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Research Article

Optimal preservation techniques for honeybee (*Apis mellifera*) eggs: A morphological study

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Abstract - Honeybees (*Apis mellifera*) are an essential species for ecology and the economy. They play a vital role in the beekeeping industry, but this industry often faces issues in apiaries. To address these problems, biologists are investigating different aspects of bee biology, including morphology, taxonomy, behavior, and genetics. Honeybee eggs are used in bee biology research, such as DNA analysis and behavioral studies like policing behavior. Preserving honeybee eggs for research purposes for extended periods is necessary. A study was conducted to investigate the optimal preservation techniques for honeybee egg morphology. The effectiveness of different preservative solutions, including 70% ethanol, 95% ethanol, and Carnoy's fixative, was compared. The samples were stored at different temperatures, including room temperature, 4°C, and -20°C, and were observed for three months. Photographs of each egg were taken every month, and the sizes of the eggs from each treatment were analyzed. The study findings suggest that the most effective preservation methods for honeybee egg morphology are 95% ethanol at all temperatures followed by Carnoy's fixative at all temperatures. Using the best preservation techniques for honeybee eggs is crucial as it allows researchers to study their morphology for an extended period without any damage.

Keywords: *Apis mellifera*, honeybee eggs, preservation techniques

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1. Introduction

Honeybee (*Apis mellifera)* eggs are used in bee biology research, such as DNA analysis and behavioral studies like policing behavior. Techniques for preservation of honeybee eggs are particularly important to keep the eggs without damage for further studies. There were several studies on egg morphology of insects (Woyke & Wongsiri, 1992; Koedam et al., 2001; Rozen et al., 2003) but preservation techniques have not been reported. Honeybee egg morphology is related to various research fields, including bee behavior (Ratnieks, 1995; Ratnieks, 1988; Ratnieks & Visscher, 1989), and egg development.

Methods of preservation of *A. mellifera* germplasm has been developed by Collins and Mazur (2006). Eggs at five ages between 24 hours and 62 hours were exposed to 0, -6.6, and/or -15°C for various times, and measured the successful hatch. The results indicated that the rate of chill injury increases dramatically with decrease in the holding temperature.

Ultrastructure and chemical characterization of egg surface on honeybee worker and queen-laid eggs (Katzav-Gozansky et al., 2003). They found that ultrastructure of surface of both types of eggs using SEM were similar, and that queen eggs are indistinguishable from worker-laid eggs. However, the diversity of compounds found on queen-laid eggs was much greater than worker-laid eggs mainly due to the number of hydrocarbons. Moreover, acetates of some fatty alcohols, alkenes and especially monomethylalkenes were characteristic to queen eggs. Thus, the police workers can discriminate both types of eggs by the differences of chemical characterization.

Pereira et al. (2006) examined whether the morphology of eggs laid by workers and queens of *Melipona scutellaris*, *M. compressipes fasciculata* and *M. asilvai* was similar to that of other *Melipona* species. Egg morphology was examined by scanning electron microscopy whereas egg size was measured by light microscopy. The chorion of queen and worker reproductive eggs showed a characteristic reticulate pattern in all species. The surface of trophic eggs was not reticulate and had an irregular appearance following fixation. Trophic eggs were also invariably smaller than queen-laid eggs and were sometimes smaller than worker-laid reproductive eggs. These findings indicate that trophic eggs can be smaller than the eggs of functional workers, which suggests that the development of this type of egg is probably associated with different physiological adaptations. Therefore, to work on honeybee egg morphology and related fields, the best method to preserve the eggs for research purpose is needed to keep them in a long period.

There are various chemicals and techniques of tissue preservation, but the commonly used solution is ethanol. Different concentration of ethanol leads to different results and effectiveness of preservation. Marquina et al. (2021) studied on the effect of ethanol concentration on the morphological and molecular preservation of insects for biodiversity studies, and they concluded that ethanol concentration at or above 90% made the insects more brittle.

Since research on honeybee eggs were not easy to manipulate due to the eggs are fragile, and there was no preservation technique reported. Although DNA can be extracted from various parts of the honeybee, extracting DNA from eggs has specific advantages for some kind of work such as social parasitism in honeybee colonies (Nanork et al., 2005). The objective of this research was to determine the best preservation techniques for honeybee eggs with a suggestion of an appropriate technique to preserve egg samples from field collection and then using them for further use, for example morphological works and DNA extraction.

2. Materials and methods

2.1 Egg collections

A. mellifera colonies were reared at Faculty of Science, Mahasarakham University. Queen-laid eggs of *A. mellifera* were carefully collected from 3 colonies using clean toothpicks. The Completely Randomized Design (CRD) was used for experimental design. Nine treatments were conducted each month for 3 months (Table 1). Twenty eggs were collected per treatment.

Table 1. Number of eggs and treatments of preservation techniques.

Temperature	Preservatives	Control (eggs)	Month 1 (eggs)	Month 2 (eggs)	Month 3 (eggs)
-	-	20	-	-	-
Room temperature	70% ethanol	-	20	20	20
	95% ethanol	-	20	20	20
	Carnoy's fixative	-	20	20	20
4°C	70% ethanol	-	20	20	20
	95% ethanol	-	20	20	20
	Carnoy's fixative	-	20	20	20
-20°C	70% ethanol	-	20	20	20
	95% ethanol	-	20	20	20
	Carnoy's fixative	-	20	20	20

2.2 Preservation and egg size measurements

The control eggs were photographed immediately after collection under a stereo microscope (Olympus SZX7). Experimental egg samples were preserved in 70% ethanol, 95% ethanol and Carnoy's fixative (95% ethanol – glacial acetic acid mixture of 3:1 (v/v)). Each preservative was subjected to three conductions: room temperature, 4º C and -20º C. Photos of the preserved eggs were taken under stereo microscope (Olympus SZX7) at month 1, 2 and 3. Two measurements were obtained for each egg: length (L) and width (W) using Axio Vision AC Release 4.1 program.

2.3 Data analysis

To compare effectiveness of different preservative solutions, the sizes of egg from each treatment were analyzed using Duncan's Multiple Range Test (DMRT).

3. Results

After honeybee eggs were preserved in different preservatives at different temperatures, the sizes of eggs changed compared to the control eggs. Length of the control eggs ranged from 1164.31-1767.86 μm which is consistent to report in Winston (1987) and Pereira et al. (2006). DMRT comparison according to CDR experimental design indicated significant differences in size of eggs preserved with some different methods. Most preserved eggs were significantly smaller than the control eggs, while eggs from some treatments did not show significant difference (Table 2). The preservation technique that resulted in measurements not significantly

different from the controls was indicated as a good preservation method.

The results of eggs preserved for 1 month suggest that the best preservation techniques were 95% ethanol at room temperature (RT) and Carnoy's fixative at -20°C (Figure 1). The sizes of eggs in the other treatments were significantly different ($P < 0.05$). The shape of the eggs was not different.

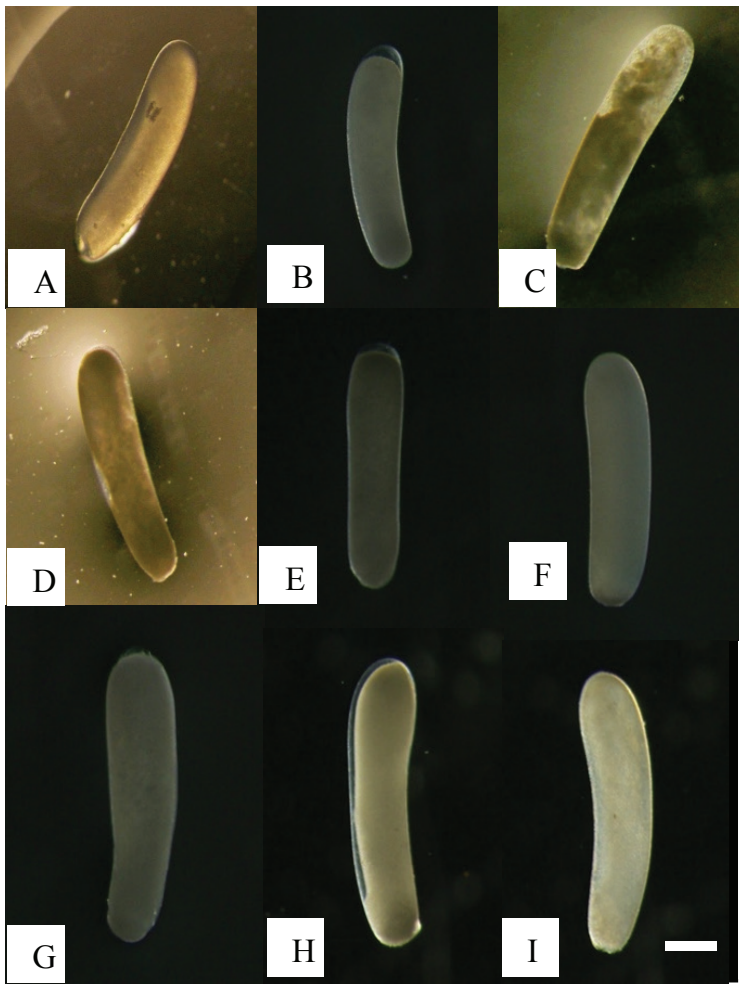

Figure 1. Honeybee eggs were preserved for 1 month. (Scale bar = 300 μm)
A: 70% ethanol, RT, B: 95% ethanol, RT, C: Carnoy's fixative, RT
D: 70% ethanol, 4°C, E: 95% ethanol, 4°C, F: Carnoy's fixative, 4°C
G: 70% ethanol, -20°C, H: 95% ethanol, -20°C, I: Carnoy's fixative, -20°C

After 2 months of eggs preservation, it was found that the best technique to preserve honeybee eggs were 70% ethanol, 95% ethanol, Carnoy's fixative at room temperature, Carnoy's fixative at 4°C and -20°C (Figure 2). The sizes of eggs in the other treatments were significantly different ($P < 0.05$). The shape of eggs preserved in 95% ethanol did not change compared to the first month of experiment. In contrast, some of the eggs preserved in 70% ethanol and Carnoy's fixative began to break (Figure 2; A, C, G).

Table 2. The width and length ± SD (μm) of honeybee (*Apis mellifera*) eggs.

Temperature	Preservatives	Control		Month 1		Month 2		Month 3	
		Egg width ± SD (μm)	Egg length ± SD (μm)	Egg width ± SD (μm)	Egg length ± SD (μm)	Egg width ± SD (μm)	Egg length ± SD (μm)	Egg width ± SD (μm)	Egg length ± SD (μm)
-	-	350.81± 32.09	1563.97 ± 137.92	-	-	-	-	-	-
Room temperature	70 % ethanol	-	-	331.64 ± 42.17*	1437.51 ± 122.66*	344.19 ± 42.83	1417.29 ± 132.90*	338.37 ± 52.79*	1419.81 ± 122.95*
	95 % ethanol	-	-	366.16 ± 36.32	1461.49 ± 57.73*	363.85 ± 48.34	1453.52 ± 50.74*	357.67 ± 49.58	1438.07 ± 51.39*
	Carnoy's fixative	-	-	320.52 ± 39.10*	1421.71 ± 91.12*	355.35 ± 35.17	1480.91 ± 100.19*	371.61 ± 58.15	1559.92 ± 291.02
4°C	70 % ethanol	-	-	249.69 ± 42.06*	1290.10 ± 106.59*	265.15 ± 36.64*	1314.59 ± 74.74*	250.70 ± 48.97*	1288.45 ± 218.77*
	95 % ethanol	-	-	295.37 ± 59.10*	1331.87 ± 297.16*	324.73 ± 36.59*	1412.72 ± 110.95*	329.80 ± 37.91*	1412.95 ± 95.33*
	Carnoy's fixative	-	-	326.41 ± 47.59*	1476.15 ± 84.57*	349.37 ± 29.46	1426.45 ± 107.90*	363.41 ± 31.63	1437.12 ± 130.18*
-20°C	70 % ethanol	-	-	302.99 ± 28.26*	1444.01 ± 81.26*	324.14 ± 22.40*	1482.71 ± 97.83*	298.38 ± 37.91*	1393.32 ± 145.52*
	95 % ethanol	-	-	309.00 ± 30.12*	1387.55 ± 65.23*	320.14 ± 29.38*	1371.91 ± 75.40*	335.42 ± 34.51*	1386.40 ± 94.74*
	Carnoy's fixative	-	-	314.76 ± 44.50	1456.18 ± 92.86*	363.82 ± 43.63	1419.80 ± 180.65*	328.12 ± 71.11*	1370.94 ± 138.73*

The asterisk (*) indicated statistical significance ($P < 0.05$) from control eggs.

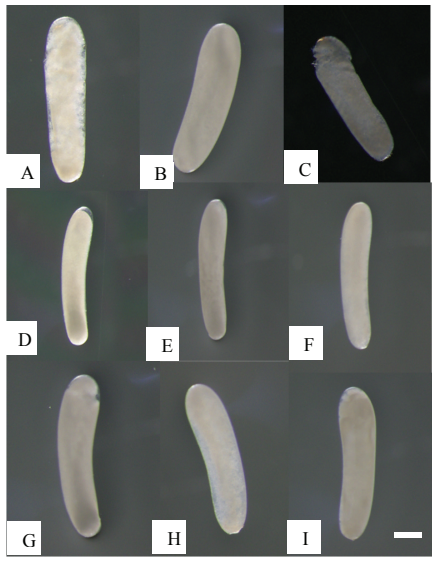

Figure 2. Honeybee eggs were preserved for 2 months. (Scale bar = 300 μm)
A: 70% ethanol, RT, B: 95% ethanol, RT, C: Carnoy's fixative, RT
D: 70% ethanol, 4°C, E: 95% ethanol, 4°C, F: Carnoy's fixative, 4°C
G: 70% ethanol, -20°C, H: 95% ethanol, -20°C, I: Carnoy's fixative, -20°C

The longest duration of honeybee egg preservation in this study was 3 months. The best technique to preserve honeybee eggs for this long was 95% ethanol and Carnoy's fixative at room temperature and Carnoy's fixative at 4°C (Figure 3). The sizes of eggs in the other treatments were significantly different ($P < 0.05$). At this stage, damage appeared in all preservatives especially in 70% ethanol and Carnoy's fixative.

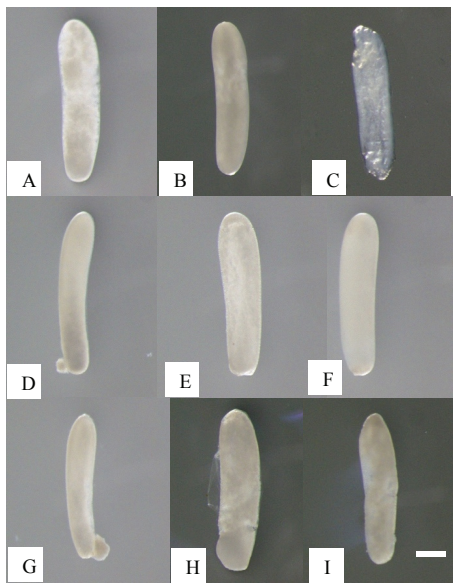

Figure 3. Honeybee eggs were preserved for 3 months. (Scale bar = 300 μm)
A: 70% ethanol, RT, B: 95% ethanol, RT, C: Carnoy's fixative, RT,
D: 70% ethanol, 4°C, E: 95% ethanol, 4°C, F: Carnoy's fixative, 4°C,
G: 70% ethanol, -20°C, H: 95% ethanol, -20°C, I: Carnoy's fixative, -20°C

4. Discussion

The experiment was conducted using common preservative solutions under different temperatures which has never been used for honeybee egg preservation. The study findings suggested that the most effective preservation methods for honeybee egg morphology were 95% ethanol at room temperature, 4°C and -20°C, followed by Carnoy's fixative at room temperature, 4°C and -20°C. However, the eggs in all preservatives had become smaller compared to the controls. This is possibly because egg tissue was dehydrated by ethanol which is also mixed in Carnoy's fixative (Troiano et al., 2009).

Honeybee egg morphology is related to several fields of research, including bee behavior which is called worker policing (Ratnieks, 1995; Ratnieks, 1988; Ratnieks & Visscher, 1989). It has been postulated that queen-laid and worker-laid eggs are discriminated based on an egg recognition pheromone; however, neither the chemistry nor the glandular source has been elucidated (Oldroyd & Osborne, 1999; Barron et al., 2001; Halling et al., 2001; Oldroyd et al., 2001; Nanork et al., 2006). To verify whether egg discrimination might be based on structural differences, researchers compared the ultrastructure of surface of queen-laid diploid and haploid eggs to that of worker-laid eggs using SEM (Katzav-Gonzansky et al., 2003). Only small differences between the different types of eggs were found. Thus, at least based on the fine structure of the egg surface, queen eggs are indistinguishable from worker-laid eggs.

Moreover, molecular genetic studies also require good preservation techniques for high quality of DNA. Muscle tissues were normally used for DNA studies in bees (Halling et al., 2001; Oldroyd et al., 2001). However, some research may require extracting DNA from eggs such as social parasitism in bee colonies (Nanork et al., 2005; Nanork et al., 2007). Interestingly, 95% ethanol is commonly used to preserve tissue for DNA work. In addition, Carnoy's fixative has been used to preserve various kinds of tissue including black flies (Pramual et al., 2011), human cells (Pereira et al., 2015) and rats (Cox et al., 2006), for morphology and molecular aspects. Therefore, preserving honeybee eggs in 95% ethanol and Carnoy's fixative could potentially serve both morphological and molecular purposes. Nevertheless, extracting DNA from preserved honeybee eggs has not been reported yet. Thus, DNA extraction from preserved eggs should be done in the future to confirm that both types of preserve solutions are suitable for molecular work.

5. Conclusion

In conclusion, the results indicated that 95% ethanol is the best preservative for honeybee eggs at least for 3 months, followed by Carnoy's fixative and 70% ethanol, respectively. 95% ethanol is not only suitable for morphological work but also good for DNA work. This report will allow researchers to choose the best preservative methods for their aspects.

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