation of diapause maintenance and termination is complex, with winter diapause induction occurring much earlier than the arrival of winter in some insects. For example, in the gypsy moth, *Lymantria dispar*, the response to temperature changes over the course of diapause development (e.g., Tauber et al., 1990; Sawyer et al., 1993).

Diapause intensity is an important factor in determining the duration of diapause. It is variable not only among different species but also within a species (Masaki, 1999, 2002). Some of the insects in which diapause is induced by short daylengths can respond to photoperiod by changing the intensity of diapause. There is an inverse relationship between diapause duration and photoperiod in insects such as *Chrysopa carnea* (Tauber & Tauber, 1972) and *Riptortus clavatus* (Nakamura & Numata, 2000) if they are reared under photoperiods below the threshold. In contrast, a positive correlation between diapause intensity and photoperiod is recorded for pupae of *Manduca sexta* (Bell et al., 1975). The latter type of photoperiodic response may be adaptive as it prevents diapausing insects from resuming post diapause morphogenesis before winter when diapause is induced early (Masaki, 2002).

In many insects that diapause during the egg stage, the regulation of offspring diapause is maternal (Mousseau & Dingle, 1991). The percentage entering diapause is often affected by the photoperiod experienced by the parents (Danks, 1987). For example, egg diapause in the lawn ground cricket, *Pteronemobius taprobanensis*, is induced by short days (Masaki, 1979). Of those insects that diapause in the egg stage however, the photoperiod dependent maternal regulation of its intensity has been shown only in a small number of insects, such as the blowfly *Calliphora vicina* (Vinogradova & Zinovjeva, 1972; Vinogradova, 1974; Saunders, 1987).

Rate of diapause development, the other important physiological process determining the duration of diapause, increases toward the end of diapause (Andrewartha, 1952; Tauber et al., 1986; Danks, 1987; Hodek, 1996; Košťál, 2006). The optimum temperatures for diapause development are often lower than those for non diapause development (e.g., Danks, 1987). For example, the optimum temperature is 10°C for the termination of egg diapause in *Locust migratoria* (Ando, 1993). As a result many insects are able to resume growth or reproduction in spring. Thus, the duration of diapause is determined by the rela-

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Table 1. Effects of the photoperiod experienced by females and their embryos and temperature on the percentage of the eggs of *Ramulus irregulariterdentatus* that hatched.

Temperature	Embryonic photoperiod	Maternal photoperiod (25°C)			
		16L : 8D		12L : 12D	
		No. of eggs	Percentage of eggs that hatched	No. of eggs	Percentage of eggs that hatched
25°C	16L : 8D	264	32.2^{d}	213	31.0^{d}
	12L : 12D	263	12.9^{e}	213	4.2^{f}
20°C	16L : 8D	265	69.1^{c}	213	72.8^{bc}
	12L : 12D	265	88.3^{a}	213	85.4^{ab}
15°C	16L : 8D	266	83.5^{ab}	214	80.4^{abc}
	12L : 12D	265	80.4^{abc}	213	76.1^{bc}

Values with same letter do not differ significantly, ANOVA/Tukey's test with $P = 0.05$ after arcsine transformation (Zar, 2010).

tion between the initial intensity and the rate of diapause development.

Among walking-stick insects there are several species that diapause in the egg stage (Bedford, 1978). Although the physiological mechanism controlling diapause has not been studied intensively, there are some species that spend over a year in the egg stage (Bedford, 1978). In *Didymuria violescens*, an Australian walking-stick insect, this means the eggs overwinter twice with a first and second diapause terminated in the first and second spring, respectively (Bedford, 1970; Readshaw & Bedford, 1971). Both are terminated by exposure to low temperatures. Consequently, this insect causes extensive defoliation every second summer (Readshaw & Bedford, 1971). In *Extatosoma tiaratum*, another Australian walking-stick insect, diapause is induced both in early embryonic development and again in the pharate first-instar larva (Yoder & Denlinger, 1992).

Ramulus irregulariterdentatus is a univoltine parthenogenetic species found on Honshu, Shikoku and Kyushu islands, Japan (Okada, 1999). In Okayama, *R. irregulariterdentatus* lays eggs mainly during summer and the nymphs hatch from eggs the next April. Because *R. irregulariterdentatus* has a univoltine life cycle, its phenology is different from the above mentioned tropical species that diapause twice. In the present study, our expectation was that *R. irregulariterdentatus* may diapause twice within the egg stage and had evolved a physiological mechanism that enables it to spend a relatively long period in the egg stage.

In the present study the effects of environmental factors on the regulation of egg diapause in *R. irregulariterdentatus* were determined. It is assumed that adults lay eggs with variable intensities of diapause in response to photoperiod: females raised under long day photoperiods may lay eggs in which diapause is more intense. To test this hypothesis, we obtained eggs that were laid by females reared from first instar nymphs under either a long or short day photoperiod. We then compared the egg periods of these two groups under both laboratory and outdoor conditions.

In addition, we expected that high temperatures in summer might prolong diapause, as recorded for insects such as *Atrachya menetriesi* (Ando, 1983). To test this hypothesis, eggs were kept at 3 moderate temperatures in the laboratory, and the percentage of eggs that hatched and the period spent in the egg stage were compared among the experimental treatments. The effect of photoperiod on diapause development was also determined by rearing eggs both under long and short day photoperiods.

It was also expected that low temperatures in winter would play a role in the termination of diapause, as observed in many insects (see Andrewartha, 1952; Lees, 1955; Tauber et al., 1986; Danks, 1987; Hodek, 1996, 2002; Košťál, 2006). Therefore we compared the percentage that hatched at two low temperatures. Given that the response to temperature changes during the course of diapause development (e.g., Hodek, 1983; Tauber et al., 1990; Sawyer et al., 1993), we also tested the effect of temperature. Eggs were kept at 25°C for different periods before exposing them to low temperatures. Subsequent hatching at 25°C were compared among the experimental treatments. Furthermore, the possibility that *R. irregulariterdentatus* has more than one diapause during embryonic development was tested.

MATERIAL AND METHODS

First-instar nymphs of *R. irregulariterdentatus* were collected in Okayama City (34.7°N, 133.9°E), Japan, in April 2005. To determine whether the mothers control the intensity of egg diapause, insects were reared under a long day (16L : 8D) or short day (12L : 12D) photoperiod at 25 ± 1°C in a group of about 10 individuals in a plastic container (35 × 30 × 38 cm in height). They were supplied with fresh leaves of cherry, *Prunus yedoensis*. After adult emergence, eggs were collected every other day. Groups of fewer than 100 eggs were placed on wet filter paper in petri dishes (90 mm diam.) and used in the experiments.

In the laboratory, eggs were kept under 16L : 8D or 12L : 12D photoperiods at 25 ± 1, 20 ± 1 or 15 ± 1°C to determine the combined effects of photoperiod and temperature on diapause development. Every other day, wet filter paper was supplied as a source of water and the number of hatchings recorded until day 210 when the experiments were discontinued. Some eggs at 25°C were transferred to 10 or 5°C and a 12L : 12D photoperiod to determine the effect of chilling. Because eggs of *R. irregulariterdentatus* are often laid before midsummer, the eggs were kept at 25°C for 30 or 60 days before chilling. After exposure to a low temperature for 30, 60 or 90 days, the eggs were returned to 25°C, and the number hatching was recorded every other day. In the chilling experiments, average egg period not including the days before chilling at 25°C and the chilling period, was calculated and presented in the Results (Table 3). The eggs were kept also under outdoor conditions from mid-June to early October 2005. Water was supplied and the number that hatched recorded

TABLE 2. Effects of the photoperiod experienced by females and their embryos and incubating temperature on the duration of the egg stage in *Ramulus irregulariterdentatus*.

Temperature	Embryonic photoperiod	Maternal photoperiod (25°C)			
		16L : 8D		12L : 12D	
		No. of eggs	Average duration of the egg stage	No. of eggs	Average duration of the egg stage
25°C	16L : 8D	85	117.0 ± 2.5^{b}	66	103.2 ± 4.3^{a}
	12L : 12D	34	118.4 ± 4.1^{b}	9	102.3 ± 5.5^{a}
20°C	16L : 8D	183	125.3 ± 4.5^{b}	155	119.5 ± 4.0^{a}
	12L : 12D	234	132.8 ± 5.7^{c}	182	126.1 ± 6.9^{a}
15°C	16L : 8D	222	148.1 ± 7.3^{ab}	172	147.4 ± 5.7^{a}
	12L : 12D	213	149.3 ± 6.4^{b}	162	148.2 ± 5.8^{ab}

Values with same letter at each temperature do not differ significantly, Tukey test with $P = 0.05$ after ANOVA (Zar, 2010).

until the following spring in the same manner as in the laboratory experiments.

The mean egg periods were subjected to an analysis of variance (ANOVA). The Tukey test with $P = 0.05$ (Zar, 2010) was used for the multiple comparison among groups. Percentage data on hatching were arcsine transformed and analyzed using ANOVA/Tukey's test with $P = 0.05$.

RESULTS

Less than half of the eggs hatched at 25°C (Table 1). The percentages that hatched under a 16L: 8D photoperiod were greater than under a 12L : 12D photoperiod, regardless of the maternal photoperiod ($p < 0.05$). The average periods spent in the egg stage were about 120 and 100 days when females were reared under a 16L : 8D or 12L : 12D photoperiod, respectively (Table 2). The periods spent in the egg stage were significantly dependent on the maternal photoperiod ($p < 0.05$) but not the embryonic photoperiod ($p > 0.05$). The hatching occurred over a period of a few weeks and then no more hatched for more than 2 months in all the experiments. At 20°C 69 to 88% of the eggs hatched (Table 1). Unlike at 25°C, significantly more eggs hatched under a 12L : 12D photoperiod than a 16L : 8D photoperiod ($p < 0.05$). Egg periods were significantly longer if the maternal generation was kept under long day conditions ($p < 0.05$) (Table 2). At 15°C, most eggs hatched synchronously between 140–160 days regardless of the maternal or embryonic photoperiod. There were no significant differences in the egg periods recorded in the four treatments.

Exposure to low temperatures also affected the percentage of eggs that hatched (Table 3). Overall, low temperatures did not have a striking effect on the termination of egg diapause. The percentages that hatched were not high, especially after keeping them at 5°C. When eggs were exposed to 10°C, egg periods after return to 25°C were negatively correlated with the duration of the low-temperature treatment, which indicates that the rate of diapause development increases at low temperatures. Keeping eggs not only at 10°C but also 25°C before chilling shortened the egg period after transfer to 25°C: 60 days at 25°C before 30 days at10°C was more effective in shortening the egg period after return to 25°C than 30 days at 25°C before 60 days at 10°C. Similar results were obtained in experiments in which the eggs were kept for an additional 30 days at 10°C. After exposure to 5°C, the average egg periods were about 60 days in all six treatments regardless of the length of the low temperature period or the maternal photoperiod. They were significantly longer than those after exposure to 10°C ($p < 0.05$).

The maternal photoperiod and when the eggs were laid affected the date of hatching outdoors (Table 4). When eggs laid by long day reared females were placed outdoors, hatching occurred mostly the next spring with only two hatching before winter. The later in the year that the eggs

TABLE 3. Effects of low temperature on the termination of egg diapause in *Ramulus irregulariterdentatus*.

Low temperature	Days before chilling (25°C)	Chilling period	Photoperiod after chilling (25°C)					
			16L : 8D			12L : 12D		
			No. of eggs	Percentage of eggs hatched	Average duration of the egg stage after chilling	No. of eggs	Percentage of eggs hatched	Average duration of the egg stage after chilling
10°C	30 days	30 days	172	45.3	53.2 ± 11.2^{fg}	173	22.5	48.7 ± 9.4^{f}
		60 days	163	54.6	30.3 ± 12.5^{e}	163	58.9	27.5 ± 4.7^{de}
		90 days	176	56.8	20.7 ± 2.9^{bc}	178	60.7	21.0 ± 4.9^{c}
	60 days	30 days	161	37.9	25.7 ± 5.0^{d}	161	25.5	23.7 ± 3.6^{cd}
		60 days	178	54.5	16.5 ± 1.5^{a}	179	29.1	15.8 ± 1.8^{ab}
5°C	30 days	30 days	158	30.4	59.7 ± 9.8^{h}	160	22.5	58.1 ± 10.5^{gh}
		60 days	189	27.0	61.5 ± 4.4^{h}	191	9.4	62.2 ± 7.6^{h}
		90 days	187	19.3	59.1 ± 7.0^{h}	187	10.2	61.5 ± 7.7^{h}

Values with same letter do not differ significantly, Tukey test with $P = 0.05$ after ANOVA (Zar, 2010). Eggs were kept under 12L : 12D photoperiod during chilling.

TABLE 4. Effects of oviposition date and maternal photoperiod on incidence and time of hatching of the eggs of *Ramulus irregulariterdentatus* kept outdoors.

Maternal photoperiod	Date of oviposition	No. of eggs	% autumnal hatching	% spring hatching	Median date of hatching
16L : 8D	June 20	9	11.1	33.3	Apr. 14
	June 23	30	3.3	20.0	Apr. 7
	June 26	45	0.0	42.2	Apr. 12
	June 29	55	0.0	74.5	Apr. 14
	July 2	57	0.0	78.9	Apr. 14
	July 5	59	0.0	83.1	Apr. 14
12L : 12D	June 11–14	3	0.0	0.0	–
	June 17	36	11.1	0.0	Oct. 14
	June 20	71	4.2	0.0	Oct. 17
	June 23	94	16.0	0.0	Oct. 14
	June 26	661	8.6	0.0	Oct. 28
	June 29	733	8.5	0.0	Oct. 21
	July 2	634	8.0	1.4	Oct. 19
	Aug. 2	142	0.0	60.6	Apr. 12
	Aug. 7	171	0.0	51.5	Apr. 14
	Aug. 15	142	0.0	55.6	Apr. 14
	Aug. 31	65	0.0	56.9	Apr. 14
	Sept. 6	40	0.0	27.5	Apr. 17
	Sept. 12	35	0.0	28.6	Apr. 19
	Sept. 17	5	0.0	60.0	Apr. 24
	Sept. 20	5	0.0	60.0	Apr. 28
	Sept. 26	7	0.0	57.1	Apr. 30
	Sept. 29–Oct. 3	3	0.0	0.0	–

were laid the greater the percentage that hatched the following spring. The median date of hatching was 14 April in four of the six treatments. Eggs of short day reared females were placed outdoors from 11 June to 2 July or from 2 August to 3 October. Most of the eggs placed outdoors in June and July hatched in autumn, except for 1.4% of 2 July eggs, which remained in diapause until the following spring. Nymphs only emerged from short day eggs placed outdoors in August or later the following April. The date of hatching was closely related to when the eggs were laid: 12 April for the eggs placed outdoors on 2 August and 30 April for those placed outdoors on 26 September.

DISCUSSION

Effects of maternal photoperiod on diapause intensity

In insects in which the mother can determine the intensity of diapause of her offspring it is more intense and occurs at a higher incidence if mothers are exposed to short photoperiods (see Mousseau & Dingle, 1991). In the blowfly *Calliphora vicina*, females kept under short days produce many larvae that need to be exposed to long periods of low temperature in order to complete diapause (Vinogradova, 1974). Diapause in the progeny of long day treated flies, however, is less intense and easily terminated by chilling. On the other hand, eggs laid by females of *R. irregulariterdentatus* under a 12L : 12D photoperiod hatched significantly earlier than those laid under a 16L : 8D photoperiod at 25 and 20°C (Table 2). The photoperiodic responses, however, reflect the life cycle of this species. When eggs of short day reared mothers were kept outdoors, an untimely hatching before winter was recorded in successive experiments from late June to early July (Table 4). The percentage hatching was extremely low in most of the treatments, suggesting that physiologically egg diapause was terminated before winter and post-diapause morphogenesis initiated but they were killed by cold during winter. In contrast, most of the long day eggs placed outdoors in early summer successfully hatched the next spring. This clearly shows that an intense diapause induced under long daylengths prevents hatching before winter. In addition, the merit of less intense diapause induced by short day rearing of females is also shown by experiments where eggs were placed outdoors in August and September. Diapause intensity is often closely correlated with the length of time between diapause induction and the onset of winter (Masaki, 1999, 2002). Diapause is more intense if it is induced early in autumn. The present results confirms that diapause intensity is correlated with the time of diapause induction.

Effects of environmental factors on diapause development

The percentage of eggs that hatched and duration of the egg stage in *R. irregulariterdentatus* were both highly dependent on the temperature at which they were incubated (Table 1, 2). A small number of nymphs emerged about 100 days after oviposition at 25°C. The higher the temperature at which the eggs were incubated within the range 15 to 25°C the shorter the egg stage. However, the duration of the egg stage at 15°C was approximately 150 days, which is about 1 month longer than at 25°C. The relatively small difference between temperatures may indicate that the rate of diapause development is relatively fast at low temperatures since post-diapause morphogenesis was faster at high temperatures. Furthermore, unlike at high temperatures, there was no difference in the duration of the egg stage in the two different maternal photoperiod treatments at 15°C. This indicates that a high rate of diapause development may mask the difference in the initial intensity of diapause. Diapause development increases over a certain range of temperatures and the optimal temperatures occurs within that range (Danks, 1987). In the false melon beetle, *Atrachya menetriesi*, the optimal temperatures are between 5 and 10°C for the termination of egg diapause although diapause development proceeds at low temperatures ranging between 1–16°C (Ando, 1983). On the basis of the present results, the optimal temperature for egg diapause in *R. irregulariterdentatus* is around 15°C.

Temperature also affected the percentage egg hatch in *R. irregulariterdentatus*. A relatively high temperature of 25°C prevented many eggs from hatching within 210 days (Table 1), which indicates that diapause development was very slow or arrested. However, the percentage that hatched was higher at the lower temperatures of 20 and 15°C. Since *R. irregulariterdentatus* lays its eggs in midsummer, they must survive a long period until the next spring. Thus, it is adaptive if the eggs can survive the summer without hatching. As already discussed in the case of the rate of diapause development, a higher temperature is less suitable for the termination of diapause development of the eggs of *R. irregulariterdentatus*.

Low temperatures often play a role in the termination of winter diapause (Andrewartha, 1952; Lees, 1955; Danilevsky, 1961, Tauber et al., 1986; Danks, 1987; Košťál, 2006). In the present study, the longer the period the eggs were kept at 10°C, the shorter the period to hatching after returning them to 25°C (Table 3). These results confirm the importance of low temperature also for *R. irregulariterdentatus*. The periods to hatching after exposing eggs to 5°C were significantly longer than after exposing them to 10°C (Table 3). Furthermore, more than 30 days of exposure to the low temperature did not further shorten the period to hatching at 25°C. These results might indicate that 5°C plays no role in the termination of diapause. However, total time to hatching at 25°C, calculated by adding 30 days before 5°C, was about 90 days in each of the three treatments, which is shorter than the durations recorded at a continuous 25°C (103.2–117.0 days on average, see Table 1). Therefore, 5°C is to some extent also suitable for diapause development. It is possible that a temperature of 5°C will only be experienced over a very limited period during diapause development. It is also possible that *R. irregulariterdentatus* has more than one period of diapause during its embryonic development. Some walking-stick insects undergo two periods of diapause during the egg stage (Readshaw & Bedford, 1971; Yoder & Denlinger, 1992). In *Extatosoma tiaratum*, a tropical walking-stick insect, diapause is induced both in an early embryonic stage and in the pharate first-instar larvae (Yoder & Denlinger, 1992). If *R. irregulariterdentatus* is assumed to have two periods of diapause, the absence of shortening of the egg period after a long exposure to 5°C may be explained as follows: the first diapause is terminated at 5°C, but the low temperature prevents eggs from developing to the second-diapause stage. Although phenologies of the Japanese and tropical walking stick insects differ, the present results may indicate there are two periods of diapause.

The period at 25°C before exposure to 10°C also affected diapause, which indicates that the timing of the change in temperature affects diapause development. Sixty days at 25°C before 30 days at 10°C was more effective in shortening the egg period after the return to 25°C than 30 days at 25°C before 60 days at 10°C (Table 3). Similar results were obtained in treatments with an additional 30 days at 10°C. These results may indicate that 25°C accelerates diapause development more than 10°C, although most eggs did not complete diapause development when kept at a moderate temperature (Table 1). In the course of diapause development there are different phases with different temperature requirements (Hodek, 1983). When adults of the heteropteran, *Pyrrhocoris apterus*, were exposed to 5°C soon after the induction of diapause and then transferred to 25°C, a small number of them terminate diapause and start ovipositing (Hodek, 1978). In contrast, most of the females laid eggs after the same exposure to low temperature late in diapause. In the gypsy moth, *L. dispar*, the lower threshold for development and optimal temperature for diapause development are less than –10°C and 5–15°C, respectively (Sawyer et al., 1993). As in *P. apterus* and *L. dispar*, diapause in *R. irregulariterdentatus* may change its temperature requirements for diapause termination.

The above responses to low temperatures in *R. irregulariterdentatus* can explain the results of hatching under outdoor conditions in spring. When eggs laid by adults reared under short day conditions were placed outdoors in August or later, the date of hatching in spring is closely correlated with when they were laid (Table 4). This positive correlation suggests that eggs of *R. irregulariterdentatus* do not terminate diapause during winter if they are laid late in the reproductive season. Thus, there may be little diapause development during mid-winter when the daily average temperature is below 5°C. In many insects that diapause overwinter, the diapause is physiologically terminated during winter (Tauber et al., 1986; Danks, 1987; Hodek, 1996, 2002; Košťál, 2006). Low temperatures in winter maintain the dormancy and development or reproduction resumes as soon as the temperature rises. This enables insects within a population to synchronize their life cycles. However, it is not clear how *R. irregulariterdentatus* synchronizes its life cycle after overwintering. One possibility is that adults reared under short day conditions and/or decreasing temperatures may lay eggs that have a less intense diapause than those laid under a constant 12L : 12D photoperiod. It is also possible that synchrony is not very important for *R. irregulariterdentatus*, because the season suitable for growth and reproduction in southwestern Japan is long enough for them to fully realize their reproductive potential. Furthermore, *R. irregulariterdentatus* is a parthenogenetic species and thus does not mate. So the synchronization of adult emergence may not be important in this species.

Photoperiod, the most important seasonal signal for many temperate insects, had unclear effects on the maintenance and termination of egg diapause in *R. irregulariterdentatus*. Constant photoperiods during the egg period did not affect the time of hatching, but affected the percentage that hatched at 25 and 20°C (Tables 1, 2). These results possibly indicate that eggs respond to photoperiod. However, opposite results were obtained at the two temperatures. Thus, it is possible that the difference in the percentage that hatched was caused by undetectable differences in temperature or other incubating conditions.

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