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Mosaic male honey bees produced by queens inseminated with frozen spermatozoa

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SEX DETERMINATION in the honey bee, *Apis mellifera* L., is similar to that of *Habrobracon*[4] except that diploid male honey bees do not normally reach adulthood; they are killed as larvae by the workers[8]. A normal male honey bee develops from an unfertilized egg and is haploid; the females are diploid.

This paper describes the genetic origin of mosaic male tissue that was produced by queens that had been inseminated with semen stored in liquid nitrogen (−196°C). Progeny from these haploid mosaics are also reported.

The mosaic male honey bees described by others apparently had origins that differed from the mosaics that I observed. Rothenbuhler[6] reported mosaic males that had zygogenetic-androgenetic origins. The zygogenetic parts were diploid male tissues derived from normal union of egg and sperm pronuclei, and the androgenetic parts were haploid male tissues that developed from an accessory sperm. Tucker[7] and Woyke[9] reported mosaic males from unfertilized binucleate eggs (gynogenetic origin). Progeny were not reported from any of these mosaics.

Methods and Materials

1978 experiment

The experimental group consisted of 13 sister queens that were homozygous for the following single-gene mutants: the body marker cordovan (*c*), the eye marker chartreuse Benson (ch^B), and diminutive wing (*di*). Each queen was inseminated with either fresh or stored semen from nonmutant males. Semen, 0.85 percent NaCl, and dimethyl sulfoxide (DMSO) was mixed in a 6:3:1 ratio and stored in liquid nitrogen (−196°C) for one day before instrumental insemination of eight of the queens. Semen used to instrumentally inseminate the other five came from the same group of males but was not diluted and was not stored in liquid nitrogen.

1979 experiment

This was a repeat of the 1978 experiment with the following modifications:

1. The stock was unrelated to that used in 1978. The queens were homozygous for the body marker cordovan (*c*) and the drones were hemizygous for the eye marker snow tan (s^t).
2. In addition to the test group (25 queens), which was inseminated with semen stored at −196°C, and the control group (6 queens), which was inseminated with fresh undiluted, unstored semen, there was a second control group (15 queens), which was inseminated with semen that had been diluted as the test semen but stored for 2 days at 12°C rather than at −196°C.
3. The diluted semen contained phosphate buffer (20 percent) and filtered egg yolk (10 percent) instead of the NaCl solution used in 1978.

I tested the possibility that some of the male tissue in the mosaics were diploid, similar to the mosaic males reported by Rothenbuhler[6]. This was done by measuring the diameters of facets in the compound eye. Facets in diploid male tissue have a diameter about 13 percent greater than the facets in haploid male tissue[6,10], and diploid worker facets are about 40 percent smaller in diameter than haploid male facets[10]. Determining the sex of eye tissue is necessary only in eyes suspected of being gynandromorphic because overall shape easily identifies the sex of eyes as well as other body parts.

Comparing eye facets is complicated by the fact that they vary in size depending on which part of the eye is measured[1,5]. Therefore, facet sizes were measured at the ventral, central, and dorsal eye surfaces to establish a position baseline for facet sizes in normal bees. The size of a single facet was calculated by counting the number of facets along a 0.36 mm line. Facets of 10 workers averaged 17.9 ± 0.9 (± SD) μm dorsally, 22.9 ± 1.3 μm centrally and 24.5 ± 2.0 μm ventrally; facets of 10 haploid males averaged 33.7 ± 1.4 (± SD) μm dorsally, 35.5 ± 2.9 μm centrally, and 22.3 ± 1.3 μm ventrally.

To determine if mosaic males produce viable spermatozoa and if the testes can be mosaic, three queens were inseminated with semen from mosaic males produced in 1978. Each queen was mated to a single mosaic male. The queens were homozygous for *c, di*, and ch^B; they were identically marked sisters of the queens that produced the mosaic males. The tissue in the three mosaic males expressed the above three mutant alleles from their mother as well as nonmutant alleles that apparently came from nonmutant, previously frozen, spermatozoa. None of the males was a perfect bilateral mosaic but all had cordovan and non-cordovan pigmentation on the abdomen.

The phenotypes of the adult worker (diploid) progeny of these mosaic males were recorded. To keep the progeny from becoming mixed with other bees in the colony, the frames of brood were caged and placed in an incubator just before the adults emerged.

Results

One or more mosaic males were produced by 3 of the 8 test queens in 1978 and by 12 of the 25 test queens in 1979. No mosaics were found among the progeny of the control group. A total of about 60 mosaic males was observed. All mosaics had some parts of their body expressing the phenotype of their mother and other parts expressing the phenotype of the frozen spermatozoa. Sometimes the mosaics were perfect bilaterals wherein the pigmentation of the entire body (even the pigmentation of the center ocellus) was sharply bisected.

The test queen with the highest proportion of mosaic male progeny produced the following from eggs laid in 1 day: 65 nonmutant workers, 2 normal gynogenetic males, 4 mosaic males, and 1 gynandromorph.

The facet sizes in the compound eyes of mosaic males were similar to the facet sizes of 10 variously sized haploid males (listed in Methods). Facets of mosaic males (Table I) averaged 34.9 ± 2.0 (± SD) μm dorsally, 35.4 ± 2.5 μm centrally, and 22.7 ± 1.6 μm ventrally. Abrupt changes in the facet sizes did

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not occur where eye colors interfaced, so it was concluded that most (perhaps all) of the mosaic males were entirely haploid.

Gynandromorphs were found among the progeny of frozen spermatozoa. Three were found in 1978 and four in 1979. Gynandromorphs occurred much less frequently than mosaic males but were significant because of their presence in both test groups.

All three matings of mosaic males to queens homozygous for three markers yielded two phenotypes of worker progeny: 1) workers expressing all three markers, and 2) nonmutant workers (Table I). None had one or two mutant markers, and no mosaics were observed.

Discussion

It was concluded that these mosaic males were caused by freezing spermatozoa in liquid nitrogen. However, other factors such as the semen diluent, a genetic tendency in the egg, and inadequate numbers of spermatozoa entering the eggs cannot be ruled out as partial contributors.

It was also concluded that the mosaic males had a gynogenetic-androgenetic origin. Apparently, a sperm pronucleus entered the egg but did not combine with the egg pronucleus. Both pronuclei then developed separately and produced a mosaic male. This is not the first example of anomalous behavior of frozen honey bee spermatozoa. Spermatozoa that have been stored in liquid nitrogen can cause reduced egg hatch in the F_1 progeny[3]. Since unfertilized honey bee eggs normally hatch, this mortality was likely caused by sperms penetrating the egg, combining with the egg pronucleus, and aborting sometime before egg hatch.

Table I. The sizes of the facets of the compound eyes of mosaic males and the progeny ratios

Drone		Eye facet diameter (μm) and phenotype: dorsal	lateral	ventral	Percent mutant progeny when mated to homozygous mutant queens*,†
			1978 Group		
1	left	35.1 ch^B	34.3 ch^B	23.2 ch^B	
	right	36.0+	34.3+	24.0+	50 (n = 206)
2	left	36.9+	34.3+	22.5 ch^B	
	right	35.1+	36.0 ch^B	22.5+	15 (n = 214)
3	left	32.7 ch^B	35.1 ch^B	23.2+	
	right	33.5 ch^B	37.5 ch^B	22.5 ch^B	75 (n = 12)
4	left	30.0+	32.7+	22.5 ch^B	
	right	32.0+	33.5 ch^B	21.8+	—
5	left	36.0+	40.0+	22.5 ch^B	
	right	36.0+	36.0+	22.5+	—
6	left	35.0+	33.6+	23.5+	
	right	33.6 ch^B	33.6 ch^B	21.2 ch^B	—
			1979 Group		
7	left	37.9 s^t	31.0+	21.7+	
	right	36.8 s^t	34.5 s^t	19.5 s^t	—
8	left	34.5 s^t	33.9+	21.1 s^t	
	right	34.2 s^t	36.1 s^t	21.7 s^t	—
9	left	38.2+	39.8+	25.5+	
	right	35.1 s^t	40.6 s^t	26.4+	—
10	left	33.6 s^t	37.1 s^t	25.2 s^t	
	right	35.5+	33.9+	21.1+	—

* The queens were homozygous for ch^B, *c*, and *di*

† The progeny either expressed all 3 markers or none of the markers in their phenotype

One might expect a combination of the two anomalous events: namely, two or more sperms entering an egg (polyspermy is the general rule with honey bees), one combining with the egg pronucleus and causing it to abort and another developing androgenetically. This would produce completely androgenetic males. If the zygote survives, gynandromorphs or zygogenetic-androgenetic males would result. Indeed, gynandromorphs were found, and test colonies produced some males that appeared to be completely androgenetic, but it was not certain. The males may have had minute gynogenetic parts and thus may have been mosaic, or they may have been produced by laying workers.

The progeny of mosaic males give further insight into the situation. Apparently all three of the mosaics used for the inseminations had mosaic testes. Thus the spermatozoa from each mosaic included identical genetic replicates of the original egg pronucleus and replicates of the sperm pronucleus that developed separately within the egg. Two types of spermatozoa from one drone was heretofore unknown but would probably be produced by some gynogenetic mosaic males as well.

Note that a sperm pronucleus contributed to a mosaic male because it did not unite with an egg pronucleus; nevertheless identical genetic replicates of this pronucleus did unite with egg pronuclei in the next generation to produce nonmutant workers. Therefore, if freezing in liquid nitrogen caused some spermatozoa to be unable to unite with an egg pronucleus, this inability was not expressed by their genetic replicates in the next generation. Thus, there is no evidence for genetic damage to the spermatozoa.

Summary

Mosaic male honey bees were found as the progeny of queens that had been inseminated with spermatozoa stored in liquid nitrogen. The origins of these mosaics and the genotype of their gametes were determined by using mutant markers. The mosaics probably developed from an egg pronucleus and a sperm pronucleus that did not unite after the latter had entered the egg. Instead, both pronuclei produced haploid tissue independently. The three mosaics that were mated to queens all had mosaic testes. Therefore, these were situations in which a male honey bee produced two types of spermatozoa.

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