

# The Use Of Egg Mortality To Detect Health Harzards To Bees

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FOR THE PAST few years much of the work done by my co-workers and me has involved honeybee eggs in some way: egg positioning by queens, eggs that fail to hatch, egg-development times, etc. The work has already been published in scientific journals, and this article is a condensed version of the results.

The work with eggs began as a natural extension of a sperm-storage project. We were developing a method of storing honeybee semen in liquid nitrogen (-320°F) that was similar to the way cattle semen is stored for 20 years and more. The objective was to store the semen of many lines of bees for a long time at a very low cost. By 1977, we had developed a storage technique that preserved the spermatozoa in viable condition (1, 2). We then wanted to evaluate the bees produced by this frozen sperm.

## Why Study Egg Mortality?

Egg death and death of the unborn are very common in the animal world and have many causes, ranging from genetic disorders to nutritional and physical stresses. It is often nature's way of saying that something is wrong. Therefore, egg mortality and spontaneous abortions are often used to measure the effects of toxic chemicals, radiation, or diet on a population of animals. For example, if x-rays cause a group of laboratory mice to produce smaller litters than a similar group that receives no x-rays, then we might conclude that x-rays (at that particular dosage) are hazardous to mice. Litter size reflects the death rate of unborn mice. Similarly, egg mortality is a measure of death rate in a population of animals at their earliest stage of life, the egg stage. (Egg mortality in bees is not related to the spotty brood pattern that results from inbreeding. A poor brood pattern from inbreeding is caused by the workers removing newly hatched larvae, not by eggs that do not hatch.)

When queens inseminated with frozen spermatozoa produced many eggs that did not hatch, I knew we had a problem. Nonhatching of honeybee eggs had been reported earlier by others, and it can be caused by damaged sperm (3, 4). Yet, if we

could produce queens from the eggs that survived and if these queens produced normal eggs and brood, our problem would be limited to the first generation. Serious genetic damage would be indicated only if nonhatching persisted in the second or later generations. My objective was to measure egg mortality to find out if genetic damage was present.

## Background Information

In a project such as this, test groups are compared with untreated or "control" groups. One tries to keep the groups as similar as possible except for the item to be tested. In these experiments, the control queens were sisters to the test queens, reared in the same cell builder, and inseminated with semen from the same group of drones. The only difference was that semen for the test group was frozen and semen for the control was not.

The control group in an experiment is as important as the test group. In fact, for beekeepers the controls in my experiments are probably more valuable because they more nearly represent normal conditions.

Before describing egg mortality in bees and the effects of freezing sperm in liquid nitrogen, I will describe normal egg laying and hatching behavior.

## Normal egg laying by queens.

Beekeepers recognize the eggs laid by a good queen. They are erect at the base of a cell, usually one per cell, and uniformly placed (Fig. 1, a and b). I found that this laying pattern depended on the insemination of a queen with live sperm (5). When queens were inseminated with dead sperm or not inseminated, the egg placement was no longer uniform; the queens seemed to drop or scatter their eggs rather than lay them uniformly. These queens also did not lay as many eggs as their mated sister queens. (Queens inseminated with dead sperm behaved just like uninseminated queens, so hereafter I will refer to mated or non-mated

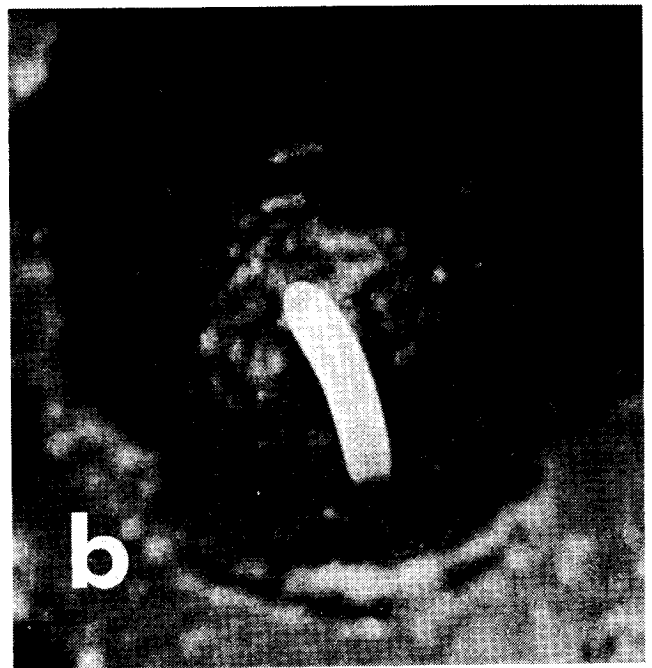
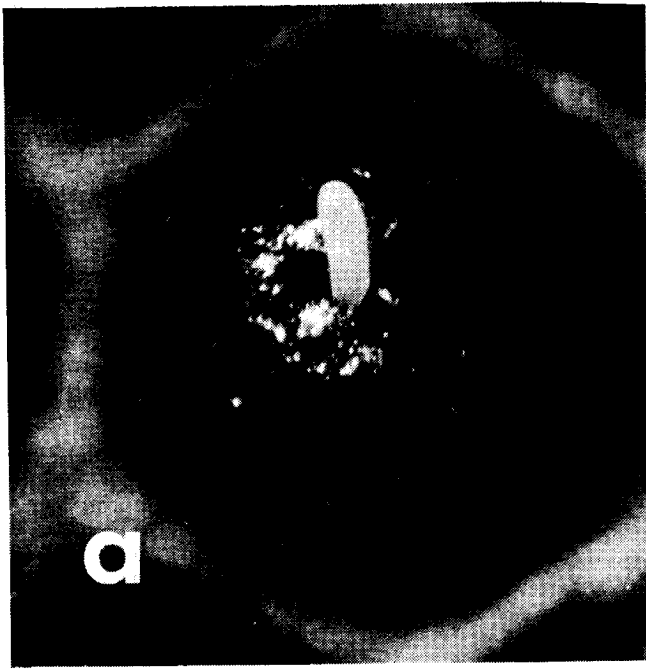
queens. The mated queens were those inseminated with live sperm; the non-mated queens were those inseminated with dead sperm or not inseminated.)

This reduced and non-uniform egg laying occurred only when non-mated queens laid an egg in worker-size cells, cells that normally receive a fertilized egg. When I counted only drone-size (cells that normally receive an unfertilized egg), the laying behavior of mated and non-mated queens did not differ.

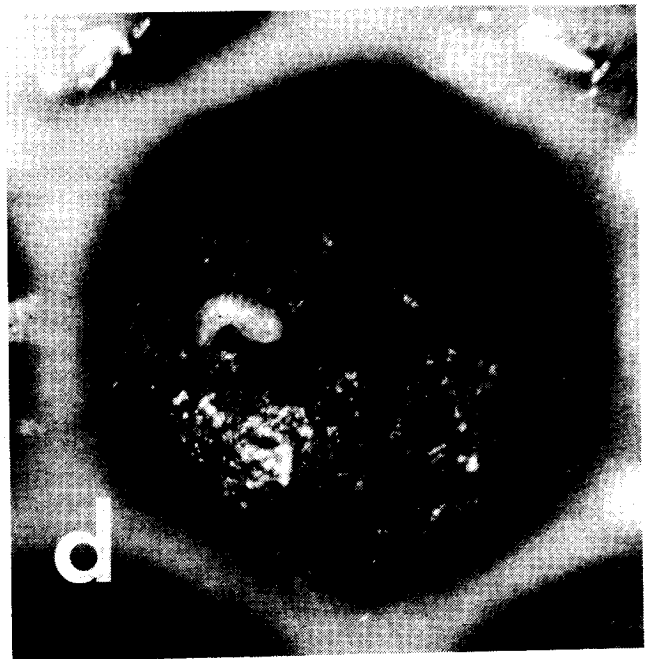
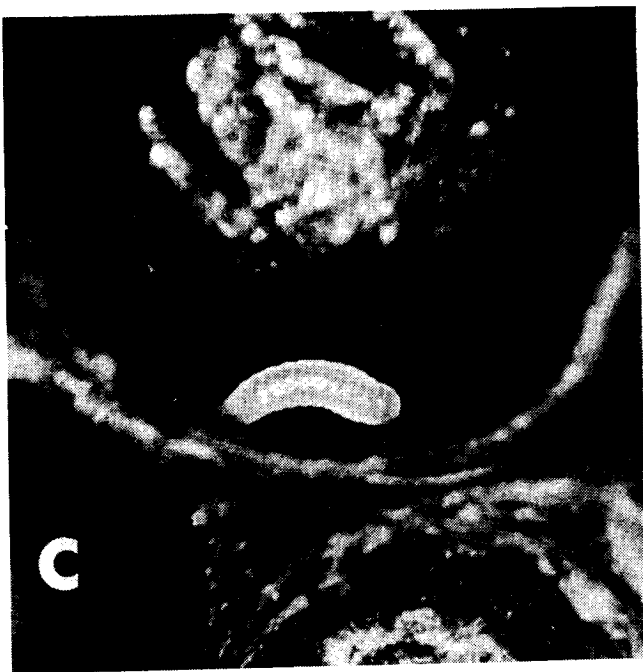
The reduced and non-uniform egg laying of non-mated queens seemed to be linked to the egg-fertilizing portion of laying behavior. Here is my interpretation: When a queen lays an egg, she needs to coordinate egg laying and subsequent body movements between the cells that receive the eggs. Nerve signals are sent that are much like those we send when probing in the dark to open a door. Nerves in our hand signal us that the key is in, the lock is turned, and the door is opening. From those signals we decide to walk in. If we miss any of those signals, progress stops. I think that when a queen lays an egg she relies on a similar series of signals. Obviously, she needs a signal to know when the egg is laid so that she can leave the cell. But, because fertilized and unfertilized eggs are placed differently when laid in worker-size cells, a queen must also have an earlier signal that tells her that the egg has been fertilized or that sperm have been released from her sperm storage organ (spermatheca). When that signal is received, the queen can move on to the next steps in the sequence, to lay the egg and then move to the next cell. When that signal is not received, the queen holds the egg, time passes, she shifts her position or leaves the cell, but eventually she must release the egg. The result, fewer eggs laid and eggs laid non-uniformly. Because queens lay unfertilized eggs in drone-size cells, a signal to fertilize the egg is not included in the sequence for laying in a drone-size cell. So, eggs in drone-size cells are positioned uniformly whether or not the queen has live sperm in her spermatheca.

## Normal egg hatching.

If kept at the proper temperature



**Figure 1. Eggs and newly hatched larvae.** Picture *a* is a top view of an egg in a cell. In *b*, the cell is cut away to show a side view of an egg. The head end is up. A side view, (*c*), shows a larva after the outer covering (chorion) of the egg has dissolved. The newly hatched larva is arching over to touch the bottom of the cell with its head (head on right). Finally, *d* shows a newly hatched, unfed larva at the base of cell (top view).



and humidity, eggs will hatch outside the bee colony without worker bees present. Contrary to some popular notions, brood food is not put on the bottom of a cell just before egg

hatch. (On rare occasions, I have seen brood food in cells with an egg, but the food was not necessarily put in the cell when the egg was near hatching age, and I have never known

such an egg to hatch).

Normal hatching of the honeybee egg was described by Dupraw in 1960 (6). Hatching begins with gentle

movements of the head end of the egg. The movement gradually becomes more intense, and then a clear water-like droplet appears on the surface. The fluid remains in place for a short time and then disperses over the surface of the egg, causing the chorion (egg shell) to dissolve. The larva, which is now visible, continues flexing until its head touches the wax (Fig. 1, c). At this point, it pauses and then falls over on its side. The entire process requires about 20 minutes.

Eggs not in their usual position hatch about as often as upright eggs (7). An egg that is lying on its side, for example, will usually hatch; but it cannot exhibit the waving motion that is seen in an upright egg. Therefore, the waving motion is apparently not a necessary step, at least not when the larva is lying on its side.

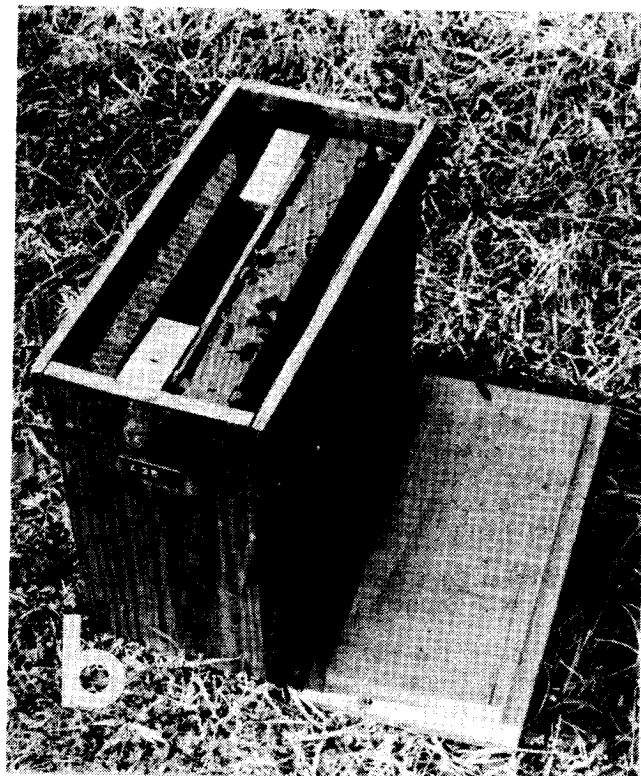
The incubation time required for eggs depends on temperature and the sex of the egg. Female eggs develop more quickly than male eggs, and eggs develop slower at cooler temperatures. At 94.6°F, female eggs in worker cells required about 71 hours from laying to hatching; male eggs required about 3 hours longer. Female eggs required about 72½ hours when kept at 93.7°F and about 100 hours when kept at 88°F. The lower limit for complete development of 50% of the eggs was between 85.6 and 88°F. Only 1% of the eggs hatched when kept at 85.6°F (8).

#### Measuring Egg Mortality

To avoid problems with workers eating some of the eggs and with temperature and humidity variations among colonies, I had all the eggs hatch in an incubator without workers present. To keep things even more uniform, the eggs were laid in the incubator too, but workers, and of course a queen, were present during the 24-hour egg-laying period.

The total procedure to measure percent egg mortality required 8 days. On day 1, I fed the colonies a pollen supplement. Then, on day 3, I put a queen and about 1,000 workers from her colony into cage (Fig. 2, a). Before putting the cage into the incubator (95°F and 50%-60% RH), I put a feeder with 15 ml (about a tablespoonful) of sugar syrup and an empty frame containing 3 grams (about a teaspoonful) of pollen into the cage (Fig. 2, b). (If too much syrup was given, bees filled the cells with syrup and had room for fewer eggs). Twenty-four hours later (on day 4), I returned the bees to their colony, and the frame of eggs was left alone in

**Figure 2. Caging bees for egg laying in an incubator. I used cages that were made by Robert van Arsdall (Bee Breeding lab., Baton Rouge, LA) for emerging bees in an incubator. The cage above happens to be built for ½-length frames, but larger cages with standard frames also work well. In picture a, the queen is caged and about 1,000 of her bees are placed in the cage. The cage of bees is then brought to the laboratory. The queen is released and the feeder and frame are added just before the cage goes into the incubator (b).**



the incubator. Since egg development requires 3 days at 95°F, eggs and larvae were counted 3½ to 4 days after the bees were removed (on day 8). All hatching was finished by then, and the percent mortality was equal to the present eggs remaining. The eggs that did not hatch were often shrivel-

ed by this time. Because the newly hatched larvae are very small (Fig. 1, d) and had no brood food around to flag the presence of a larva, counting was done with 10X magnification of a dissecting microscope. A total of 200 (live or dead) eggs and larvae were counted from each queen.

### Survey of Egg Mortality in Baton Rouge

The purpose of this survey was to estimate the rate of egg mortality that exists in a normal population and to estimate the variance. Since each experimental group consisted of uniform sister queens, they may not be typical of bees in general. Thus, the survey served as a baseline to compare the experimental controls.

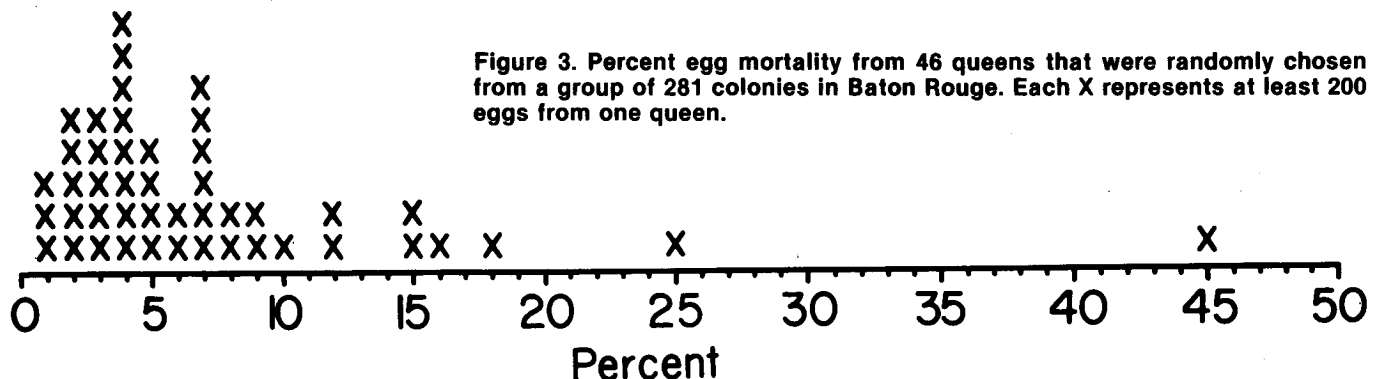
Forty-six colonies were randomly chosen from a group of 281 colonies in the Baton Rouge area. Neither the size of the colony nor the age of the queen was controlled. Fig. 3 shows the egg mortalities as measured by egg development in an incubator. Half of the colonies had egg mortalities of 5% or less; 80% had egg mortalities below 10%. The average mortality was 7% (9).

The very high egg mortalities were re-checked by switching the queens in colonies that had very high (12%-45%) and very low (1%-2%) egg mortalities (see Fig. 3). Eggs were again collected 2 weeks after the switch. The 6 "high" queens measured 2%, 2%, 5%, 7%, 7%, and 18% egg mortality after the switch. The "low" queens had mostly higher mortalities after the switch: 2%, 2%, 6%, 6%, 9%, and 24%. So, in some cases, the present egg mortality was more influenced by the colony, in others by the individual queen. Apparently, then egg mortality in bees has many causes. This variety of causes is an important reason for keeping test and control groups alike in every way.

All experimental controls were well within the normal range predicted in the survey. Except for this comparison, the survey was not used in any experimental analyses.

#### Worker Eggs Compared With Drone Eggs

Drone eggs (normal, unfertilized eggs) had a higher mortality than



worker eggs (9). Four groups of queens that produced fertilized eggs (not including the queens in the survey above) averaged 5%, 6.7%, 6.7%, and 4.6% egg mortality. Four groups of queens that produced unfertilized eggs averaged 18.4%, 51.0%, 61.3%, and 15.9%. (Queens within each group were sisters, and they were sisters to the four respective groups that produced fertilized eggs).

Because of this high mortality in drone eggs and because of the great variation within each group of queens that produced drone eggs, I concluded that a test of egg mortality should not involve drone eggs. With such a great variance in normal drone eggs, experimental differences as great as 10% would be difficult to detect. Of course, when checking frozen sperm, one must use fertilized eggs in the first generation; but, for the next generation, I had considered using unfertilized eggs.

#### Is Frozen Sperm Safe to Use?

In my opinion, frozen sperm is not yet safe enough. Queens inseminated with frozen sperm produced eggs with a higher rate of non-hatching than control queens inseminated with nonfrozen sperm (10). This non-hatching was still detected in the next generation, but at a much lower rate (9). There is some evidence, then, that freezing causes genetic damage.

Is slight genetic damage a serious problem? One could argue that genetic damage occurs naturally and that all animal and plant populations carry imperfect genes and chromosomes. It is very possible that freezing sperm would cause no serious problem. In fact, the risk of a serious problem is probably small. Yet, I am not willing to take this risk.

#### Conclusion

Because of the results with progeny testing, the practice of storing hundreds of lines of bees in liquid nitrogen is delayed. Developing this

method of progeny testing was tedious, but it yielded a useful technique plus information about egg laying, egg-development periods, and normal egg mortality. The progeny test can help ensure that future sperm storage techniques as well as other bee treatments or medications are safe to use. □

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Figure 3. Percent egg mortality from 46 queens that were randomly chosen from a group of 281 colonies in Baton Rouge. Each X represents at least 200 eggs from one queen.