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Regulation of Oviposition in *Anopheles gambiae s.s.*: Role of Inter- and Intra-Specific Signals

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Abstract Females of *Anopheles gambiae* Giles normally oviposit in a large number of fresh, small, sunlit, and spatially spread temporary pools. Such pools are associated with lower levels of predation compared to large, longer-lasting habitats. We compared oviposition levels on preferred (water collected from natural anopheline larval habitats) and non-preferred (distilled water) aqueous substrates by gravid females that contained different densities of conspecific eggs or early and late instar larvae. The presence of conspecific larvae, but not eggs, had a positive or negative effect on the ovipositional responses of gravid *An. gambiae* females, depending on the quality (preferred or non-preferred by the mosquito) of the oviposition water and the density of larvae. Presence of larvae, at all densities, in distilled water deterred oviposition. However, in natural anopheline pool water, a low density of larvae increased oviposition, whereas a higher density inhibited oviposition. Our results suggest that two signals produced by this mosquito may be involved in regulating oviposition: a volatile pheromone emitted by conspecific larvae, which augments the effect of a volatile signal emitted by preferred habitats, and a non-olfactory cue associated with high densities of larvae that deters oviposition.

Keywords *Anopheles gambiae* Giles · Oviposition · Intra-specific signals · Larval pheromone

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Introduction

The effect of conspecific immatures on the oviposition behavior of gravid mosquitoes varies according to species. Aggregated oviposition has been well documented in culicines, such as *Culex quinquefasciatus* (Say) and *Culex tarsalis* (Coquillett) (Osgood 1971; Clements 1999); volatile pheromones associated with the egg rafts mediate this behavior (Osgood 1971; Laurence and Pickett 1985; Blackwell et al. 1993). Among aedine mosquitoes, different studies have implicated either larval-produced attractants (or stimulants) (Kalpage and Brust 1973; Bentley et al. 1976; Reisen and Siddiqui 1978; Zahiri et al. 1997; Allan and Kline 1998; Zahiri and Rau 1998) or repellents (or deterrents) (Benzon and Apperson, 1988; Chadee 1993). However, in the case of enhanced oviposition in water conditioned by larvae, it is unclear whether the attraction/stimulation is caused by larval-produced chemical signals or by bacterial contamination of the water (Trimble and Wellington 1980; Benzon and Apperson 1988). In addition, eggs in the water may also influence oviposition (e.g., by *Aedes aegypti*, but not by *Aedes albopictus*), although the nature of any signal has not been established (Allan and Kline 1998).

Similar studies have been reported for anophelines. McCrae (1984) found that ovipositing *An. gambiae* were repelled/deterred by conspecific larvae at a concentration

of 1.5 larvae ml^{-1} in larval rearing water. Recently, Munga et al. (2006) compared oviposition choices of *An. gambiae* to (1) rainwater conditioned with different numbers (none, five or 50 in 200ml) of conspecific larvae but had been removed prior to assays and (2) rainwater with different densities of larvae (none, five, 40, 70 and 100 in 200ml). Fewer eggs were laid in rainwater conditioned with larvae than in unconditioned rainwater. In the presence of different densities of larvae, more eggs were laid in rainwater that had the fewest or no larvae. Additionally, the greatest number of eggs were laid in rainwater that contained the lowest concentration of larvae. These authors proposed that *An. gambiae* females were not influenced by the presence of conspecific larvae, but by other habitat characteristics associated with food quality and quantity, such as algal and microbial populations (Munga et al. 2006). In our previous studies on *An. gambiae*, we found that more eggs were laid in water from natural anopheline habitats than in distilled water or water from natural culicine habitats (Sumba et al. 2004a), and that volatile emissions associated with microbial populations from these habitats mediate this preference (Sumba et al. 2004b). We suggested that production of intra-specific cues from eggs or immature stages of the insect may occur only in habitats favorable for the optimal development of larvae and that rearing water (McCrae 1984) and rainwater (Munga et al. 2006) do not contain the full range of chemical signals present in natural anopheline larval habitats.

The present study was undertaken with two objectives in mind: (1) to compare *An. gambiae* oviposition levels in water collected from natural anopheline larval habitats with those in distilled water by using different densities of conspecific eggs or larvae and (2) to establish whether or not intra-specific olfactory signals mediate any of the observed effects.

Methods and Materials

Mosquitoes An. gambiae s.s. Mbita strain larvae were initially collected from anopheline pools at Mbita Point, Suba District, western Kenya, and reared in a screenhouse (11.5×7.1×4.4 m) (Seyoum et al. 2002) at a density of about 500 larvae in 3 l of water obtained from a natural ground pool containing predominantly anopheline larvae. Average temperature in the screenhouse was 29±2°C during the day and 24±2°C during the night, and RH ranged from 57±4% (day) to 72±5% (night). These conditions approximated the natural conditions prevailing in the Suba district in western Kenya. Larvae were fed daily on tetramin fish food (Seyoum et al. 2002). Adult mosquitoes were kept in standard 30×30×30 cm cages in an adult insectary at 27±2°C, 65–70% RH, a photoperiod of 12:12 h (L–D), and were offered 6% glucose solution on which to feed ad libitum. Three to four-days-old females were starved for 12 h and allowed to feed on human arms for a 10-min period on two consecutive evenings at 18:00 h. Unfed mosquitoes were removed from the cage after each blood meal. Fully engorged females were left in the cages until they were gravid and used in oviposition assays on the second night after their last blood meal. Approval for feeding mosquitoes on human subjects was obtained from the Kenya National Ethical Review Board (protocol number KEMRI/RES/7/3/1).

Collection of Anopheline Habitat Water Anopheline habitat water was collected at the start of the assays from natural ground pools around Mbita Point (Minakawa et al. 1999; Sumba et al. 2004a). Presence of anopheline larvae in these pools was confirmed by randomly sampling the water five times with a 350 ml standard dipper and inspecting it for larvae. Collected water was normally turbid with an average pH of 7.4±0.1 and was sieved to remove mosquito larvae or pupae.

Oviposition Response to Conspecific Larvae Oviposition assays were carried out in 25×25×25 cm Plexi®-glass cages under ambient conditions in the screenhouse. A gravid mosquito was placed in each cage and provided with a choice of two artificial oviposition sites, each in a black plastic cup (2 cm deep, 4 cm diameter), placed diagonally at opposite corners of the cage, 30 cm apart. One cup contained test water and the other control water. The test waters were prepared by placing different numbers (0, 1, 5, 10, 20, 30, or 40) of early (first and second) or late (third and fourth) instars in 20 ml of either (1) water taken from freshly collected natural ground pool colonized by anopheline larvae (pool water), or (2) distilled water. Larvae in distilled water were left in the cage for at least 24 h before the assay was started. Control water was either pool water or distilled water without larvae. Mosquitoes were released into the cages at about 17:00 h, and the number of eggs on each cup was counted the following morning. Two or three replicates of each treatment were performed on the same night and the experiment repeated on 10 different nights.

Oviposition Response to Conspecific Eggs Varying number of eggs (0, 1, 5, 10, 20, or 30 eggs) laid the previous night were placed in 20 ml of either a fresh batch of pool water or distilled water in a black plastic cup. Each was paired with another cup with control water (distilled or pool water) without eggs in a Plexi®-glass cage, and a gravid female was introduced into the cage 24 h later, as for the larval assays. The numbers of eggs laid in the two cups were recorded the following morning. Treatments were replicated from 22–27 times.

Nature of Signal(s) Mediating Larval Effects In the 2-choice assays, the greatest increase in oviposition occurred at a concentration of about 10 (early instar) larvae in 20 ml of pool water, and the greatest reduction in oviposition occurred at a concentration of about 40 (late instar) larvae in the same volume. These densities were used in 4-choice experiments carried out in 60×60×60 cm Plexi®-glass cages to determine whether the stimulus was olfactory or otherwise (tactile, contact chemical, or visual) in nature. 'Double cup' oviposition setups (Sumba et al. 2004b), placed at the corners of the cage, were used. Each setup consisted of an outer opaque plastic cup (8 cm deep, 6 cm diameter) containing 20 ml of either pool or distilled water, with or without larvae, and a smaller inner black plastic cup (2 cm deep, 4 cm diameter) containing 20 ml of distilled water that was floating on the water in the outer cup. In a setup designed to restrict exposure of gravid females to volatile chemicals from test substrates, a 6-cm diameter cone of white folded polyester cloth was placed so that it fit the inside of the plastic cup neatly and acted as a barrier against direct tarsal or visual contact of the test water in the outer cup by mosquitoes. This setup allowed olfactory perception of any volatile signal emanating from the test water. Four sets of 4-choice assays were carried out: (1) a choice of distilled water, distilled water with 10 early instars, pool water, and pool water with 10 early instars, with no cone barriers in any set-ups; (2) same as (1) with polyester cones in all setups; (3) same as in (1) but with 40 late instars; and (4) same as (3) with cone barriers. In a given replicate, the four treatments were randomly assigned to a corner of the Plexi®-glass cage. In all experiments, gravid mosquitoes were released into the cages in groups of five at about 17:00 h and the numbers of eggs laid on each treatment (outer and inner cups in treatments without cone barrier and cone in treatments with barrier) were counted the next morning. Fresh gravid mosquitoes and treatments were used on each experimental night. The assays were replicated from 16–22 times (Table 1).

Data Analysis An oviposition index (OI) for each replicate in the dual-choice assays was calculated according to the formula $OI=N_t-N_s/N_t+N_s$ (Kramer and Mulla 1979), with N_t=number of eggs on the test substrate (distilled water or pool water with larvae or eggs) and N_s=number of eggs on the control substrate (distilled water or pool water without larvae or eggs). Thus, OI indices can range from +1 to −1, with positive values indicating that more eggs were laid on the treatment than on the control and negative values the converse. Generally, a substance with an OI of +0.3 or above is considered attractive, whereas one of −0.3 or below is considered a deterrent or repellent (Hwang 1980; Poonam et al. 2002). In the present study, the statistical significance of the OI of each treatment (with larvae or eggs in one of the cup pairs) relative to the control (no larvae or eggs in a cup) was determined by a one-sample *t* test. In the 4-choice experiments, the number of eggs laid on each oviposition site was arcsine transformed (Gomez and Gomez 1984) and the angular values were subjected to ANOVA (SAS Institute Inc. 2003). Means were compared by a Student-Newman-Keuls (SNK) test at a 5% level of significance.

Results

Oviposition Responses to Different Densities of Conspecific Larvae Figure 1 shows OIs of treatments with different densities of early (Fig. 1a,c, and e) or late (Fig. 1b,d, and f) instars, in choices between pool water vs. distilled water control (Fig. 1a,b), pool water vs. pool water control (Fig. 1c,d), and distilled water vs. distilled water control (Fig. 1e, f). Water without larvae, obtained from a natural anopheline pool habitat was significantly more attractive (OI>+0.38; $P<0.05$, $N=27$, *t*-statistics) than the distilled water control (Fig 1a,b), confirming previous results (Sumba et al. 2004a, b). Irrespective of larval age, oviposition responses of gravid mosquitoes in the presence of conspecific larvae in pool

Table 1 Mean numbers of eggs (±SE) laid by gravid *Anopheles gambiae s.s.* given choices of four aqueous substrates: (1) using low density (10) early instars with no cone barriers; (2) same as (1) but with polyester cones preventing contact with aqueous substrate cues; (3) same as (1) but with high density (40) late instars; and (4) same as (3) with polyester cones

	Low larval density		High larval density	
Substrate	(1) (N=16)	(2) (N=22)	(3) (N=19)	(4) (N=22)
Distilled water	23.1±12.9a	36.1±11.5a	72.4±15.5b	27.1±10.8a
Distilled water+larvae	22.8±13.7a	21.4±8.8a	19.3±7.6a	25.4±12.4a
Pool water	96.3±19.8b	93.2±15.1b	232.9±36.7c	79.6±20.1b
Pool water+larvae	197.9±23.9c	198.2±21.7c	37.6±15.7a	169.2±22.9c

Means in the same column sharing a common letter are not significantly different at an alpha of P=0.05 level (SNK test)

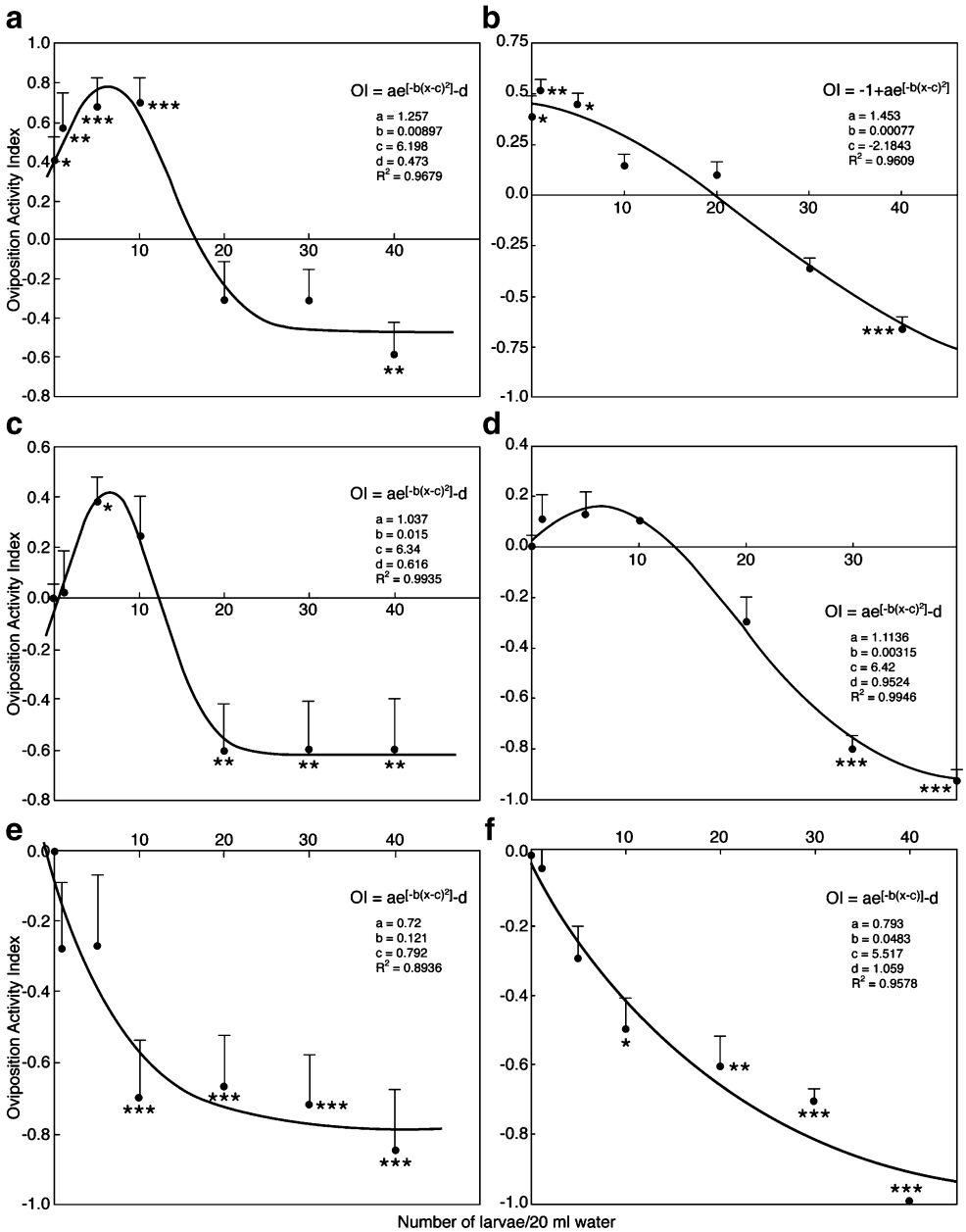

Fig. 1 Oviposition indices (OIs, mean±SE) and regression relationships showing oviposition responses of *Anopheles gambiae s.s.* to different densities of early (**a**, **c**, **e**) and late (**b**, **d**, **f**) conspecific larvae in: (1) pool water with distilled water control (**a** and **b**); (2) pool water with pool water control (**c** and **d**); and (3) distilled water with distilled water control (**e** and **f**). Starred OIs are significantly different from zero (one-sample *t* test) at **P*<0.05, ***P*<0.01 or ****P*<0.001

water were density-dependent, with test substrates with lower densities being preferred. Oviposition indices >+0.3 or <−0.3 were significantly different from zero (one sample *t*-test), indicating the test water was either stimulatory or deterrent, respectively, to females. The presence of larvae, particularly early instars (compare Fig. 1a and c with Fig. 1b and d), at relatively low density in pool water, stimulated oviposition by females (although not all increases were statistically significant). On the other hand, high larval densities, particularly late instars (compare Fig. 1b and d with Fig. 1a and c), deterred oviposition. Interestingly, the presence of larvae in distilled water deterred oviposition by gravid females at most densities (at low densities this was not significant) with late instars appearing particularly effective in this regard (Fig. 1e and f).

Oviposition Responses to Conspecific Eggs In choice assays between distilled water with eggs and a distilled water control, OIs (±SE) were (egg numbers in parentheses): 0.0±0 (0), 0.14±0.20 (1), 0.10±0.20 (5), −0.03±0.20 (10), 0.13±0.10 (20), and −0.12±0.10 (30). In assays involving a batch of pool water with eggs and pool water control, OIs were: 0.0±0 (0), 0.0±0.20 (1), 0.27±0.20 (5), 0.14±0.20 (10), 0.27±0.20 (20), and 0.06±0.10 (30). In assays with pool water with eggs and distilled water control, OIs were: 0.82±0.18 (0), 0.64±0.24 (1), 0.82±0.18 (5), 0.64±0.24 (10), 0.64±0.24 (20), and 0.82±0.18 (30). In all three sets of assays, the presence of 1-day-old eggs had no significant effect (*P*>0.05, *t*-test) on oviposition by *An. gambiae* females at any density tested. The high positive OIs (≥0.64) in the third set of assays between

natural pool and distilled water confirmed the preference for the former by *An. gambiae* females.

Roles of Olfactory and Non-olfactory Cues In all the 4-choice assays, pool water was significantly more attractive than distilled water (Table 1). The presence of larvae at low density (10 early instars in 20 ml) in pool water gave increased oviposition compared to pool water alone (Table 1 (1) and (2)), regardless of whether gravid females had contact with non-olfactory cues or not ($P<0.05$, SNK test). Distilled water showed no effect of low-density larvae (Table 1 (1) and (2)) for both situations. In assays involving the higher density of larvae (40 late instars in 20 ml), in which contact with non-olfactory cues was possible (Table 1 (3)), females laid significantly ($P<0.05$, SNK test) fewer eggs in water with, than in water without, larvae for both pool and distilled water. In contrast, in the situation in which only perception of volatile cues was possible (Table 1 (4)), the number of eggs laid in pool water with larvae was significantly ($P<0.05$, SNK test) greater than in pool water without larvae; there was no such difference observed with distilled water.

Discussion

Immatures of *An. gambiae* occur largely in fresh, sunlit, relatively small, and transient water pools with sparse vegetation (Muirhead-Thomson 1951; Gillies and De Meillon 1968; Service 1993; Minakawa et al. 1999, 2005a, b; Gimnig et al. 2001). Such pools are associated with lower levels of predation compared to large, long-lasting habitats (Service 1977; Washburn 1995; Sunahara et al. 2002). These pools are colonized rapidly within a few days of formation, suggesting that gravid females of this mosquito may actively search for and select such habitats for oviposition (Minakawa et al. 2005a). Laboratory studies have shown that volatile emissions associated with microbial activities in these habitats may partly mediate location of such habitats (Sumba et al. 2004b). Gravid females of two other anopheline species, *Anopheles albimanus* Wiedemann and *Anopheles vesttipennis* Dyar & Knab, are attracted similarly to volatile chemicals from their respective larval habitats (Rejmankova et al. 2005). The effect of the presence of *An. gambiae* larvae in the pools has been explored as an additional mechanism that influences attraction of conspecific females to a preferred oviposition site. Munga et al. (2006) compared oviposition levels of *An. gambiae* in rainwater with and without different numbers of larvae and found that, in all larval densities, the mosquito preferred to lay in clean rainwater over rainwater with larvae. This led the authors to suggest that conspecific larvae play no role in oviposition selection in this mosquito (Munga et al. 2006).

The results of our study demonstrate the role that conspecific larvae play in oviposition by *An. gambiae* and help to clarify some of the mechanisms that underlie the selection and spatial spread of larval habitats of this mosquito. First, the presence of conspecific larvae (but not eggs) influenced oviposition by gravid females. Preferences for ovipositing in water with larvae changed depending on the quality of the water and density of the larvae. In natural anopheline pool water, low densities of larvae (particularly early instars) resulted in increased oviposition, whereas higher densities resulted in decreased oviposition in a dose-dependent manner. Thus, contrary to previous work (Munga et al. 2006), our results show that, depending upon larval density, conspecific larvae of *An. gambiae* may play a dual role of augmenting the inter-specific signal emitted by preferred *An. gambiae* habitats and also of limiting the number of eggs laid in a particular habitat. Thus, conspecific larvae may fine-tune the balance between allowing exploitation of a healthy breeding site and avoiding intra-specific competition and other effects of overcrowding (Gimnig et al. 2002; Spencer et al. 2002; Kiflawi et al. 2003; Koenraadt and Takken 2003; Munga et al. 2006).

The 4-choice assays allowed us to clarify the roles played by inter- and intra-specific signals in oviposition by *An. gambiae*. At the lower larval density, with or without cone barriers, and the higher larval density with cone barriers, more eggs were laid on anopheline pool water substrates with larvae than on those without. This suggests that larvae in a favorable habitat emit a volatile intra-specific signal (pheromone) that augments the attraction to inter-specific volatiles (kairomone) associated with microbial activity in natural anopheline pools (Sumba et al. 2004b; Rejmankova et al. 2005). The presence of larvae, even at low density, in distilled water did not increase oviposition compared to distilled water alone, consistent with the observations made by Munga et al. (2006) in their study of the effects of different densities of larvae in rainwater. This suggests that either the larval pheromone is not stimulatory by itself (i.e., in the absence of the kairomone) or that production of the pheromone by larvae occurs only in *An. gambiae* preferred habitats with suitable organic matter, microbes, and algae (Merritt et al. 1992; Gimnig et al. 2001; Sumba et al. 2004b). The present study then represents the first demonstration of a larval pheromone on the oviposition behavior of *An. gambiae*. Chemical characterization of the pheromone and the kairomone associated with anopheline larval habitats will facilitate further studies on the relative roles of the two semiochemicals and possible manipulation of the oviposition behavior of this mosquito.

Our study of the effects of high larval density in assays with and without cone barriers suggests the presence of

intraspecific non-olfactory cues in addition to the olfactory signal. In the situation (no cone barriers) that allowed contact of females with the substrate, gravid *An. gambiae* females laid significantly fewer eggs in pool water with larvae than in that without larvae, suggesting an inhibitory effect. However, in the situation (with cone barriers) in which only olfactory cues could be perceived, the relative number of eggs laid in pool water with or without larvae resembled that with lower density of larvae. This suggests that gravid *An. gambiae* females are attracted to suitable pools with conspecific larvae, but that the intensity of non-olfactory, close range or contact cues, related to the density of larvae present, influences whether they oviposit or not. The nature of this cue, i.e., whether it is the physical disturbance of the water surface by the feeding larvae, contact with the larvae and/or visual effects, or a contact chemical from larval secretions, remains to be established. However, the work of Munga et al. (2006), who found that rainwater conditioned by different numbers of larvae deterred oviposition by gravid females, suggests the mediation of a chemical (non-volatile pheromone).

Finally, some differences between the effects of early and late instars on oviposition were apparent (Fig. 1). In anopheline pool water, in which gravid females were exposed to both olfactory and non-olfactory cues, comparison of OI values suggests that older instars appeared less effective in increasing oviposition at low density and more effective in deterring oviposition at high density (compare Fig. 1b,d and f with Fig. 1a,c and e). The results may indicate that ovipositing females are less inclined to lay in pools that have late instars compared with those with early instars. Follow-up field and laboratory studies will confirm whether these differences are real or not and, if so, elucidate the underlying factors.

In summary, the present study demonstrates the role that larvae of *An. gambiae* play in attracting and regulating oviposition by conspecific gravid females. Work is needed to elucidate the chemicals involved.

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