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ARTIFICIAL MIXING OF SPERMATOZOA FROM HONEYBEES AND EVIDENCE FOR SPERM COMPETITION[1,2]

JOHN R. HARBO

Agricultural Research Service, USDA, Honey Bee Breeding, Genetics & Physiology Research, Baton Rouge, Louisiana 70820, USA

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Summary

In each of 6 experiments, about 300 µl of honeybee (*Apis mellifera* L.) semen was diluted, mixed, and used to inseminate about 20 queens. The mixture for each experiment contained a portion of genetically marked sperm. Since the queens were also genetically marked, one could estimate the ratio of marked and unmarked sperm in each queen by counting her marked and unmarked progeny. The objective was to determine if each queen received equal proportions of marked sperm. In two experiments having semen mixtures with about 50% marked sperm, the 95% confidence interval for percent marked progeny was ±9·7% among the 22 queens in group 1 (semen was diluted 1:1 and stirred for 3–5 min) and ±9·0% among the 16 queens in group 6 (semen diluted 1:40 and centrifuged). The confidence intervals were similar and both were significantly greater than ±4·5% (expected 95% confidence interval when sampling a perfect mix with 500 workers from each queen) ($P<0·01$). In experiments having a very low frequency of marked spermatozoa in the mixture (1%, 2%, and 4%), at least one marked worker was found among the 500 progeny from each queen (n = 68 queens), even when insemination doses were <0·2% of the total semen mixture. Nonrandom changes in progeny frequencies were related to insemination volume, time, and sperm handling procedures. Since nonmixing would cause random changes in progeny frequencies, much of the variability may have been caused by sperm competition rather than nonmixing.

Introduction

Work by Kaftanoglu & Peng (1980) generated interest in the technique of mixing the spermatozoa of honeybees (*Apis mellifera* L.). They demonstrated that honeybee sperm could be diluted, reconcentrated (with centrifugation), and then used to successfully inseminate queens.

Mixing of honeybee sperm has practical potential for use in germplasm storage, selective breeding, and experimentation. Sperm mixing allows the storage of germplasm from hundreds of drones in a single queen. Also, the work of maintaining a closed breeding population (a system of germplasm storage) can be reduced by mixing sperm. For selective breeding, one can inseminate all test queens with sperm from a single mixture, and the queens can then be compared and evaluated against a uniform genetic background. This technique has been used in a selection program in Australia (Kühnert et al., 1989). Depending on the breeding strategy, the uniform genetic background may be narrow (drones collected from a single queen), intermediate, or very diverse (drones collected from hundreds of queens). In experimentation, mixing can simply provide uniform matings for a group of test queens, or it can be used in a specific experimental design such as used by Moritz et al. (1987).

Previous studies have evaluated the uniformity of mixed spermatozoa. Most have studied mixing within a queen after mating with several different drones (Taber, 1955; Martinho & Gonçalves, 1979; Kerr et al., 1980). Moritz (1983) also evaluated the homogeneity of spermatozoa that were artificially mixed with dilution and centrifugation. Based on chi-squared analysis, he concluded that queens inseminated with mixed spermatozoa produced genetically marked progeny at a more uniform frequency than control queens inseminated with unmixed semen.

In this study, the genetic uniformity of artificially mixed spermatozoa was evaluated. The overall objective was to mix large quantities of spermatozoa before insemination so that a population of spermatozoa received by one queen would be equal in its genetic proportions (breeding value) to that received by other queens inseminated with the same mixture. To

[1]In cooperation with the Louisiana Agricultural Experiment Station.

[2]Mention of a proprietary product or company name does not constitute an endorsement by the US Department of Agriculture.

evaluate a mixture, the frequency of marked progeny produced by each queen was measured and the percent marked progeny for each queen was determined.

Interest centered on the variation among queens: the 'between-queen variance'. However, the total variance includes not only a between-queen variance but a 'within-queen variance'. Within-queen variance is a function of sampling error. For example, samples of 100 workers would produce a greater variance than samples of 500. Thus, if the spermatozoa were perfectly mixed, the total variance would equal the expected sampling error, leaving the between-queen variance equal to zero.

The null hypothesis in four experiments was that the between-queen variance is equal to zero. This would indicate that the spermatozoa were uniformly mixed. However, the between-queen variance was greater than zero in every experiment. The data also showed that semen volume, time, and sperm handling procedures can cause a group of queens to make nonrandom changes in progeny frequency. Since nonmixing would cause random changes, it was concluded that much of the variance may be caused by sperm competition.

Materials and Methods

General procedure

To evaluate the uniformity of mixed spermatozoa, 6 experiments were conducted. The general approach was to mix genetically marked and unmarked spermatozoa, inseminate genetically marked queens, and then compare the frequencies of the genetically marked workers produced by the different queens.

Spermatozoa carrying a recessive gene for a body or an eye mutant were mixed with wild-type spermatozoa. The sperm representing the different phenotypes were kept separate during collection in the syringe. For example, in experiment 3, 290 µl of wild-type semen was collected in the syringe followed by 3 µl of cordovan (*cd*) semen.

Except for experiment 6, mixing of spermatozoa was done in a 3 ml conical centrifuge tube (Pyrex no. 8060). Saline (1·25% NaCl and 0·3% dihydrostreptomycin sulphate), equal in volume to the semen, was put into the centrifuged tube. The semen from the syringe was then injected into the saline, and the mixture (1:1, semen:saline) was stirred for 3–5 min with a thin glass rod at a rate of *c.* 2 rounds per second.

The mixture was then collected into a clean insemination syringe and used to inseminate queens. Each mixture was used to inseminate a group of sister queens that were carrying the same single gene recessive mutant that was present in the mixture. In experiments 1–3, each queen was inseminated with about 1% of the mixture.

Worker progeny were sampled from each queen to determine the sperm parentage of each worker and thus estimate the proportion of marked spermatozoa in the spermatheca of that queen. After the queens had produced brood, frames of nearly mature worker cells were placed in cages in an incubator. Each day, all the adult workers were removed from each cage and scored as mutant or nonmutant. Four hundred or more worker progeny were counted from each queen.

Experiment 1

The test began with 230 µl of semen, about equal parts chartreuse Benson (ch^B) and wild-type spermatozoa. The exact proportion does not matter since I only compared progeny frequencies among the queens receiving the sperm. A drone produces about 1 µl of semen (Harbo, 1985), so semen for this experiment required over 200 drones. Since the mutant and wild-type drones in this experiment were brothers, the populations of marked and unmarked sperm differed only in the presence or absence of the mutant mark and any genes that are closely linked to that mark. The queens receiving the mixed semen were homozygous for the eye marker, ch^B, and were each inseminated with 6 µl of the diluted mixture.

Experiment 2

The mixture consisted of 355 µl of wild-type semen and 25 µl of semen that carried the body marker cordovan (*cd*). The queens were heterozygous with *cd*.

Experiment 3

This test was designed to measure if spermatozoa that are present at a very low frequency (1%)

would be detected in the progeny of all 23 queens. There were 290 µl of wild-type and 3 µl of *cd* semen. The queens were homozygous for *cd*.

Experiment 4

This experiment tested the effect of taking very small samples of spermatozoa from the mixture. There were 55 µl of wild-type semen and 5 µl of semen that carried the eye marker, snow (*s*). The queens were homozygous for the eye marker, tan (s^t), which is allelic to *s*. This was done to distinguish between possible parthenogenetic workers and workers sired by the *s* spermatozoa. Any parthenogenetic workers (s^t/s^t) would have pinkish-white eyes on emergence; workers sired by the *s* spermatozoa (s^t/s) would have red eyes. Queens were inseminated in random sequence with either 0·2 or 6 µl (0·017 or 5·0%) of the diluted mixture. With imperfect mixing, a group of queens receiving the very small amounts of semen ought to show more queen to queen variability in their percent mutant progeny than those queens receiving the larger amounts.

Experiment 5

This experiment was designed to test whether insemination volume or time affects progeny frequency. Snow (*s*) and tan (s^t) markers were used as in experiment 4, but equal volumes (30 µl) of marked (with the *s* marker) and unmarked semen were mixed. In subset 5*a*, a single queen produced the marked and unmarked drones (semen sources), so the drones were brothers as in experiment 1; in 5*b* the drones came from 2 unrelated queens.

After diluting and mixing the semen on 23 May, queens were randomly given a very small dose of 0·2 µl of diluted semen or a more normal dose of 6 µl. Progeny scored between 23 June and 7 July were classified as early; those scored between 27 July and 8 August were classified as late. Results were analyzed with analysis of variance as a complete randomized design with time as a repeated measure.

Spermatozoa were transferred from the spermathecae of previously evaluated queens to the spermathecae of a new group of queens (supersisters to the donor queens). The purpose of this procedure was to determine if the sperm frequencies would change because of the substantial loss of spermatozoa that occurs during such a transfer (Harbo, 1985). On 19 July, spermatozoa were collected from the spermathecae of the 10 queens that received a large insemination dose in subset 5b. A similar collection was made on 4 August from 5 queens that received large inseminations in subset 5a. About 0·5 µl of spermathecal contents was collected from each queen by removing the spermatheca, puncturing it with a needle, inserting the insemination tip into the puncture hole, and then suctioning the contents. Three and 13 queens were inseminated with the spermatozoa from 5a and 5b, respectively. Progeny frequencies between donor and receptor queens were compared using Student's *t*-Test.

Experiment 6

The purpose of this experiment was to determine if high dilution, followed by mixing, and then reconcentrating the spermatozoa with centrifugation would produce a mixture that the evaluation system would identify as uniform. Snow (*s*) and wild-type spermatozoa (31 µl of each) were mixed with 2·5 ml of saline to make 1:40 dilution. The saline (2·43 g trisodium citrate·$2H_2O$, 0·21 g $NaHCO_3$, 0·41 g KCl, 0·3 g sulfanilamide, 0·3 g D-glucose, and water to make 100 ml) was the Kiev diluent described by Ruttner & Tryasko (1976), but with more KCl. The osmolarity of the saline was 0·422 as measured by a Wescor osmometer. After 2 minutes of stirring the mixture and repeated inversion of the container, the mixture was centrifuged at 8700 G for 10 minutes. The supernatant was then removed and 92 µl of reconcentrated spermatozoa was collected in an insemination syringe. A group of supersister queens, homozygous for the eye marker, tan (s^t), was inseminated with the reconcentrated spermatozoa.

Statistical Analyses

SAS software was used for all statistical analyses.

Analysis of variance (general linear models, SAS 1982) was used to calculate the within-queen variance (measurement error) and the between-queen variance (mixture variance) in experiments 1, 2, 3, and 6. A parametric procedure is valid because the binominal

sampling (presence or absence of a mutant mark) approaches a normal distribution as the number of observations becomes large (Steele & Torrie, 1980). For example, successive counts of percent marked workers from the same queen would produce a variance and a mean; that variance would be much larger with sample sizes of 50 than with samples sizes of 500. Thus the within-queen variance is a sampling error and is a function of sample size. When the sampling errors in these experiments, which were estimated with analysis of variance, were compared with the expected sampling errors derived from a table of binominal confidence limits (Steele & Torrie, 1980, sample size of 500), the two estimates were nearly equal.

The objective of the analysis is to measure the between-queen variance and to determine if it is different from zero. This between-queen variance (δ_q^2) measures the deviation from homogeneity of the sperm mixture plus unknown factors that affect the proportion of marked progeny. The following equation was used with between-queen variance (δ_q^2) : mean square queen = mean square error + $N \times \delta_q^2$. N equals the mean number of worker progeny counted per queen.

Results and Discussion

Experiment 1

This experiment showed that queens inseminated with the same mixture of spermatozoa produced significantly different proportions of marked workers. If the mix were perfect (with a sample size of 497 workers per queen), one would expect a standard deviation of 2·2% (Table 1). However, the overall standard deviation was 4·7%, and the between-queen variance of 0·00176 (SD = 4·2%) was significantly greater than zero ($F = 4{\cdot}59$, $df = 21$ and 10917, $P < 0{\cdot}0001$).

This significant between-queen variance occurred in spite of collecting all the drones from the same colony. Thus, unless the genetic basis is closely linked to the mutant mark, the between-queen variance in this experiment did not have a genetic basis. Experiments 2, 3, and 6, unlike experiment 1, did not have marked and unmarked drones from the same mother, yet their results were similar to that of experiment 1. In each experiment, the between-queen variance was significantly larger than zero (Table 1).

Experiment 3

All 23 queens inseminated with 7 µl of this semen mixture produced progeny from a marker that was present at a 1% frequency. The frequencies of marked workers ranged from 0·6 to 3·8%. Each insemination consisted of about 1% of the total volume of the mixture, and the cordovan marker was present in all 23. Moreover, it was always present in sufficient numbers to enter the spermatheca and to be represented in a sample of 500 progeny.

A significant change was noted in the frequency of marked progeny from the first measurement in September to a second measurement in June. Of the 18 queens that survived until June, their mean frequency of marked workers was 1·5% in September and 2·4% in June. Analysis with a paired *t*-test indicated that the differences were significant (mean difference = 0·85%, $t = 4{\cdot}54$, $P = 0{\cdot}0003$). Only one of the 18 queens produced a lower percentage of cordovan workers in June than in September. Ten queens survived until the following September and the frequencies of marked workers were measured for those queens for the third time. From June to September 1987, the frequency of marked workers decreased in 8 of 10 queens and the difference was marginally significant (mean difference = 0·46, $t = 2{\cdot}25$, $P = 0{\cdot}05$).

There are many possible causes for such shifting in the percent mutant progeny. However, nonmixing of spermatozoa is not a likely cause because nonmixing would produce random rather than unidirectional frequency shifts. A possible cause is sperm competition. Competition between spermatozoa from different sources may be enhanced by their genetic differences, by prior environmental conditions, or by environmental conditions at the time of egg fertilization. The latter could include egg laying rate, the number of spermatozoa in the spermatheca, or season of the year.

While working with low frequencies of a marker, measuring a low frequency of parthenogenetic workers rather than the presence of cordovan sperm may have occurred. This possibility was not totally disproved, but with identical evaluation techniques (>500 workers per queen), it was found that 5 of 8 naturally mated cordovan queens (sisters to the test

TABLE 1. Progeny counts from queens inseminated with a mixture of genetically marked and unmarked spermatozoa that had been diluted 1 : 1 with saline and stirred for 3–5 min. In Expt. 6, the spermatozoa were diluted 1 : 40 and reconcentrated by centrifugation.

Expt. No.	*No. of queens*	*Mean no. of workers counted per queen*	*Mean frequency of marked workers*	$\sqrt{\text{Variance}}$ *Total variance*[a]	$\sqrt{\text{Variance}}$ *Within-queen*[b]	*95% conf. interval*[c]	*F*	*P*[d]
1	22	497	55·5	±4·7%	±2·2%	55·5±8·7%	4·59	<0·0001
2	23	511	3·3	±1·8%	±0·8%	3·3±3·3%	5·21	<0·0001
3	23	678	1·5	±0·8%	±0·5%	1·5±1·2%	2·64	<0·0001
6	16	900	53·8	±4·2%	±1·66%	53·8±8·1%	6·34	<0·0001

[a]Within-queen variance plus between-queen variance ($\delta^2_{wq}/n + \delta^2_q$)
[b]Sampling error calculated as SE/*n* or δ^2_{wq}/n where n = no. of workers counted in column 3.
[c]This is a measure of the 'between-queen' variance assuming no sampling error (sample size = ∞, so there is no within-queen variance). The data project that 95% of the queens produce marked workers at a frequency that falls within this range.
[d]Probability that the total variance was not significantly greater than the within-queen variance (*df* = 21 and 10917, 22 and 11765, 22 and 15631, 15 and 14466, for experiments 1, 2, 3, and 6, respectively (General Linear Models: *SAS*, 1982).

queens) produced no cordovan workers. The other 3 had probably mated with one or more cordovan drones. Since this was done in June when the frequency of cordovan workers was greatest, it was concluded that parthenogenetic workers did not account for this increase and that parthenogenetic workers are not common enough to account for the presence of at least one cordovan per 500 workers from every queen. All subsequent experiments used combinations of genetic markers that could detect the presence of parthenogenetic workers, and such workers were extremely rare (see results in experiment 5).

Experiment 4

This experiment showed that even very small samples of a mixture (0·2 µl, which was <0·2% of the total volume) gave a good representation of the two sperm types, even though one type was present at a low frequency.

Sample size is important in evaluating a mixture. For example, if a sample contains 200 unmixed clumps of marked sperm, each of 20 samples (each 5% of the total) would probably contain the marker and an evaluation of the progeny would likely show the samples to be quite uniform. However, if 20 tiny samples were taken (each 0·2% of the total), one would expect greater variation in the progeny and a good chance of finding one sample that did not contain the marker.

Neither occurred. Marked spermatozoa were not only present in all the microsamples, but were represented more uniformly in the smaller samples ($\bar{x} \pm SD = 1{\cdot}00 \pm 0{\cdot}31\%$ mutant progeny) than in the larger samples (2·48 ± 1·22% mutant progeny). The variances were significantly different ($F = 15{\cdot}5$; $df = 10,10$; $P = 0{\cdot}001$).

Thus, the smaller dose of semen produced less variation and a different mean. A *t*-test for samples with unequal variances indicated a significant difference between the two means ($t = 3{\cdot}7$, $df = 10{\cdot}6$, $P = 0{\cdot}004$). Again, this is a nonrandom shift in progeny frequency, so it is probably not caused by nonmixing of spermatozoa (see discussion of experiment 3, above).

Experiment 5

The results of experiment 5b indicated that both semen volume and sampling time can affect progeny frequency (Table 2). Insemination volume was previously seen to affect progeny frequency in experiment 4, and others have noted time-related, unidirectional shifts in

TABLE 2. The effect of insemination volume and time on progeny frequency. Each experiment was independent and consisted of one mixture of spermatozoa.

Expt. No.	*Insem. vol.*	*percent marked workers mean ± SE(n)*[a] *early*	*late*[b]	*transfer to spermatheca*[c]
4[d]	small	1·00±0·12(11)	—	—
	large	2·48±0·39(11)	—	—
5a[e,f]	small	49·0±5·2(2)	39·3±5·2(2)	
	large	54·7±3·3(4)	46·2±4·0(4)	54·5±3·7(2)
5b[e]	small	44·2±1·4(5)	59·6±1·7(4)	
	large	59·2±0·9(10)	43·5±0·9(10)	76·9±4·9(5)
6[f]	centri. large	53·4±1·1(16)	55·2±1·8(9)	51·2±3·3(5)

[a]An observation consists of the percent marked workers in a sample ≥400 workers from a single queen. *n* = number of such observations (*i.e.* number of queens tested).
[b]Eggs for the 'early' progeny were laid within 3 weeks of initial egg laying; 'late' progeny were 1 month later.
[c]Early and late measurements from a queen were combined and their progeny frequencies compared with those of sister queens that received spermatozoa from their spermatheca. Some observations in this category consisted of as few as 243 bees, so this may account for the higher variance. Based on *t* tests, only experiment 5*b* showed a significant difference ($t = 1{\cdot}7$, $df = 5$, $P = 0{\cdot}14$ for experiment 5*a*; $t = 4{\cdot}9$, $df = 4{\cdot}8$, $P<0{\cdot}005$ for 5*b*; and $t = 0{\cdot}2$, $df = 4.9$, $P = 0{\cdot}8$ for 6).
[d]In experiment 4, the queens receiving large insemination doses produced significantly more marked progeny than the queens receiving the small doses ($t = 3{\cdot}7$, $df = 10{\cdot}6$, $P = 0{\cdot}004$).
[e]See Table 3 for ANOVA.
[f]No significant differences were found in experiments 5*a* and 6.

progeny frequency (Taber et al., 1979; Kerr et al., 1980).

In experiment 5b, when spermatozoa were transferred from the spermathecae of one group of queens to the spermathecae of a second group of queens (supersisters to the donor queens), there was s significant change in progeny frequency. The 10 donor queens (large insemination volume, Table 2) had produced 51·5 ± 4·8% (mean ± SD) mutant progeny (*c.* 1100 progeny were counted from each queen). Four recipient queens that produced 500 or more worker progeny produced 73·7 ± 9·9% mutants. The remaining six recipient queens each produced only 19 to 243 workers, but had a similarly high proportion of mutants (82·9 ± 11·1%).

In experiment 5*a*, insemination volume, time, and sperm transfer did not cause significant differences in progeny frequency (Table 2). Marked and unmarked drones in 5*a* had the same mother and were taken from the same colony, whereas, marked and unmarked drones in 5*b* were not related and were reared in different colonies. As in experiment 1, genetic uniformity between marked and unmarked spermatozoa did not eliminate between-queen variance in experiment 5*a*, but it may have reduced sperm competition.

Two of 29 000 workers observed in experiment 5 were produced parthenogenetically. Experiments 4 and 6 were also designed to detect parthenogenetic workers, and none of the 28 000 workers from those experiments was parthenogenetic. Both parthenogenetic workers came from queens that were producing some unfertilized eggs because they had been given very small doses of semen. This rate of 2/58000 would be very low for unmated queens, so it was suspected that the presence of spermatozoa produces a biparental female (a normal worker) from an egg that would have developed into a parthenogenetic female in the absence of sperm. Since many parthenogenetic females have been observed from virgin queens, a few from poorly mated queens, and none from adequately mated queens, it is concluded that parthenogenetic females are rarely produced by an adequately mated queen.

Experiment 6

High dilution and centrifugation produced results that were similar to those of stirred spermatozoa in experiments 1–3 (Table 1). Therefore, it is suspected that much of the variation observed in this and the other 3 experiments was caused by factors other than nonmixing of spermatozoa.

Table 2 shows the effects of time and the transfer of sperm from the spermatheca. Neither effect was statistically significant. Some of the centrifuged sperm was stored for 2 days at 20°C before insemination. The six queens inseminated with the stored sperm produced 49·4 ± 4·7% (mean ± SD) marked workers. This was not statistically different from the 53·8 ± 4·5% produced by the 16 queens inseminated with unstored sperm ($t = 2·0$, $df = 20$, $P = 0·058$), but with these results, it was not concluded that they are not different.

Conclusions

Honeybee spermatozoa do mix. In this paper, spermatozoa have been artificially mixed and then subdivided into insemination doses. These doses, even micro-quantities, retained the genetic proportions of the whole.

TABLE 3. Analysis of variance for experiments 5*a* and 5*b*.

Source	df	Mean square	F	P
5a				
tmt	1	106·3	2·27	0·2
queen (tmt)	5	46·8	0·86	0·6
time	1	220·2	4·04	0·1
tmt × time	1	1·0	0·02	0·9
error	4	54·5		
5b				
tmt	1	0·89	0·02	0·89
queen (tmt)	14	38·5	4·68	0·01
time	1	0·0	0·00	0·99
tmt × time	1	1145·4	139·19	0·0001
error	11	8·4		

However, the mixtures were statistically imperfect, and a group of queens often showed unidirectional changes in the frequency of certain marked workers. Since (1) inseminations with micro-quantities of sperm showed less between-queen variation than inseminations with volumes that were 30 times larger and (2) nonrandom changes in progeny frequency did occur (nonmixing would show random changes), it was concluded that much of the variation in progeny frequency may be a result of sperm competition rather than nonmixing.

Sperm competition is a broad category. It may include competition for leaving the spermatheca, for reaching the egg, for penetrating the egg, or for reaching the egg pronucleus when inside the egg. There could also be differential survival of sperm, such as during spermathecal transfer in experiment 5b. Moreover, all of these events may be modified by factors such as season, egg-laying rate, age, and nutrition.

Queens inseminated with mixed supermatozoa perform well enough to use in bee breeding or stock maintenance programs. Therefore, the mixing technique is ready to use, either with stirring or centrifugation. This breeding program involves establishing instrumentally inseminated queens in colonies in July or August and evaluating the resulting colonies from October to April. After initial acceptance by the colony, the 8-month survival rate for queens inseminated with mixed spermatozoa (diluted 1 : 1 and stirred) was 78% in 1986 (n = 18), 76% in 1987 (n = 33), and 83% in 1988 (n = 36). Each year had 11 or 12 naturally mated, sister queens as controls, and their survival rate was 94% (n = 35). Although the survival rate of queens inseminated with mixed sperm was not as high as that of naturally mated queens, it was similar to that of instrumentally inseminated queens inseminated with undiluted and unmixed semen (during a September to May time period, Harbo & Szabo (1984) reported 76% (n = 49) and 89% (n = 56) survival for instrumentally and naturally mated queens, respectively). Kühnert et al. (1989) also report that the survival of queens inseminated with mixed semen was similar to that of queens inseminated with unmixed semen.

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