Optimized Histological Preparation of Ovary for Ovariole Counting in Africanized Honey Bee Queens (Hymenoptera: Apidae)

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Abstract

Techniques for counting ovariole number in virgin and mated Apis mellifera L. queens have been described in previous studies. Having a systematic and fast way to collect this measurement can help accelerate bee breeding programs, because selection decisions can be taken faster. The aim of this work was to develop an efficient histological method to preserve ovaries that allows assessing the number of ovarioles in newly emerged virgin queens, and also in mated queens, in a shorter time than the methods already published. The proposed method resulted in images suitable for ovariole counting in both newly emerged and mated queens, and the total histological process took less than 10 h. This method provides the optimization of the histological procedure for research breeding programs that use ovariole number as selection criteria for improving reproduction and production traits.

Keys words: histotechnique, honey bee breeding, insect ovary, protocol time

In honey bee breeding programs, selective breeding can be planned in order to genetically improve the traits of interest. For that, high-quality phenotypes are needed, given that the traditional genetic evaluation depends not only on pedigrees, but on phenotypes as well. The genetic gain is inversely proportional to generation interval; therefore, if phenotypes can be collected earlier, the selection decisions will be taken faster, increasing the genetic gain.

Reproduction traits in honey bee queens are of special importance, because the ability to produce more eggs can be phenotypically related to the increased productivity in the colony (Akyol et al. 2008), and the colony can be partially considered as the ‘expanded phenotype’ of its queen (Rangel et al. 2013). The number of ovarioles in a honey bee queen is found to be a phenotypic indicator of her reproductive potential (Delaney et al. 2011, Jackson et al. 2011, Chuda-Mickiewicz and Samborski 2015). If there is phenotypic variation in the number of ovarioles, part of this variation could be attributed to the genetic differences. The genetic variation is the base of breeding programs, and studying the genetic variance components of number of ovarioles can help to improve queen fertility and other economically important traits in honey bees.

The quantification of ovarioles can be assessed in a freshly dissected ovary (Corbella and Gonçalves 1982); however, the process of counting ovarioles is destructive and the ovary cannot be kept after the procedure. Histological techniques can also be used for counting the number of ovarioles (Jackson et al. 2011, Hassona and Mourad 2016). Those techniques are useful when less detail is required, as to quantify the ovarioles in mated queens from commercial populations.

The ovary of virgin queens is not as developed as in mated queens. Virgin queens’ ovaries and ovarioles are more sensitive to dissection (Cruz-Landim 2009). However, if the number of ovarioles can be accurately assessed in virgin queens, there is no need to wait for the queen to be mated, and there is no need to dissect a laying queen.

Efficient methods for ovariole counting have been developed for mated (Jackson et al. 2011, Hassona and Mourad 2016) and virgins queens (Hassona and Mourad 2016); however, all of them require at least 24 h before the histological cut is ready to be analyzed. When a large number of queens are going to be evaluated, especially at emergence, a faster procedure may optimize ovariole counting. Therefore, the aim of this work was to develop a feasible, easy, and inexpensive histological method to preserve ovaries that allows assessing the number of ovarioles in newly emerged virgin queens, and also in mated queens, in a shorter period of time.
Materials and Methods

The queen rearing and histological procedures were performed in the Honey Bee Breeding Laboratory (UNEPE-Apicultura) and in the Biological Control Laboratory, at Federal Technological University of Parana, Dois Vizinhos (25°69'05" W, 53°09'92" W), Brazil. Fifty Africanized queens were reared using the Doolittle method (Doolittle 1889), which consists in grafting (i.e., transferring) 24-h-old larvae and putting them into mini-hives for 10 d. After that, the queen alveoles were transferred to an incubator (34°C, 60% humidity) and the emergence was monitored for 24 h.

At emergence, the virgin queens were dissected to remove the ovaries. The number of queens to be dissected (n = 50) was chosen based on the amount of information needed for the breeding program and the capacity of processing samples simultaneously in our laboratory. Additionally, five mated queens with an average of 5 mo of productive life (i.e., laying eggs) were randomly picked from colonies at UNEPE-Apicultura to test the feasibility of the proposed method in nonvirgin, older queens. A total of 100 ovaries from 50 newly emerged queens and 10 ovaries from five mated queens were histologically processed. Among the 50 newly emerged queens, six had damaged ovaries during initial dissection, then these queens were removed from the study.

The queens were euthanized by freezing for approximately 10 min at −18°C. Thorax and abdomen were pinned in a waxed-bottom dissecting Petri dish. The abdomen of each queen was dissected, and the right and left ovaries were removed with forceps and fine curved pointed scissors. The definition adopted for right ovary (Jackson et al. 2011) was the ovary on the right side of the queen pinned ventral side down and anterior forward on the dissecting Petri dish.

The ovaries were immersed immediately in Bouin fixative (150 ml 80% ethanol, ≥99.3% purity; 60 ml formaldehyde 40%, ≥40% purity; 15 ml glacial acetic acid, ≥99.7% purity; and 1 g of saturated picric acid solution; ≥98% purity) (Presnell and Schreibman 1997), at room temperature for 1 h. Then the ovaries were immersed in 70% ethanol for 15 min, three times (Table 1). Dehydration, diaphanization, and infiltration were the next stages (Table 1).

After infiltration, the ovaries were placed in base molds (diameter and height of 1.5 cm) and embedded in paraffin. To facilitate sectioning the tissue, the ovaries were carefully oriented in the base molds using heated forceps (Jackson et al. 2011). The histological sections were performed in the middle zone using a manual rotary microtome (Eikonal, Micro/São Paulo, Brazil) at 5 μm thick and distended in a gelatinized histological slide (Kiernan 1999), then dried at room temperature for 1 h. Paraffin removal, rehydration, staining (hematoxilin, ≥95.0% purity; and alcoholic eosin, Ribeiro et al. 2012) and dehydration were the next steps as described in Table 2.

The sections were dried for 1 h at room temperature (±25°C), then the slides were mounted with cover slip and Canada Balsam (synthetic). For ovariole counting, the sections were photographed in digital camera coupled to a binocular light microscope (Carl Zeiss Microscopy GmbH, Jena, Germany). The images were processed and the ovarioles counted by using the software Zen 2 Blue Edition (2014).

To summarize the dataset and to present a numerical reference, mean, SD, and CV were presented for the total number of ovarioles in each ovary and for each queen, in 88 ovaries of virgin queens and 10 ovaries of mated queens. The descriptive statistics were obtained using the pastecs package (Grosjean et al. 2018) available in R (R Core Team 2016).

Results

Well-preserved and well-individualized ovarioles (Fig. 1A and B) were obtained from the ovary sections. The proposed method resulted in images suitable for ovariole counting in both newly emerged and mated
queens (Fig. 1C and D). All 98 ovaries were efficiently visualized and the ovarioles were counted. SD and CV% were high for ovariole count in the five mated queens probably due to the lower sample size (Table 3).

In the histological method we proposed, the ovariole counting efficiency was linked to a shorter time of the total histological process (Table 4), except for infiltration (Jackson et al. 2011). As shown in Table 4, our method saved 8 h and 15 min (Jackson et al. 2011), and 22 h and 15 min (Hassona and Mourad 2016) in fixation; 58 h and 25 min in infiltration (Hassona and Mourad 2016); 9 h in slide drying (Jackson et al. 2011, Hassona and Mourad 2016); 4 min and 20 s (Jackson et al. 2011), and 5 h, 17 min and 15 s in slide staining stage (Hassona and Mourad 2016). The total histological process took less than 10 h (Table 4); however, the time spent to remove the ovariole, microtomy, and slide assembly were not accounted for because a large variation can exist based on the adopted method, equipment, and availability of qualified personnel.

Discussion

Histological techniques for tissue preparation are widely used to study structures in insects. When the technique is appropriate, structures of interest and surrounding tissues are well-preserved. In our method, it was possible to distinguish the ovarioles from adjacent structures (Fig. 1A and B), showing that the method is as applicable as the ones described in Jackson et al. (2011) and Hassona and Mourad (2016) for counting ovarioles. Therefore, the proposed method, in addition to the photomicrography and the software used, provided a successful ovariole count (Fig. 1C and D), and the average number of ovarioles per ovary and ovaries in newly emerged and mated queens (Table 3) are in agreement with the findings in the literature (Snodgrass 1956, Patrício and Cruz-Landim 2002). The proposed method seems to be feasible for mated queens; however, for honey bee breeding programs, it would be desirable to test the method in a group of queens as large as the newly emerged one.

The main benefit of our method is that the histological procedure is considerably shorter than other published methods, especially time required for fixation, slide drying, and slide staining (Table 4). Hassona and Mourad (2016) method becomes unfeasible when the objective is to have a rapid assessment of the histological cut, because the processes of infiltration alone take over 2 d. Thus, the method of Jackson et al. (2011) was taken as the benchmark for discussion, with only few references to the Hassona and Mourad (2016) method.

The considerable reduced time for the fixation stage in our method (Table 4) is because the ovaries of queens are mesodermic structures, therefore, do not present cuticle (Cruz-Landim 2009) which facilitates the fast penetration of the fixative solution and other reagents. The slide drying stage in our method was shortened by 9 h, because gelatinized histological slides were used, which allowed a faster drying when compared to those used by Jackson et al. (2011) (Superfrost Plus slides, Fisher; www.fishersci.com; last accessed 08/01/2018). The choice of dyes in our investigation was done in order to emphasize only the ovariole structures. The proposed method shortened the slide staining stage when compared with Hassona and Mourad (2016) method, which aimed not only the ovariole count but the detailed identification of cellular components. Jackson et al. (2011) showed that a detailed identification of cellular components is also possible in a short period of time as in the proposed method, but using different dyes.

As the method was primarily proposed for newly emerged virgin queens, which have poorly developed ovaries composed of fragile tissues (Cruz-Landim 2009), the dehydration, diaphanization and
infiltration stages (Tables 1 and 4) were 3 h slower than in Jackson et al. (2011) who worked with laying queens.

In the proposed method, the dehydration was gradual (Table 1) and aimed to preserve the ovaries from the negative action of the manipulation and histological preparation, taking 1 h more than in Jackson et al. (2011). Additionally, an extra half an hour was spent in the diaphanization process and one and a half hours in the infiltration, because the histological paraffin used required more time to infiltrate the tissue in comparison to Paraplast (Leica, www.leica-microsystems.com; last accessed 08/01/2018). Overall, the use of histological paraffin was adequate for the purpose of the study, even with a longer infiltration time, because it has a lower cost enabling the use of the technique in a large sample size.

The method we proposed is advantageous when a large number of queens have to be measured, and the ovariole count must be done in a short period of time. For example, in genetic studies, where the choice of the next generation of queens depends on information from the current generation, and a shorter time between generations is required to maximize the genetic gain.

In addition to being feasible and fast, the proposed method for ovariole counting can easily be replicated, uses commonly available and inexpensive materials and can easily be taught to laboratory technicians. New methods, with alternative reagents and stains, can also be tested in order to further optimize the histological procedure for research breeding programs that use ovary measurements as selection criteria for improving reproduction and production traits.

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**Table 3.** Descriptive statistics for total number of ovarioles counted per ovary in newly emerged virgin and mated queens

| Queen     | Trait    | Mean (SD) | CV (%) |
|-----------|----------|-----------|--------|
| Virgin    | Right ovary | 151.7 (±18.9) | 12.5   |
|           | Left ovary | 149.3 (±28.6) | 19.1   |
|           | Ovaries    | 301.0 (±37.7) | 12.5   |
| Mated     | Right ovary | 115.2 (±64.3) | 55.8   |
|           | Left ovary | 118.0 (±40.2) | 34.0   |
|           | Ovaries    | 233.2 (±85.1) | 36.5   |

**Table 4.** Time comparison for all histological steps used for ovary preparation in two already published methods and in our proposed method

| Step       | Jackson et al. (2011) | Hassona and Mourad (2016) | Proposed method |
|------------|-----------------------|---------------------------|-----------------|
| Fixation   | 10 h                  | 24 h                      | 1 h 45 min      |
| Infiltration | 3 h 30 min            | 64 h 55 min               | 6 h 30 min      |
| Slide drying | 10 h                  | 10 h                      | 1 h             |
| Slide staining | 47 min               | 5 h 59 min 15 s           | 42 min 40 s     |
| Total time | 24 h 17 min           | 104 h 54 min 15 s         | 9 h 57 min 40 s |

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Supplementary Data

Supplementary data are available at *Journal of Insect Science* online.

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