Olfactory attraction of Scaptotrigona mexicana drones to their virgin queen volatiles

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Original article

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Abstract – Drone congregations are a ubiquitous phenomenon described in several species of stingless bees and extensively studied in the honeybee, *Apis mellifera*. In meliponaries of *Scaptotrigona mexicana*, it is usual to observe drones forming such congregations close to the nests, apparently waiting for a virgin queen for mating. We hypothesize that drones of this species, similarly to those of *A. mellifera* and the stingless bee *Scaptotrigona postica*, use olfactory signals to detect queens and evaluate their reproductive status. In both field and laboratory experiments, our results showed that *S. mexicana* drones were able to differentiate between virgin and physogastric queens. It seems that this ability to discriminate depends on the amount of 2-alcohols, since even though no differences were observed in the qualitative content between virgin and physogastric queens, these compounds were found in larger quantities in the virgin queens. Attraction is due to compounds found in the queen head, mainly 2-alcohols, where 2-nonanol is the most significant for drone attraction in field and laboratory bioassays and for eliciting a high drone antennal response.

Scaptotrigona mexicana **/ drones / behavior / EAG**

1. INTRODUCTION

Bee species commonly exhibit highly elaborated mating systems (Alcock et al. 1978; Duffield et al. 1984; Wheeler and Duffield 1988) which are thought to be mediated largely by pheromones produced either by the male or the female in specific glands (Ayasse et al. 2001). Sex pheromones are also located on the surface of the cuticle (Ayasse et al. 1999). The females' mandibular glands are an important source of sex pheromones in eusocial stingless bees (Engels et al. 1990, 1993), bumble bees (van Honk et al. 1978), and carpenter bees (Gerling et al. 1989). Male bees can also be attracted by other glandular secretions. For example, in *Lasioglossum zephryum* (Halictidae), macrocyclic lactones produced by the Dufour's gland appear to function as a sex pheromone (Smith et al. 1985). Focusing on stingless bees, the only reported case is that of *Scaptotrigona postica* (Engels 1987; Engels et al. 1990), where in field tests, the attractiveness of males to an artificial scent mixture, reflecting the main compounds of the cephalic volatiles found in virgin females, was shown. In the present study, we contribute to knowledge of olfactory sex attraction in bees by studying a stingless bee species widely distributed in Mexico and of considerable ecological and economic importance, *Scaptotrigona mexicana* (Ayala 1999). In established colonies in a meliponary in the Soconusco region of Chiapas, Mexico, *S. mexicana* drone congregations have been observed close to the nests very often when a

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virgin queen is present. Although no observations have been registered on *S. mexicana* mating, Galindo-López (2008) observed that drones flew up apparently influenced by a virgin queen flight that had already been observed in a colony close to a drone congregation. Subsequently, the virgin queen was found to have mated. Therefore, our main hypothesis in this study is that *S. mexicana* drones use olfactory cues to detect virgin queens.

2. MATERIALS AND METHODS

2.1. Biological material

Virgin queens (n=10), 1-month-old physogastric queens (n=10), and drones of *S. mexicana* of unknown age were collected from different colonies at the meliponary of El Colegio de la Frontera Sur (ECOSUR) located in Tuxtla Chico, Chiapas (14°54′N and 92°11′W). Bees were collected between January and May 2007. Immediately after collection, queens were transferred to 2-mL glass vials and placed at −20°C for 10 min to prevent them from emptying their glands during dissection. Males were collected directly from the drone congregation located near the colonies and placed in plastic bottles (20×10 cm) with both ends covered with a thin plastic net and transported to the laboratory for bioassays.

2.2. Sample preparation

The head and abdomen of the queens were carefully removed from the thorax and crushed individually in 2 mL of hexane. Then, they were concentrated to 100 μL by a standard nitrogen-based procedure. Concentrates were stored at −20°C for further analysis and bioassays. Whole individuals were placed in 2 mL of hexane for 1 min and then removed. Extracts were concentrated and stored as described above.

2.3. Chemical analysis

Gas chromatographic separation was performed on a Varian Star model 3400 CX gas chromatograph (GC; Palo Alto, CA., USA). A DB-5 column (30 m× 0.25 mm ID) was temperature programmed from 50°C (held for 2 min) to 250°C at 15°C min^{-1}. The injection port temperature was held at 200°C. The GC was coupled to a Varian Saturn 4D mass spectrometer and integrated data system (Palo Alto, CA, USA). Ionization was carried out by electron impact at 70 eV, 230°C. Mass spectral identifications of at least 50% of the compounds were confirmed by comparison of retention times and mass spectra with those of synthetic standards. Other compounds were tentatively identified based on comparison with spectra from the computer library NIST 2002. The relative amount of each compound was calculated from the peak area, while the relative percentage of the components was calculated from the sum of all recorded peak areas. Quantification of the cephalic secretion was performed using tridecane (1 μg/sample) as an internal standard.

2.4. Field tests

We performed field experiments with two drone congregations in the ECOSUR meliponary. The experiments were performed between January and May 2007, using a similar protocol described by Engels et al. (1990). Virgin queen cephalic extract (VQCE), physogastric queen cephalic extract (PQCE), whole virgin queen extract (WVQE), whole physogastric queen extract (WQPE), abdominal virgin queen extract, abdominal physogastric queen extract were impregnated at a concentration of one queen equivalent in pieces of filter paper, which were exposed in random order with a fishing rod in front of a drone aggregation during 15 min. Following chemical analysis of the extracts, single compounds 2-heptanol, 2-nonanol, 2-undecanol, and a blend of synthetic (2-heptanol (23%), 2-nonanol (72%), 2-undecanol (5%)) in hexane as solvent all at a concentration of one queen equivalent were tested in the same way as described above. Controls consisted of 10 μL of hexane, and in all cases, the solvent was allowed to evaporate for 20 s before each test. The number of drones attracted was recorded. Bioassays were repeated over 5 days between 1100 and 1300 hours, local time, with 20–40-min intervals between stimuli to allow drones to return to their original position.

2.5. Olfactometer bioassays

In this experiment, drone responses to extracts that elicited positive responses in the field tests were evaluated. VQCE, PQCE, WVQE, WQPE, and single

compounds 2-heptanol, 2-nonanol, 2-undecanol, and a blend of synthetic 2-alcohols (2-heptanol (23%), 2-nonanol (72%), 2-undecanol (5%)) in hexane as solvent all at a concentration of one queen equivalent were evaluated in a Y-tube olfactometer similar in design and operation to that of Cruz-López and Morgan (1995). Activated charcoal-filtered air was pushed at a rate of 200 mL/min into each sample chamber. One chamber held the test material (2-alcohols hexane solutions or hexane glandular extracts were applied onto a 1-cm^2 piece of filter paper, Whatmann No. 2, Mildstone, England) and the other held a similar piece of filter paper, onto which hexane was applied. In all cases, the solvent was allowed to evaporate for 20 s before each test. Drones were introduced individually into the central section of the olfactometer and observed for a period of 5 min. An arm was considered chosen when a drone reached the sample chamber. Drones that failed to choose an arm in 5 min were recorded as non-responders. The assignment of odor sources to each arm was reversed after every trail to eliminate directional bias. Possible bias between the two olfactometer arms was evaluated by running blank tests (i.e., empty arms). After each set of trails, the olfactometer was rinsed with acetone and dried in an oven at 100°C for at least 30 s. All bioassays were conducted at 25±1°C and 55±5% RH. Illumination was provided by six fluorescent bulbs (General Electric, 39 W) at an intensity of 1,676 lx located at 120 cm directly over the olfactometer. Thirty replicates were carried out for each treatment.

2.6. Electroantennography

Antennal receptivity of drones to the cephalic extract and solutions of selected individual synthetic compounds (2-heptanol, 2-nonanol, and 2-undecanol) and a blend of these synthetics was determined by electroantennography (EAG). A drone's head was cut off carefully and the reference glass capillary electrode inserted into its base. The distal end of the antenna was inserted into the tip of the recording glass capillary electrode. The capillaries were filled with saline solution (Malo et al. 2004). The signals generated by the antenna were passed through a high-impedance amplifier (Syntech NL 1200, Hilversum, The Netherlands) and displayed on a monitor using Syntech software for processing EAG signals. Solutions of individual synthetic compounds (2-heptanol, 2-nonanol, and 2-undecanol) at 1 μg/μL, a blend of these synthetics each with a concentration of 1 μg/μL, and cephalic extract (1 GE) were prepared in HPLC-grade hexane. For each solution, 1 μL was applied to a filter paper (0.5×3.0 cm, Whatman no. 1), left for 20 s to allow solvent evaporation, and then inserted into a glass Pasteur pipette or sample cartridge for 40 s before testing. New cartridges were prepared for every insect tested. A stimulus controller (CS-05, Syntech) was used to generate stimuli at 1-min intervals. A current of humidified pure air flow (0.7 L/min) was constantly directed onto the antenna through a 10-mm diameter glass tube to ensure that odors were immediately removed from the antennal preparation. To present a stimulus, the pipette tip containing the test compounds or the extracts was inserted through a side hole located at the midpoint of the glass tube through which humidified pure air flowed at 0.5 L/min. The duration of the stimulus was 1 s. Control stimuli (hexane) were presented at the beginning, followed by stimuli of the chemical products and gland extracts in random order. Finally, a control stimulus was applied again. In order to analyze the EAG recordings, we used the amplitude

Table I. Field responses of *S. mexicana* drones to queen extracts, individual synthetics and blend of synthetics. $n=5$.

Treatment	Response
Hexane	A
Virgin queen cephalic extract	B
Virgin queen abdominal extract	C
Virgin queen whole extract	B
Physogastric cephalic extract	D
Physogastric abdominal extract	A
Physogastric queen whole extract	D
2-Heptanol	C
2-Nonanol	C
2-Undecanol	C
2-Alcohols synthetic blend	C

A no response, *B* numerous drones touching the lure, *C* occasional contact of drones with the lure, *D* drones move away from the congregation

value in millivolts. We used one drone antenna for each series of the chemical products and the extracts tested. Ten drones were used.

2.7. Data analysis

Data from drone responses in the "Y" tube olfactomer were analyzed using a *G* test for goodness-of-fit with William correction. Drones that did not respond were excluded from the analysis. EAG data were analyzed by a one-way analysis of variance (ANOVA; Program GLM, SAS Institute 2001). In some cases, original data were transformed by √x to stabilize variances before ANOVA. Data were analyzed by normality by a Komolgorov–Smirnov test. Post hoc comparisons were done by a Tukey test.

3. RESULTS

3.1. Field bioassays

Behavioral responses of a large number of *S. mexicana* drones include vigorous flying and strong attraction or repellence towards extracts of heads, abdomens, and whole queens of both virgin and physogastric queens, loaded on filter paper and attached to a fishing rod, when these approached the congregation area (Table I). Whole queen and head of virgin queen extracts showed a higher attraction; abdomen extracts showed a lesser attraction, while physogastric queen extract elicit variable responses (either no response or repellence).

3.2. Chemical analysis

Typical gas chromatogram of the cephalic secretion of physogastric and virgin queens of *S. mexicana* is shown in Figure 1. All secretions contain the same 2-alcohols composition with 2-nonanol as the major component. However, there are several differences: physogastric queens are characterized by the presence of hexyl hexanoate, while virgin queen secretion is distinguished by nonen-2-ol, which was tentatively identified according to the mass spectrum shown by Kozlov et al. (1996), and peak 7

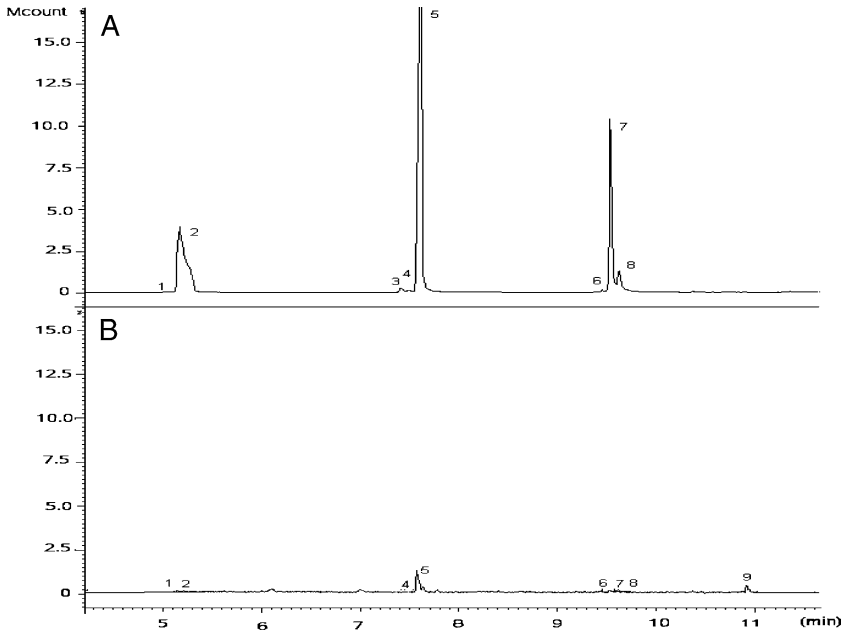

Figure 1. Chromatograms of queen cephalic extracts of *S. mexicana* queens. **a** Virgin queen, **b** physogastric queen. *1* 2-Heptanone, *2* 2-heptanol, *3* nonen-2-ol, *4* 2-nonanone, *5* 2-nonanol, *6* isomer of 7, *7* undecen-2-ol, *8* 2-undecanol, *9* hexyl hexanoate. *Peak numbers* correspond to those in Table II.

which resembles the mass spectrum of a nonen-2-ol isomer. Other compounds such as 2-heptanone, 2-nonanone, and undecen-2-ol, the latter identified according to the mass spectrum provided by Baeckström et al. (1989), were found in both virgin and physogastric queens (Table II).

3.3. Laboratory olfactometer

In laboratory bioassays, males were more attracted to virgin queen extracts than to the control (G=4.8, df=1, P=0.02). In contrast, males preferred the control than physogastric queen extract (Figure 2). When individual synthetics or blend were tested, drones showed significant attraction to 2-nonanol (G=21.7, df=1, P=0.00001) followed by 2-undecanol (G=8.8, df=1, P=0.002) and synthetic blend (G=4.8, df=1, P=0.02), while 2-heptanol did not elicit any attraction (G=2.1, df=1, P=0.1; Figure 3).

3.4. Antennal response (EAG)

Male antennal response was affected by different queen gland extracts (F=5.8, df=3,32, P=0.003). The highest response was elicited by the VQCE extract, followed by PQCE extract, both significantly different to controls (Figure 4). Drones exhibited a significantly different response (F=6.3, df=4,40, P< 0.0001) to individual synthetic compounds and synthetic blend (Figure 5), being 2-nonanol that elicited the highest antennal response.

4. DISCUSSION

Our field experiments show that drones of *S. mexicana* are able to differentiate the odor between virgin and physiogastric queens. Whole or cephalic extracts of virgin queens attracted drones, however, abdominal extracts were less attractive. Whilst whole or cephalic extracts of physogastric queens repelled drones, they were indifferent to the abdominal extract. These results imply that virgin queen's secretions contain volatile compounds that alter the sexual behavior of drones. Thus, the chemical composition of queen cephalic secretions of *S. mexicana* varied with their physiological status. Traces of nonen-2-ol were present only in virgin queens, while hexyl hexanoate was identified only in physogastric queens, the latter as one of the compounds that confer the physiological status of queens, but it could also be responsible for the non-attraction of drones. However, it was not tested because hexyl hexanoate was not available at the time of the experiment. There were no differences in the content of 2-alcohols

Table II. Mean (±SE) composition (percent) of the cephalic volatiles of *S. mexicana* queens.

Peak	Compound	Source of supply (chemical purity)	VQ	PQ
1	2-Heptanone	Aldrich (99%)	0.5 (±0.14)	1.5 (±0.22)
2	2-Heptanol	Aldrich (98%)	20.32 (±1.17)	19.15(±0.46)
3	Nonen-2-ol	–[a]	(t)	(−)
4	2-Nonanone	Aldrich (≥99%)	1.15 (±0.08)	1.5 (±0.19)
5	2-Nonanol	Aldrich (99%)	62.05 (±4.8)	60.13 (±2.9)
6	Isomer of peak 7	–[a]	1.5 (±0.22)	0.15 (±0.02)
7	Undecen-2-ol	–[a]	11.02 (±0.98)	8.7 (±0.35)
8	2-undecanol	Aldrich (97%)	4.2 (±0.24)	3.45 (±0.18)
9	Hexyl hexanoate	–[a]	(−)	(t)
Total amount (μg)/queen			10.5	0.6

VQ virgin queens, *PQ* physogastric queens

[a] Tentatively identified

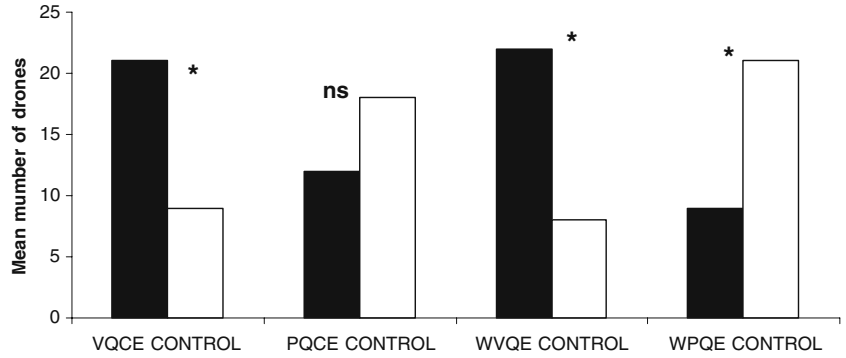

Figure 2. *S. mexicana* drones attraction to virgin queen cephalic extract (*CVQE*), physiogastric queen cephalic entract (*CPQE*), whole virgin queen extract (*WVQE*), and whole physogastric queen extracts (*WPQE*). Clean air passed over filter paper with hexane was used as control. Differences between *paired bars* indicate *ns* not significant ($P>0.05$); *$P<0.05$; ***$P<0.001$, ($n=30$).

between virgin and physogastric queens with the exception that these compounds are in larger amounts in virgin queens (i.e., there are only quantitative and not qualitative differences). Similarly, Engels et al. (1993) showed that 2-alcohols were also present in the cephalic secretions of *S. postica*, but additionally, they showed that chemical composition of the virgin queen cephalic secretion varied with age. Further field, laboratory, and electroantenography tests showed that 2-nonanol is the most significant extract involved in *S. mexicana* drone attraction. However, the abdominal compounds of virgin queens elicited a slight attraction from drones, which could be attributed to the chemical composition of the Dufour's gland secretion, which is the main abdomen volatile source of *S. mexicana* (Grajales-Conesa et al. 2007) or to compounds originated from head glands and spread over to bee's body surface. According to the latter author, the Dufour's gland secretions of virgin queens consisted mainly of 2-alcohols, ketones, hydrocarbons, and esters. It is possible that the presence of 2-nonanol resulted in the slight attraction of drones. Physogastric abdominal queen extracts showed some repellence of drones, which again could be explained by the composition of their Dufour's gland volatiles which contained esters and hexyl hexanoate as

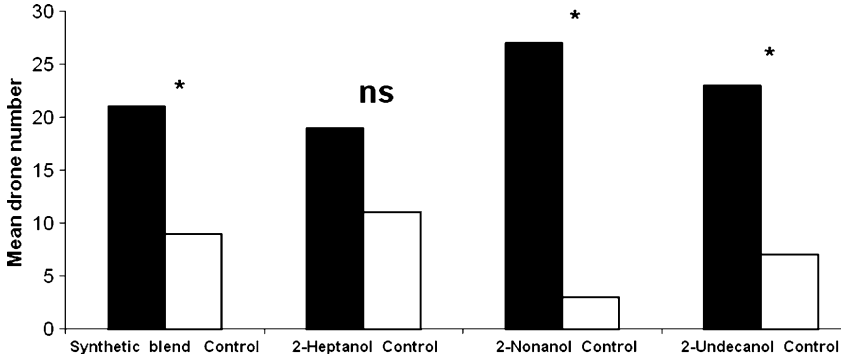

Figure 3. *S. mexicana* drones attraction to 2-heptanol, 2-nonanol, 2-undecanol, and synthetic blend (2-heptanol, 2-nonanol, 2-undecanol). Clean air passed over filter paper with hexane was used as control. Differences between *paired bars* indicate *ns* not significant ($P>0.05$); *$P<0.05$; ***$P<0.001$, ($n=30$).

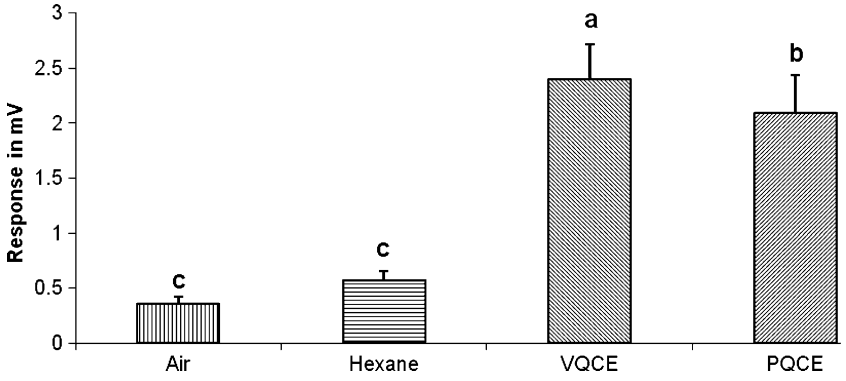

Figure 4. EAG response (millivolts) of *S. mexicana* drones to virgin queen cephalic extract (*VQCE*), physiogastric queen cephalic extract (*PQCE*). *Different lowercase letters* imply significant differences based on the ANOVA test ($P<0.05$), ($n=10$).

the major compounds (Grajales-Conesa et al. 2007). Hexyl hexanoate was also identified as a major compound of cephalic secretion of physogastric queens of *S. postica* (Engels et al. 1990). Few studies have investigated the chemical composition and biological activity of Dufour's glands of queens in stingless bees so far (Abdalla et al. 2004; Grajales-Conesa et al. 2007), but none related to sexual behavior. It is interesting to note that the pattern of volatile compounds identified in queen cephalic secretions qualitatively resembles those of *S. postica* (Engels et al. 1990). Therefore, this could be a genus-specific pattern in stingless bees.

In summary, we have shown empirically that *S. mexicana* young virgin queen volatiles affect the behavior of drones, which are attracted to virgin queen lures. This attraction is due to queen cephalic glands producing a mixture of compounds, mainly 2-alcohols, where 2-nonanol is the most significant for drone attraction, also eliciting a high drone antennal response.

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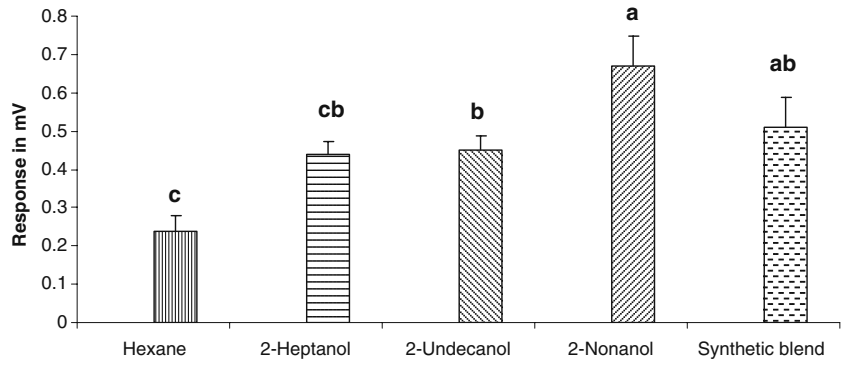

Figure 5. EAG response (millivolts) of *S. mexicana* drones to 2-heptanol, 2-nonanol, 2-undecanol, and synthetic blend (2-heptanol, 2-nonanol, 2-undecanol). *Different lowercase letters* imply significant differences based on the ANOVA test ($P<0.05$), ($n=10$).

Attraction olfactive des mâles de *Scaptotrigona mexicana* envers les substances volatiles émises par les reines vierges.

***Scaptotrigona mexicana* / mâle / comportement sexuel / signal olfactif / EAG / Meliponinae**

Olfaktorische Anlockung von Drohnen von *Scaptotrigona mexicana* durch von jungfräulichen Königinnen produzierte flüchtige Substanzen

***Scaptotrigona mexicana* / Drohnen / Verhalten / EAG /**

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