Original Article

SIZE AND SHAPE DIFFERENCES IN FORE WINGS OF HONEY BEE (*APIS MELLIFERA*) QUEENS, WORKERS AND DRONES

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Abstract

The three honey bee phenotypes - queens, workers, and drones differ in the size and shape of body parts. We used a large dataset to describe how the three phenotypes differed with regard to fore wings and applied the methodology of geometric morphometrics to measure the wings using the coordinates of nineteen landmarks. On average, queens had wings larger than workers, but the two castes had a broad overlap. Drones, on the other hand, had distinctly larger wings. Wing shape differed markedly among queens, workers and drones and can be used to reliably differentiate them from one other. Surprisingly, the fore wing shape of workers was more similar to that of drones than to that of queens. Small queens were not more similar to workers than large queens, and large workers were not more similar to queens than small workers. Because wing size, unlike body mass, does not change over the life of the queen, it can be used to evaluate the quality of a queen throughout its life and not only during a short period after emergence. We provide a large number of wing images of queens and drones, which can be used as a reference in future studies.

Keywords: *Apis mellifera*, drone, honey bee, queen, shape, size, wing, worker

INTRODUCTION

A honey bee (*Apis mellifera*) colony usually has only one queen. Apart from the small fraction of eggs produced by laying workers, the queen is the only source of eggs in the colony. Because the colonies consist of tens of thousands of bees, the queen's egg-laying rate can be up to 2000 per day (Free & Williams, 1972), which can be a limiting factor for colony growth. The potential to lay a large number of eggs during one day and their whole lives varies markedly among queens and can determine colony success both in nature and in beekeeping (Delaney et al., 2011; Büchler et al., 2013; Rangel et al., 2013). Problems with queen bees were predicted to be one of the causes of bee colony mortality (Gray et al., 2019).

The quality of queens can be assessed on the basis of various physical and behavioural traits (Hatjina et al., 2014). Among others, the quality assessment can be based on the health of queens (Czekońska, 2001; Gregorc & Bakonyi, 2012; Amiri et al., 2017), but most often body size is used for this purpose. Large queens have more ovarioles (Woyke, 1971; Yi et al., 2020, 2021), have larger spermathecae (Woyke, 1971; Akyol et al., 2008), store more spermatozoa (Delaney et al., 2011) and mate with a larger number of drones (Delaney et al., 2011). Colonies headed by large queens build more combs and store more food (Rangel et al., 2013), and their brood area is large (Akyol et al., 2008; De Souza et al., 2013).

Queen body size can be affected by various factors related to breeding (Gregorc & Smodiš Škerl, 2015; Prešern & Smodiš Škerl, 2019). These include the age of grafted larvae (Woyke, 1971), size of cell cups (Mattiello et al., 2022; Wu et al., 2018), position of the queen cell in the nest, month of year (Koç & Karacaoglu, 2004) and presence of supplementation (Mahbobi et al., 2012; De Souza et al., 2019; Dolasevic et al., 2020). Furthermore, the body size of the queen is reduced in the presence of pesticides (Collins et al., 2004; Gajger et al., 2017; but see also Milone & Tarpy, 2021).

The presence of the queen in the nurse colony does not affect body weight of reared queens (Cengiz et al., 2009). Queen body weight depends mainly on environmental factors, but to some degree, it is determined genetically and differs between geographic locations (Gromisz, 1969), subspecies (Konopacka et al., 1977; Gromisz & Jabłońska, 1978) and breeding lines (Skowronek et al., 2004).

Beekeepers generally accept that large queens are of better quality because they have more ovarioles and a larger spermatheca. Consequently, large queens can lay more eggs both daily and over their lifetime. Queens with a body mass below 175 mg are not suitable for instrumental insemination because the size of their reproductive organs does not match the insemination syringe, and this can lead to queen injuries (Vanicek, 1975). In Poland, there was an industry standard that obliged queen breeders to control the size of queens they produced. The minimum requirements differed between subspecies; in the case of uninseminated *A. m. carnica* queens, the minimum body mass was 185 mg (Konopacka et al., 1977).

The non-lethal assessment of honeybee queen quality is often based on queen weighing. This method is fast and requires no sophisticated equipment besides a precision balance. However, the body weight of the queen changes markedly during her life (Skowronek et al., 2004; Kahya et al., 2008). Between emergence from the queen cell and mating, it decreases at a rate of more than 1 mg per day. After mating, the body mass increases; in an egg-laying queen, it is greater than at emergence (Kahya et al., 2008). In laying queens, body mass probably fluctuates and depends on the egg lying rate, which changes during the season. In order to increase the repeatability of the measurements, the queen bee is usually weighed at the beginning of her life, immediately after she emerges from the queen cell. At a later stage, the assessment of queen quality based on wet weight is less precise. The problem of assessing the quality of the queen regardless of age can be solved through the use of external morphology measurements. Various body parts' dimensions were measured for this purpose, including thorax width and length, wing length and width, and head width (Hatch et al., 1999; Delaney et al., 2011; Tarpy et al., 2011). Those measurements correlated with one another and with wet weight (Woyke, 1971; Delaney et al., 2011; Tarpy et al., 2011).

Nowadays, morphological measurements are usually made with computer software using scaled images (Nawrocka et al., 2018). The quality of the images is crucial for obtaining precise measurements. It is relatively easier to take a good-quality image of a wing because it is flat and has a clear pattern of venation. Wings can be assumed to be two-dimensional. If they are mounted between two microscopic glasses perpendicular to the optical axis of the camera, they can be measured with high repeatability. Measurements of the thorax or head are more difficult because those body parts are rounded and need to be correctly oriented. If the image used to make measurements is taken at an incorrect angle, the results will be inaccurate. Additionally, the problem during the measurements can be a small depth of field. In contrast to wings, the thorax or head is usually not entirely in focus, which makes measurements more difficult.

Honey bee queen measurements are also interesting in the context of caste determination and development regulation. One possible method of caste determination is allometric growth (Wilson, 1953). Different body parts grow at different rates, and consequently there can be distinct phenotypes that differ in body size. If queens, workers, and drones are determined by allometric growth, the largest workers should be similar to the smallest queens.

The study aimed to compare the fore wings of queens, workers and drones. Both the size and shape of the wings were used to verify if the three phenotypes overlapped with one another. This is particularly interesting in the case of queens and workers because their development is determined by the environment. We predict that the smaller queens will be more similar to workers, and the larger workers will be more similar to queens. We discussed our results in the context of queen quality.

MATERIAL AND METHODS

In this study, we used 18561 individuals, representing 2086 queens, 12373 workers and 4102 drones. Most individuals were represented by both the left and right wings; however, damaged wings were discarded. Thus, the number of investigated wings was 31514. Most queens (1481 individuals) were obtained from queen breeders and beekeepers in Poland, who had responded to an advertisement in a beekeeping journal and sent us the queens by post. The queen breeders and beekeepers sent us queens which were of inadequate quality, were not accepted by the honey bee colony during introduction or were removed from the colony during replacement with the young queen. For those queens, we have no additional information apart from wing measurements. Another part of the queen sample (605 individuals) was reared experimentally with the use of the standard method of transferring larvae from the worker cell to the artificial queen cell. To obtain higher variability in body sizes, the larvae used for queen rearing were one day old, two days old or three days old. The queens reared from younger larvae should be larger and have a large body mass. On the other hand, the queens reared from older larvae should be smaller and have a smaller body mass. Sealed queen cells were moved in individual cages to an incubator at 35°C, where they emerged. Immediately after emergence, the queens were weighed to the nearest 1 mg.

The drones were collected from over twenty colonies in Poland. Most of the drones originated from queenright colonies, where they developed in drone cells. Some of the drones (374 individuals) originated from permanently queenless colonies with lying workers. About sixty of those drones were markedly smaller and probably developed in worker cells.

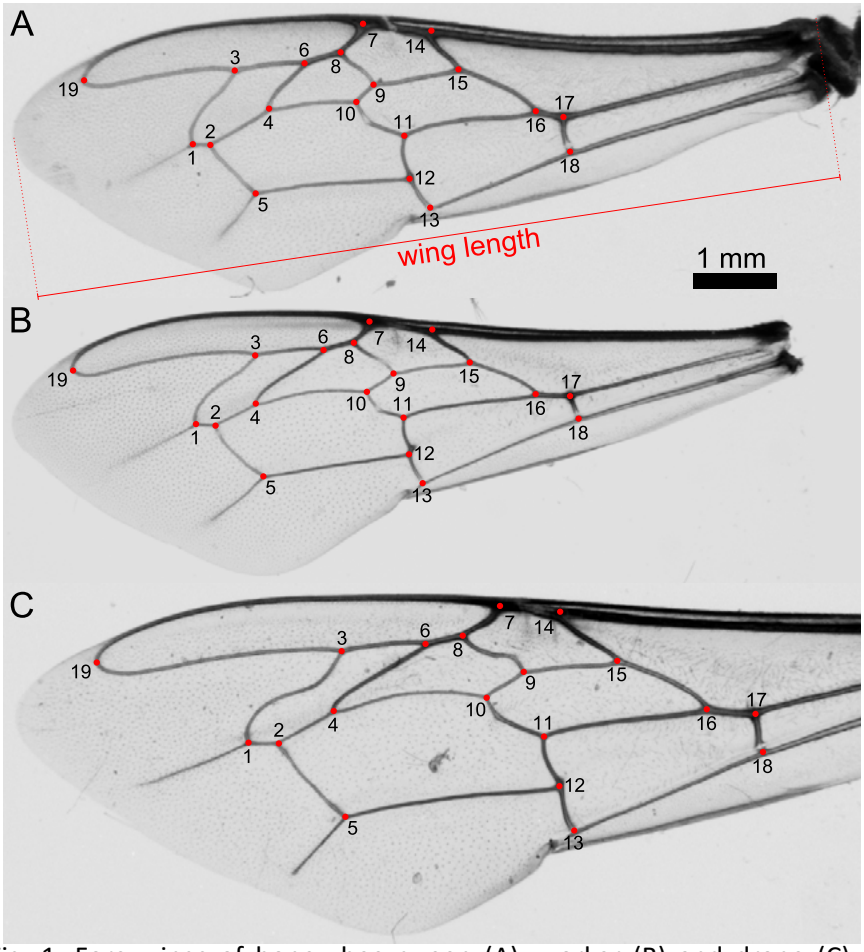


Fig. 1. Fore wings of honey bee queen (A), worker (B) and drone (C). Numbered red dots indicate the position of nineteen landmarks used for wing measurements. In queens, wing length was also measured. The scale bar indicates 1 mm and refers to all three images.

The queens and drones were stored in a freezer until the preparation of the wings. The left and right fore wing of each queen were dissected and mounted between two microscope slides, and their images were obtained using a USB camera equipped with a lens. The wing images were saved as PNG files at a resolution of 94488 pixels per metre (2400 dpi). The wings were measured with the use of the methodology of geometric morphometrics. The position of nineteen landmarks (Fig. 1) was determined on the wings with the use of IdentiFly (v. 1.8) software (Nawrocka et al., 2018). Some wing images were discarded because they were damaged, dirty or had missing veins (Łopuch & Tofilski, 2016). In queen images with a well-visible wing base and wing tip (2645 wings), wing length

was also measured (Fig. 1A). The dataset related to queens and drones is available on the Zenodo website at https://zenodo.org/record/8396176.

Images of worker fore wings were obtained from an earlier study (Oleksa et al., 2022, 2023). We used only part of the dataset from Poland (Oleksa et al., 2021), Romania (Tofilski et al., 2021), Slovenia and Croatia (Puškadija et al., 2020) because for those wing images there was a known image resolution, which was the same as in the present study. The worker wings were already measured in the previous study, and the configuration of landmarks was compatible with the present study. The statistical analysis was performed in R (v. 4.3.2) with the use of RStudio (v. 2023.09.1). The script was prepared in R Markdown and together with the results of the analysis was saved as an HTML file available as Supplementary Document S1. The Cartesian coordinates of the landmarks were superimposed with the use of generalised Procrustes analysis in the geomorph package (v. 4.0.6) (Baken et al., 2021). The superimposition allowed for the separation of wing size from wing shape, which were analysed separately. The wing size was described with the centroid size, a standard measure used in geometric morphometrics that is defined as the square root of the sum of the squared distances of all landmarks from their centroid (Dryden & Mardia, 1998). The centroid size was expressed in millimetres as the landmark coordinates were converted from pixels to this unit. The aligned coordinates of the left and right wing were averaged within individuals, and the averages were used in the subsequent analysis. Principal component analysis was used to extract the first thirty-four principal components, which were used to describe wing shape. Canonical variate analysis (CVA) from the Morpho package (v. 2.11) (Schlager, 2017) was used to classify wings as queens, workers and drones. Leave-one-out cross-validation was used to verify how reliable the classification was. The identification data were saved in a DW.XML file (available at https://zenodo.org/record/10512712), which can be easily used with IdentiFly (v. 1.8) software to classify an unknown wing as one of the three phenotypes. The differences in wing shape were described with the use of Mahalanobis distance. Because we were not controlling wing length, centroid size or body weight and those variables were collected randomly, we used the reduced major axis (RMA) using the lmodel2 package (1.7-3) to obtain regression equation coefficients. The association between centroid size and Mahalanobis distance was analysed with the use of generalised additive model (GAM) regression.

RESULTS

All the relationships between different measures of queen body size were highly positively correlated (Pearson correlation; centroid size versus wing length: $r=0.9048$, $P<2.2x10^{-16}$, Fig. 2A; centroid size versus body weight: $r=0.5527$, $P<2.2x10^{-16}$, Fig. 2B; wing length versus body weight: $r=0.6190$, $P<2.2x10^{-16}$, Fig. 2C).

Centroid size, further referred to as "wing size", differed significantly among queens, workers and drones ($F=3752$, $P<2x10^{-16}$). In pair-wise comparisons, queens, workers, and drones differed significantly in wing size from one another. As expected, the mean wing size was the smallest in workers and the largest in drones. On average, queens were larger than workers, but there was a large overlap between the two phenotypes (Fig. 3). The wing size distribution was symmetrical and steeper in the case of workers. In queens and drones, the wing size distribution was more flat and skewed to the left, with a relatively large proportion of unusually small individuals. The median centroid size of queens, workers and drones was 7.311, 6.948, and 9.422, respectively.

Wing shape differed significantly among queens, workers and drones ($F=7462$, $P<0.001$). In the graph of the first two canonical variates, the three groups formed separate clusters of points (Fig. 4). The wing shape can be used to discriminate among queens, workers and drones without error. Queens were more similar to workers (MD=10.98) than to drones (MD=16.11). Surprisingly, workers were more similar to drones (MD=10.10) than to queens (MD=10.98). The relatively high similarity between workers and drones was present even if the small drones, developing in workers cells, were removed from the analysis (Supplementary

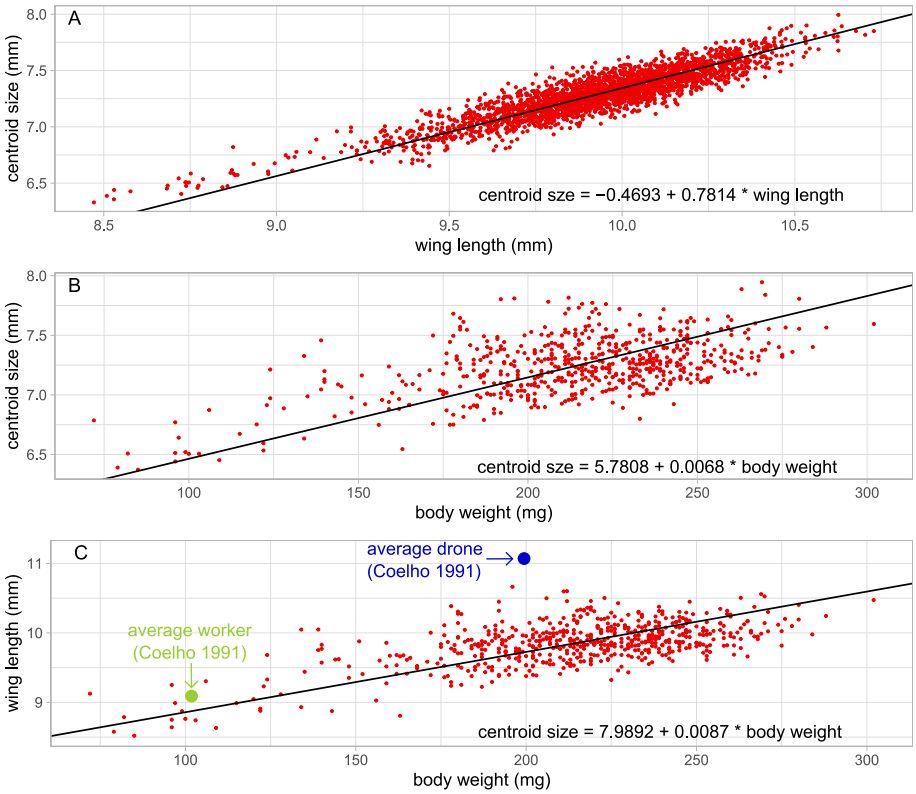


Fig. 2. Relationship between centroid size and wing length (A), centroid size and body weight (B), and wing length and body weight (C) of honey bee queens (red dots). Green and blue dots indicate data for the average worker and average drone obtained from Coelho (1991). Reduced major axis regression lines and equations are provided for each relationship.

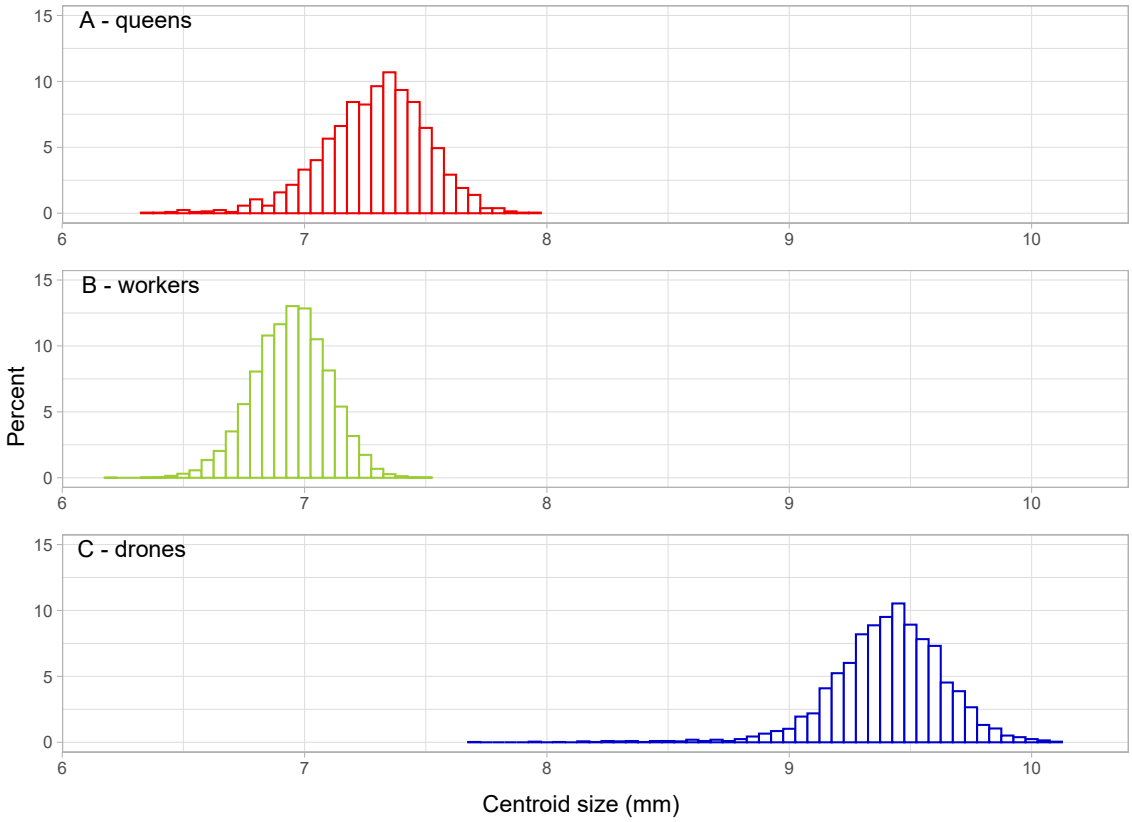


Fig. 3. Distribution of wing centroid size in queens (A), workers (B) and drones (C).

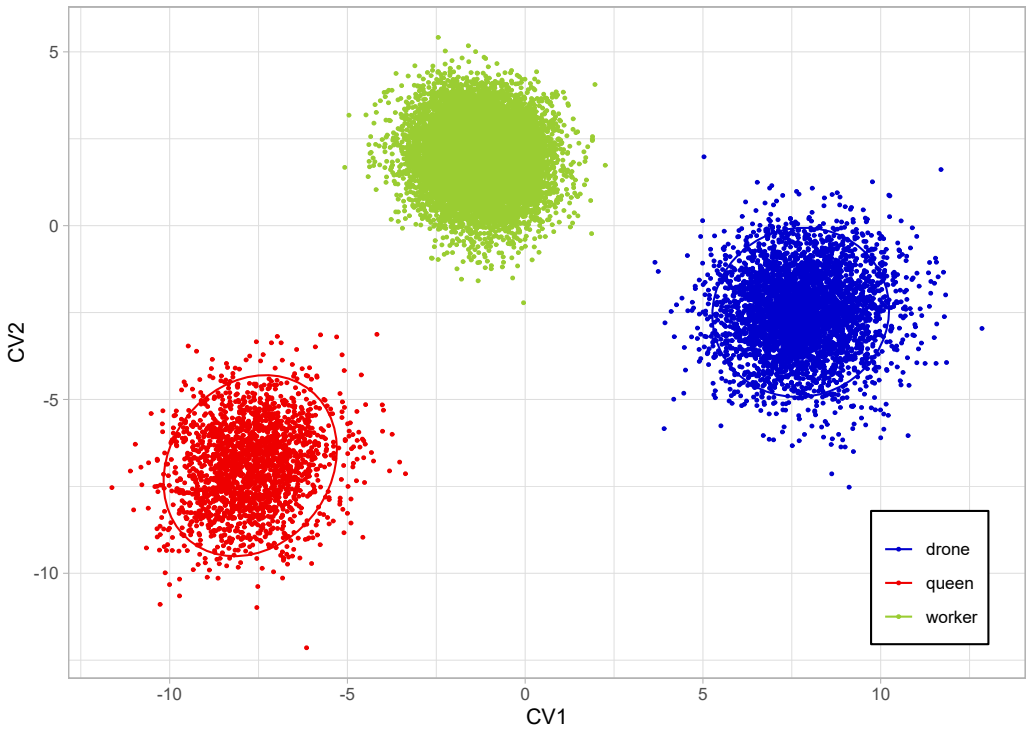


Fig. 4. Differences in wing shape among queens, workers and drones are depicted by the first two canonical variates.

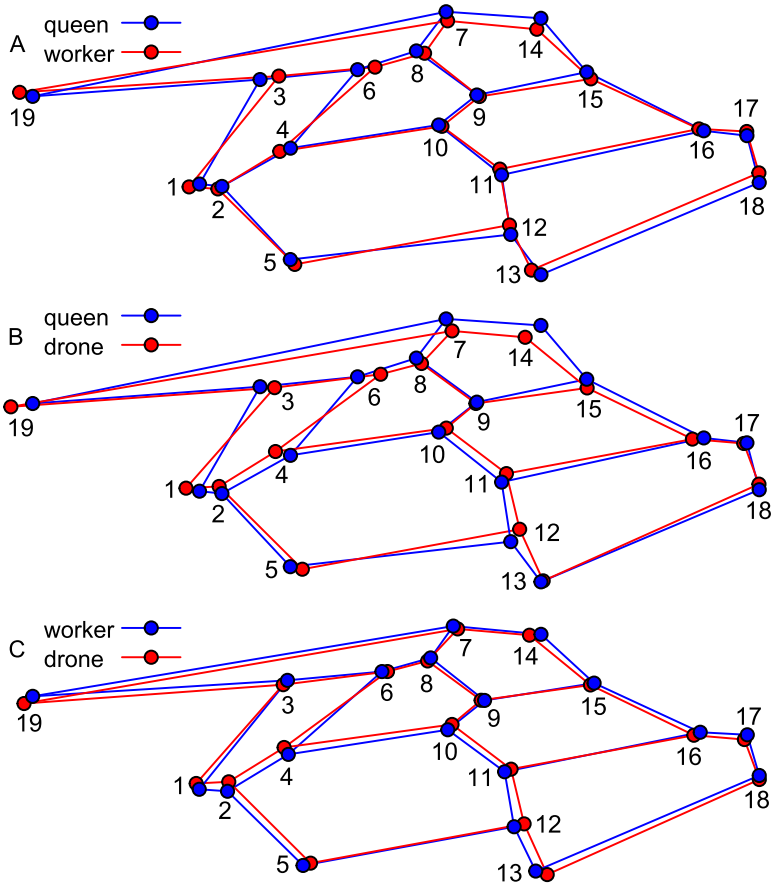


Fig. 5. Wireframe graphs show differences in wing shape between queens and workers (A), between queens and drones (B) and between workers and drones (C). The configurations of landmarks were scaled to the same size. In order to compare wing size see Fig. 3.

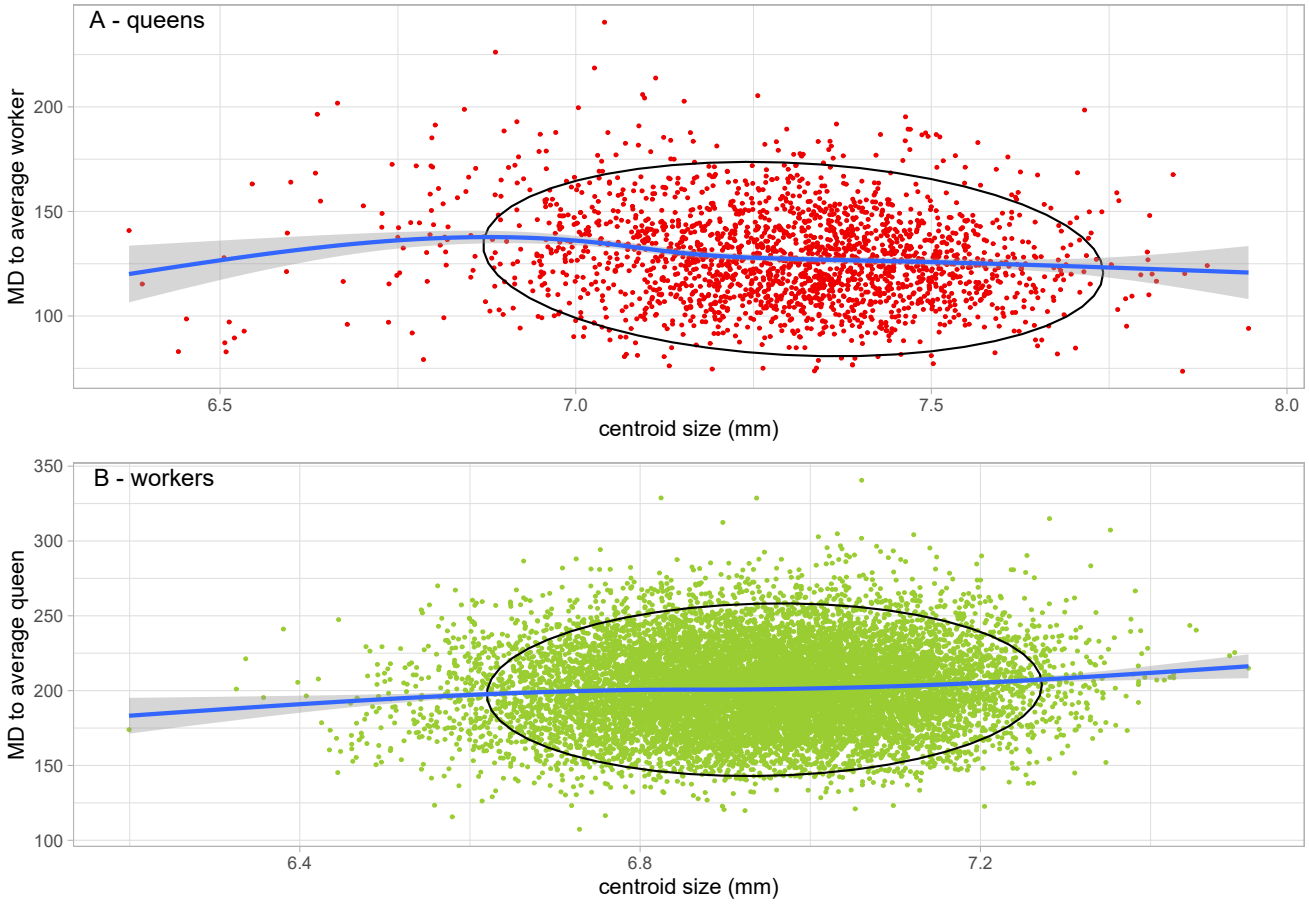


Fig. 6. Relationship between centroid size of queens and dissimilarity with average worker (A) and relationship between centroid size of workers and dissimilarity with average queen (B). The dissimilarity was expressed as Mahalanobis distance (MD). The lines were fitted to the data with the use of generalised additive models.

Document S1). After being scaled to the same size, the wings of queens were proportionally wider than the wings of workers (Fig. 5A). Differences between queens and drones were similar to those between queens and workers but larger (Fig. 5B). Differences between workers and drones were located mainly in the posterior and distal parts of the fore wing (landmarks 1, 2, 4, 5, 10-13) which in workers shifted towards the posterodorsal wing margin (Fig. 5C).

There was a significant negative correlation between queen wing size and Mahalanobis distance to the average wing shape of workers (Pearson correlation: r=-0.1371, $P=3.14x10^{-10}$; Fig. 6A). Although the relationships were highly significant, the effect sizes were relatively small, and the relationships were to some degree nonlinear at extremely small queen wing sizes. There was a significant positive correlation between worker wing size and Mahalanobis distance to the average shape of queens (Pearson correlation: r=0.0659, $P=2.04x10^{-13}$; Fig. 6B). There was a significant relationship between wing shape and wing size (allometry) in all three phenotypes (queens: F=54.83, P=0.001; workers: F=118.96, P=0.001; drones: F=41.35, P=0.001).

DISCUSSION

The results presented here show that queens had wings larger than workers, but there is a broad overlap between the two phenotypes. Drones, on the other hand, had distinctly larger wings. Workers were less variable regarding wing size, and their distribution was symmetrical. On the other hand, the distribution of queens and drones was skewed to the left, with a small fraction of unusually small individuals (Fig. 3). The unusually small individuals represent underfed queens and drones developed in worker cells (Berg et al.,

1997; Schlüns et al., 2003; Gençer & Firatli, 2005; Gençer & Kahya, 2011; Metz & Tarpy, 2022).

Queens had disproportionally small fore wings compared to their body mass (Fig. 2C). Drones with a body mass of 199.4 mg had a fore wing that is 11.07 mm long (Tab. 1 in Coelho, 1991). The queen wing length estimated for the same body mass from the regression equation (Fig. 2C) was 9.72 mm, which was 1.35 mm (12%) shorter. When queens were compared to workers, the difference was smaller. Workers with a body mass of 101.8 mg had wings 9.09 mm long (Tab. 1 in Coelho, 1991). The queen wing length estimated for the same body mass was 8.87 mm, which was 0.21 mm (2%) shorter. Smaller wings may be an adaptation to life in a crowded nest where queens spend most of their lives.

Measurements of honey bee wings can be used to estimate body size, which is often used as a measure of queen quality. We showed a high correlation between queen body mass at emergence and both centroid size (Fig. 2B) and wing length (Fig. 2C). We provide equations that can be used to estimate body mass from the wing measurements. Compared to centroid size, the wing length proved to be a better predictor of the queen's body mass, as the correlation coefficient for this relationship was higher. However, wing length cannot be measured when the wing tip is frayed or when the wing base is not visible or destroyed. Body size based on wing size can be estimated at all stages of a queen's life, but it requires obtaining a wing image. In the case of dead individuals, the wings can be detached and mounted between microscopic classes. It is also possible to take a wing image of a living bee, but this requires anaesthesia for the bee. If both wing tip and wing base are visible in the image, wing length can be measured; otherwise, only centroid size can be obtained. Problems with wing length measurements of damaged wings motivated earlier researchers to use some distances along the venation (Dedej & Nazzi, 1994). The centroid size should be better for this purpose because it contains information about most parts of the wing.

Queens, workers, and drones differ markedly in wing shape. Those differences are so significant that they allow reliable discrimination among the three phenotypes. It had been earlier reported that the cubital index, the proportion of two wing vein lengths (Ruttner, 1988), differed among honey bee queens, workers and drones (Ruttner, 1988; Pathania et al., 2022). Here, we confirm that the position of landmarks 1, 2 and 4, which are used to calculate cubital index, differs among the phenotypes (Fig. 5). Additionally, we provided data about the whole fore wing shape and compared the variation among and within phenotypes for the first time. At the moment, the reference samples for the identification of honey bee subspecies consist only of workers' wings (Gerula et al., 2009; Nawrocka et al., 2018). Queens or drones have markedly different wings and therefore cannot be used for the identification of subspecies using workers' wings as a reference. In the future, such identification will be possible if reference samples consisting of queens' and drones' wings are available.

When honey bees are reared in vitro, they often produce intercastes, females with phenotypes intermediate between queens and workers (De Souza et al., 2015). In this study, we provided data about individuals reared naturally by bees inside a honey bee colony, but we could not detect any clear intercastes. We made some efforts to produce low-quality queens and consequently obtained queens with small bodies, but they did not differ markedly from typical queens. The cluster of points representing the wings of queens in CVA graphs (Fig. 4) formed a single ellipse, which suggests no apparent polymorphism within queens. We predicted that smaller queens would be more similar to workers but were not able to confirm this. However, the opposite relationship was true, and among queens there was a significant negative correlation between wing size and distance to the average worker (Fig. 6A). Very small queens, with centroid sizes below 6.6, did not follow this relationship (Fig. 6A). We also predicted that larger workers would be more similar to queens. Again, we could not confirm this prediction, as among workers there was a significant positive relationship between wing size and distance to the average queen (Fig. 6B).

The developmental path of honey bees is determined at the early stage of larval development, and as a result, in natural circumstances, there are distinct phenotypes. The tasks performed by the three phenotypes were entirely different, and therefore they probably require particular morphological adaptations. The intermediate phenotypes among the three phenotypes are probably maladaptive, and therefore there is a disruptive selection to eliminate them. In honey bees, evolution proved to be very effective in this respect. Similar clear discrimination of queens, workers and males was reported for the stingless bee *Scaptotrigona postica* (Hartfelder & Engels, 1992).

Because queens and workers are females, in contrast to drones, which are males, queens and workers can be expected to be similar. However, we show here that workers are more similar to drones than to queens. Allometry cannot explain this surprising result because queens and workers have clearly smaller wings than drones, and workers are on average smaller than queens. The similarity of workers to drones agrees with the hypothesis that workers are masculinized females (Kerr, 1974). This hypothesis was not widely accepted (Hartfelder & Engels, 1992), but some recent studies show that experimental manipulation of some genes in workers can induce some traits normally present in drones (Roth et al., 2019). Another explanation for the similarity between workers and drones is their adaptation to flight, which is much stronger in workers and drones than in queens. As mentioned before, the wings of queens can be selected for small size and not for optimal flight. Flying with disproportionally small wings can be associated with different aerodynamic forces, consequently the optimal shape of smaller wings under heavy loading can be different.

In contrast to wing size, wing shape was not very helpful in evaluating queen quality. Wing shape can be used to discriminate between queens and the two other phenotypes; this is of little practical value to beekeepers and queen breeders, as they can recognise queens at first sight without any measurements. The wing measurements can be more useful in scientific research on caste determination. In laboratory conditions, under various experimental manipulations, individuals often have intermediate phenotypes (De Souza et al., 2015; He et al., 2017; Roth et al., 2019). The data provided here, including wing images, their measurements and identification data, can be used to classify the intermediate individuals as queens, workers and drones and quantify the similarity to each of those phenotypes.

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