

Inheritance of Traits Associated with Reproductive Potential in *Apis mellifera capensis* and *Apis mellifera scutellata* Workers

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Abstract

When workers of the thelytokous Cape honeybee, *Apis mellifera capensis*, come into contact with colonies of the neighboring arrhenotokous subspecies *Apis mellifera scutellata*, they can become lethal social parasites. We examined the inheritance of 3 traits (number of ovarioles, number of basitarsal hairs, and size of spermatheca) that are thought to be associated with reproductive potential in *A. m. capensis* workers. To do so, we produced hybrid *A. m. scutellata*/*A. m. capensis* queens and backcrossed them to either *A. m. capensis* or *A. m. scutellata* drones. We then measured the 3 traits in parental, hybrid, and backcross offspring. We show that the 3 traits are phenotypically correlated. We also show that the expression of ovariole number, basitarsal hairs, and size of spermatheca is influenced by the genotype of the individual and the rearing environment but that the influence of the rearing environment is less important to the number of ovarioles. We hypothesize a single recessive allele (*l*), present at high frequency in natural *A. m. capensis* populations, which when homozygous causes larvae to elicit more food. This increased feeding as larvae causes resulting adult workers to develop more queen-like morphology and increased reproductive potential. The number of ovarioles, in contrast, appears to be under independent genetic control.

The Cape honeybee, *Apis mellifera capensis* (hereafter *capensis*), of South Africa is unique among honeybees in that a large proportion of eggs produced by unmated workers develop as females by thelytokous parthenogenesis (Verma and Ruttner 1983). This is in contrast to all other honeybee species and subspecies in which, when queenless, workers may produce male-destined eggs by arrhenotokous parthenogenesis (Winston 1987; Oldroyd and Wongsiri 2006), although in some cases about 1% of eggs may be thelytokous (Mackensen 1943; Tucker 1958). Ratnieks (1988) argued that in arrhenotokous honeybees, worker reproduction is largely absent in the presence of a queen because of the higher average relatedness of workers to sons produced by the queen compared with sons produced by workers ($r = 0.25$ vs. ≈ 0.125 , respectively, due to multiple mating by the queen). In contrast, thelytokous *capensis* workers are related to their own female-producing eggs by unity and are equally related to

the female progeny of their sister workers ($r \approx 0.25$) as they are to the progeny of their queen. Thus, the kin structure of *capensis* colonies is altered in ways that strongly favor selection for direct worker reproduction (Greeff 1996). This arises because, if a worker produces a daughter worker, there is limited cost to the colony (Hamilton 1972), and individual workers can increase their personal reproductive success hugely if they can lay thelytokous eggs in queen cells (Beekman and Oldroyd 2008; Jordan et al. 2008).

As predicted by theory, *capensis* workers often oviposit in queen cells during reproductive swarming (Jordan et al. 2008) and show physiological traits that are suggestive of higher reproductive potential than is typical for workers of the arrhenotokous subspecies. First, *capensis* workers have larger numbers of ovarioles (10–20) rather than the 3–5 typically observed in arrhenotokous subspecies (Ruttner 1977; Allsopp et al. 2003). Second, many *capensis* workers

possess a spermatheca (an organ used for sperm storage in mated queens), which is absent in workers of all other honeybees (Ruttner 1988). Third, queenless *capensis* workers produce queen-like mandibular pheromones in large amounts that prevent ovary activation in other workers (Moritz et al. 2000; Wossler 2002).

Expression of reproductive traits in *capensis* workers is strongly influenced by the amount of food a larva receives (Beekman et al. 2000; Calis et al. 2002; Allsopp et al. 2003). Overfed larvae are more likely to possess a spermatheca and reduced pollen combs (as measured by an increased number of basitarsal hairs) as adults, traits that indicate that these workers are more queen-like than workers that are fed normally (Beekman et al. 2000; Calis et al. 2002; Allsopp et al. 2003). Interestingly, however, the number of ovarioles appears to be less influenced by larval feeding, indicating that this trait is more canalized than the other 2 (Allsopp et al. 2003).

Thelytoky in *capensis* is reported to be controlled by a single gene (Lattorff et al. 2005), the location of which has been fine mapped to a small region of chromosome 13 (Lattorff et al. 2007). The *Thelytoky* gene, possibly homologous to the transcription factor “grainy head” of *Drosophila* (Lee and Adler 2004), acts pleiotropically on 2 other important reproductive traits of *capensis* workers: the production of large amounts of the queen pheromone 9-oxo-2-decanoic acid (9-ODA) and early onset of oviposition (Lattorff et al. 2007). However, the genetic architecture of the other traits expressed by *capensis* workers is not known. Here we study the inheritance of 3 such traits: 1) ovariole number, 2) basitarsal hair number, and 3) spermathecal size. We were particularly interested to see how these 3 traits covary in backcross progeny, which would suggest that they are strongly influenced by the same genetic or environmental switch, or if the traits are expressed independently, which would suggest that multiple genetic factors control these traits independently.

Our initial hypothesis was that the traits studied here are influenced by a single locus, *Larva*, which, when homozygous recessive, results in increased expression of these traits via increased larval feeding. Such a hypothesis is suggested by the coexpression of presence of spermatheca, large number of ovarioles and basitarsal hairs in *capensis*, and the crucial role of larval feeding for the full expression of these traits (Allsopp et al. 2003). The recessive allele *l* is hypothesized to be present at high frequency in the *capensis* population and the dominant allele, *L*, in the *Apis mellifera scutellata* (hereafter *scutellata*) population. We tested this hypothesis by crossing *scutellata* queens with *capensis* males and then backcrossing progeny of these F₁ queens with either *capensis* or *scutellata* males (Figure 1). If our hypothesis is correct, then we can make the following predictions: 1) *capensis* parentals (mainly *ll*) should show higher expression of the traits than *scutellata* parentals (*LL*); 2) the F₁'s (mainly *Ll*) should be phenotypically more similar to the *scutellata* parentals than the *capensis* parentals; and 3) in colonies backcrossed to *scutellata* males, workers would then be either *Ll* or *LL* and should be more similar to *scutellata*

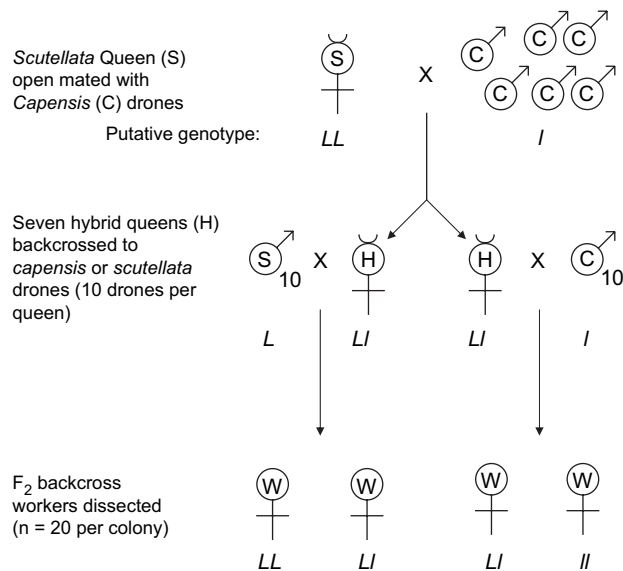


Figure 1. Backcross scheme and hypothesized genotypes of workers and queens at a hypothesized locus *Larva*, which influences the rate at which larvae are fed and thereby their reproductive morphology.

parentals than *capensis* parentals. In colonies backcrossed to *capensis* males, the workers would be either *ll* or *Ll* and should express *capensis*-like traits or *scutellata*-like traits. Depending on the dominance relationship between the 2 alleles, these colonies should show intermediate phenotypes between *capensis* and *scutellata* parentals or should have much greater variance in phenotypic scores than any other group.

Methods

Scutellata virgin queens were reared in 3 *scutellata* colonies that originated from Douglas (lat $-26^{\circ}02'$, long $29^{\circ}37'$), Northern Cape, South Africa. These queens were open mated from drone-free *scutellata* mating hives with *capensis* drones in Stellenbosch (lat $-33^{\circ}93'$, long $18^{\circ}85'$), Western Cape, a region where *scutellata* drones do not occur naturally (Allsopp MH, personal observations). The mating site contained 8 *capensis* colonies that were producing drones. We raised 3 hybrid virgin queens from these colonies. These virgin queens were then backcrossed with either *scutellata* (originating from Douglas) or *capensis* (originating from Stellenbosch) drones using artificial insemination (Harbo 1986). We used an average of 10 males per queen, collected from 1 *capensis* and 1 *scutellata* colony (Figure 1). (We chose not to use single drones for the matings because queens mated to single males have much reduced longevity.) Backcrossed queens were then introduced into insemination nucleus colonies (which comprised *scutellata* workers to reduce the incidence of absconding and queen rejection) and allowed to lay eggs for a month so that all emerging workers were offspring of the backcrossed queens, but had been

reared by *scutellata* workers used to populate the insemination nuclei. We then collected adult workers from each backcross colony (3 *capensis* backcross colonies and 4 *scutellata* backcross colonies), 3 parental *scutellata* colonies, 3 F₁ colonies, and 3 randomly selected parental *capensis* colonies.

We dissected 20–40 of these adult workers from each colony. We pinned each worker onto a wax plate through the thorax and separated the fifth and sixth dorsal tergites using fine forceps to expose the reproductive organs, under irrigation with water. In workers, the section of the ovary containing ovarioles is positioned above the hindgut and spermatheca below the hindgut (Dade 1977). To count an individual's ovarioles, both ovaries were gently removed with forceps, placed onto a microscope slide, and covered with a drop of water and a coverslip and the number of ovarioles were counted under a dissecting microscope. The number of ovarioles in the left and right ovaries was highly correlated (Pearson's correlation; $r = 0.865$, $P < 0.01$). Therefore, only left ovarioles were used in further analysis. Spermathecae were exposed by removing the hindgut and were then measured in situ using an eyepiece graticule fitted to a dissecting microscope. Finally, we counted the number of basitarsal hairs between the most posterior and second-most posterior pollen combs on the left hind leg, again using a dissecting microscope.

Results

Workers in the parental *capensis* colonies had significantly larger numbers of ovarioles and more basitarsal hairs than the *scutellata* parental workers (Figure 2). Spermathecae were completely absent from all *scutellata* parental workers but were present in 8.3% of workers from the *capensis* parental colonies.

The F₁ colonies were intermediate between the 2 parentals for the number of ovarioles and the number of basitarsal hairs but had significantly larger spermathecae than either parental (Figure 2), with 20.0% of F₁ workers showing spermathecae.

Workers of the *capensis* backcross colonies had the highest number of basitarsal hairs and showed the greatest expression of spermathecae (Figure 2), with 75.0% of individuals having spermathecae. Workers of the *scutellata* backcross colonies were intermediate between the *capensis* and *scutellata* parental colonies for number of ovarioles and basitarsal hairs and not significantly different from the F₁ colonies. In one *scutellata* backcross colony, 75.0% of workers had a spermatheca, but no workers had a spermatheca in the other 2 *scutellata* backcross colonies. Thus, overall, 27.1% of workers in *scutellata* backcross colonies had a spermatheca present, and the *scutellata* backcross colonies were not significantly different from the F₁ colonies (Figure 2).

The variance of phenotypes observed among worker progeny was higher in the *capensis* backcross colonies than in the *scutellata* backcross colonies for the number of basitarsal hairs and the size of the spermatheca, but not for the

number of ovarioles (see standard error bars in Figure 2). To determine if workers in the *capensis* backcross colonies showed a significantly greater range of phenotypes than did workers in the *scutellata* backcross colonies, we performed 1-way analyses of variance (ANOVAs) of untransformed data for both backcross sire groups separately for each character. The residual mean square from these ANOVAs provides an estimate of interworker variability, σ^2 s, after removing colony effects, which include maternal and paternal genetic effects and any colony-specific environmental effects (Oldroyd et al. 1991; Moritz and Southwick 1992). We then determined if the *capensis* backcross workers were more variable than the *scutellata* backcross workers by calculating $L = \sigma_c^2 / \sigma_s^2$, where the subscript c denotes *capensis* and s *scutellata* and σ^2 is the error mean square. L is distributed as F with the respective degrees of freedom of the 2 σ^2 s. By this measure, *capensis* backcrossed workers were significantly more variable than *scutellata* backcross colonies for number of basitarsal hairs ($F_{57,96} = 14.55$, $P < 0.001$) and the size of spermatheca ($F_{57,96} = 2.08$, $P < 0.001$), but not for the number of ovarioles ($F_{57,96} = 1.01$, $P = 0.49$).

Pairwise Spearman's rank correlations were significant for all pairs of characters (ovariole–basitarsal hairs: $r = 0.600$, $P < 0.01$; ovariole–spermatheca: $r = 0.649$, $P < 0.01$; spermatheca–basitarsal hairs: $r = 0.859$, $P < 0.01$), showing that the 3 traits are phenotypically correlated. As a test of pleiotropy, we designated individuals with >6 ovarioles, >5 basitarsal hairs, or the presence of any spermatheca as showing “high” expression of these traits and all individuals with less than these thresholds as showing “low” expression. We then analyzed the data from backcross colonies only (with standard rearing environment) to determine if the 3 traits were coinherited.

For all pairs of characters, we constructed 2×2 contingency tables to determine if individuals showing high or low expression of one trait also showed high or low expression of the other. There was a strong coexpression of the 3 traits: $\chi_1^2 = 10.24$, $P < 0.001$, for ovarioles and basitarsal hairs; $\chi_1^2 = 17.98$, $P < 0.001$, for ovarioles and spermatheca; and $\chi_1^2 = 76.33$, $P < 0.001$, for basitarsal hairs and spermatheca.

Discussion

Our results not only are consistent with a strong genetic component to the expression of traits associated with reproductive potential in *capensis* workers but also suggest a strong environmental (rearing environment) component. The number of basitarsal hairs and the size of the spermatheca were strongly phenotypically correlated across the experimental populations. The number of ovarioles was also correlated with the other 2 traits, but the correlation was less strong. This suggests that the number of basitarsal hairs and the presence and size of the spermatheca are influenced pleiotropically by the same genetic determinants or by similar environmental influences. The number of ovarioles is also influenced by these same factors, but to a lesser degree.

Consistent with earlier studies (Ruttner 1988; Allsopp et al. 2003), *capensis* parental workers had nearly 3 times the number of ovarioles as *scutellata* parental workers. Allsopp et al. (2003) showed that the rearing environment has little effect on the number of ovarioles found in the workers. Thus, variance in the number of ovarioles between *capensis* and other honeybees is primarily genetic. F_1 and *scutellata*

backcross workers were intermediate between the 2 parentals, whereas the *capensis* backcross individuals approached—but did not equal—their parental type. This pattern of inheritance is consistent with an additive, possibly quantitative pattern of inheritance of ovariole number. It is not consistent with a simple Mendelian pattern based on a single locus with complete dominance because F_1 's were intermediate between the parentals. It also suggests a limited role of rearing environment in the expression of ovariole number.

The inheritance of the other 2 traits, the number of basitarsal hairs, and the presence and size of the spermatheca show a very different pattern. Workers from *capensis* backcross colonies had nearly 4 times the number of basitarsal hairs than the highest parental (*capensis*). Similarly, spermathecae were absent in *scutellata* parentals, present in low frequency in *capensis* parentals, and appeared at 10 times the frequency in *capensis* backcross colonies. Expression of this trait was also highly variable among *scutellata* backcross colonies. Combined, these results suggest a strong genotype by environment interaction affecting the expression of these traits. Possibly, expression of these traits is influenced by some sort of epigenetic factor that is itself influenced by larval nutrition. The honeybee has a functioning DNA methylation system that may turn out to be important in the regulation of reproductive characters (Wang et al. 2006).

We suggest that these latter results for the spermatheca and basitarsal hairs are not inconsistent with our hypothesized single locus (*Larva*) that affects the expression of queen-like characteristics in honeybee workers, in which *ll* individuals develop more queen-like traits than either *Ll* or *LL* individuals. Considering first the backcross colonies, we note that, in colonies backcrossed to *capensis*, males, workers showed significantly higher expression and variance among individuals than did workers in the *scutellata* backcross colonies. This is consistent with individuals in the *scutellata* backcross colonies being either *LL* or *Ll* and therefore failing to express the more queen-like phenotypes. In colonies that were sired by *capensis* males, workers were either *Ll* or *ll* and thus expressed a much greater range of phenotypes (Figure 2), with some individuals showing large numbers of basitarsal hairs and well-developed spermathecae.

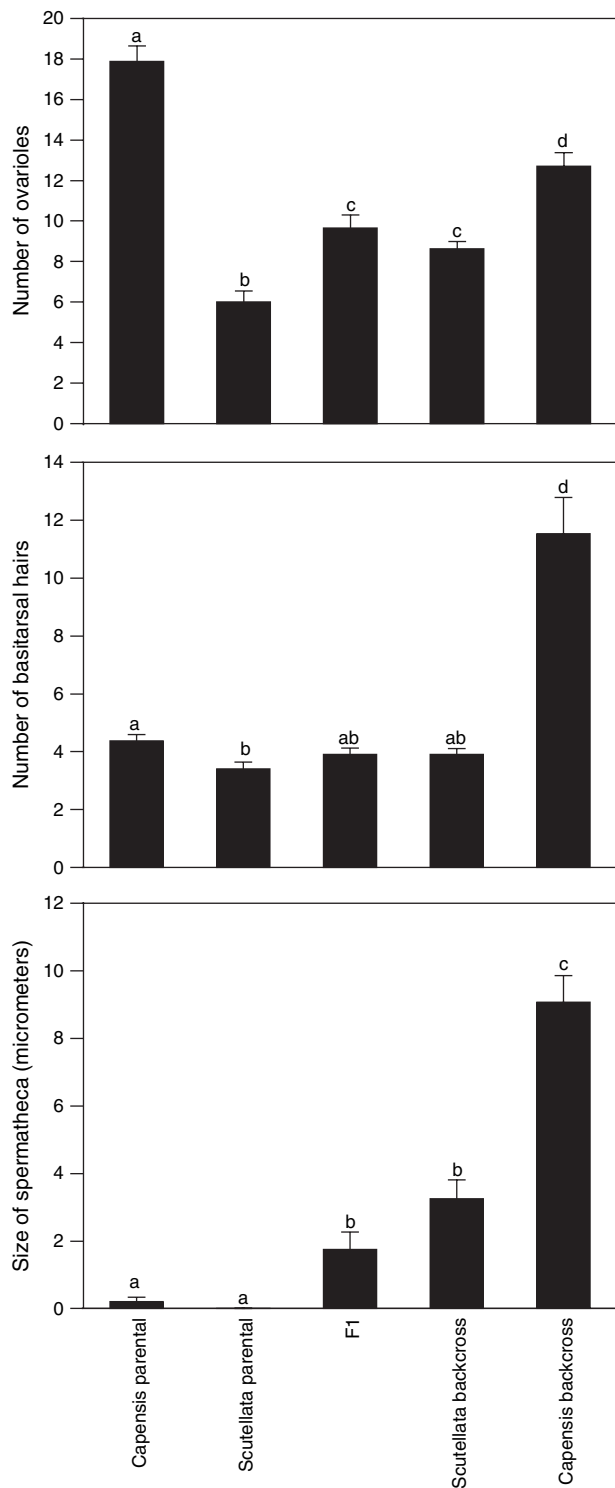


Figure 2 Mean number of ovarioles and basitarsal hairs and size of spermatheca in parental *Apis mellifera capensis* (3 colonies, 20 workers per colony), parental *Apis mellifera scutellata* (3 colonies, 20 workers per colony), F_1 (3 colonies, 20 workers per colony), *A. m. scutellata* backcross (4 colonies, 20 workers per colony for 3 colonies, 40 workers for 1 colony), and *A. m. capensis* backcross (3 colonies, 20 workers per colony). Error bars indicate standard errors of the means. Bars with a different letter are significantly different at the 5% level (after Bonferroni correction for 30 independent comparisons) based on Mann–Whitney *U* tests. Individuals without a spermatheca were scored as zero.

How might the putative *Larva* locus act? A plausible suggestion is that the *Larva* locus influences the amount of food a larva receives, which in turn influences the degree to which the individual expresses queen-like traits (Haydak 1943; Dietz and Haydak 1971). We hypothesize that // larvae release cues (probably pheromonal) that strongly signal nurse workers to feed them. *LL* and *Ll* larvae do not elicit feeding responses in nurse workers to the same degree. This hypothesis is consistent with the fact that *capensis* worker larvae solicit more larval food than do *scutellata* larvae (Beekman et al. 2000; Calis et al. 2002; Allsopp et al. 2003). A locus that influences the amount of food a larva receives explains how a single locus can influence more than one trait pleiotropically: // individuals become more queen-like because they are fed more like queens.

The genotype of nurse workers is also known to be important to the amount of food that larvae receive: *scutellata* workers have a lower threshold for feeding larvae, and so *capensis* larvae receive more food when fed by *scutellata* workers than when fed by *capensis* workers (Allsopp et al. 2003). This difference may explain why workers in *capensis* backcross colonies showed higher mean than either parental or the F_1 's: these workers had been reared by *scutellata* nurse workers and hence both spermathecae size and basitarsal hairs are more strongly expressed. This demonstrates that a 2-fold process is necessary for the full expression of *capensis*' reproductive potential: the excessive "feed-me" pheromonal signal of *capensis* larvae and the overzealous response of *scutellata* workers.

We need to point out that the observed larger within-colony variance in *capensis* backcross colonies relative to *scutellata* backcross colonies should be treated with some caution. The larger intracolony variance in *capensis* backcross colonies may be due in part to the larger mean. Indeed, if we correct for the larger mean by comparing the squared coefficients of variation (Lewontin 1966; Lande 1977), the *scutellata* backcross colonies show greater within-colony variance than the *capensis* backcross colonies. This is most likely caused by the coefficients of variation being large: they should not exceed 30% for this test to be valid (Lewontin 1966; Lande 1977). In addition, our test of variance assumes a normal distribution, and this assumption is violated with respect to spermathecal size, which is primarily a threshold character (presence or absence).

Lattorff et al. (2007) demonstrated that a single gene, *Thelytoky*, pleiotropically controls thelytoky, the production of 9-ODA and age at onset of oviposition. It seems plausible that this same gene might affect the number of ovarioles, another important component of reproductive potential in *capensis*. This possibility remains to be investigated. It is less likely that *Thelytoky* affects the size of the spermatheca or the number of basitarsal hairs (i.e., *Larva* and *Thelytoky* are the same) because expression of these genes, while clearly having a large genetic component, is also strongly influenced by larval feeding.

Our study emphasizes the complexity of inheritance in social insects. More so than in nonsocial animals, genotype not only affects individuals directly but also affects the social

environment in which individuals are reared. The 2 may strongly interact, as we suspect happens in this case.

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References

- Allsopp MH, Calis JNM, Boot WJ. 2003. Differential feeding of worker larvae affects caste characters in the Cape honeybee, *Apis mellifera capensis*. *Behav Ecol Sociobiol.* 54:555–561.
- Beekman M, Calis JNM, Boot WJ. 2000. Parasitic honeybees get royal treatment. *Nature.* 404:723.
- Beekman M, Oldroyd BP. 2008. When workers disunite: intraspecific parasitism in eusocial bees. *Annu Rev Entomol.* 53:19–37.
- Calis JNM, Boot WJ, Allsopp MH, Beekman M. 2002. Getting more than a fair share: nutrition of worker larvae related to social parasitism in the Cape honey bee *Apis mellifera capensis*. *Apidologie.* 33:193–202.
- Dade HA. 1977. Anatomy and dissection of the honeybee. London: International Bee Research Association.
- Dietz A, Haydak MH. 1971. Caste determination in honey bees. 1. The significance of moisture in larval food. *J Exp Biol.* 177:353–358.
- Greeff JM. 1996. Effects of thelytokous worker reproduction on kin-selection and conflict in the Cape honeybee, *Apis mellifera capensis*. *Philos Trans R Soc Lond B.* 351:617–625.
- Hamilton WD. 1972. Altruism and related phenomena, mainly in social insects. *Annu Rev Ecol Syst.* 3:193–232.
- Harbo JR. 1986. Propagation and instrumental insemination. In: Rinderer TE, editor. *Bee genetics and breeding*. Orlando (FL): Academic Press. p. 361–389.
- Haydak MH. 1943. Larval food and development of castes in the honeybee. *J Econ Entomol.* 36:778–792.
- Jordan LA, Allsopp MH, Oldroyd BP, Wossler TC, Beekman M. 2008. Cheating honeybee workers produce royal offspring. *Proc R Soc Lond B.* 275:345–351.
- Lande R. 1977. On comparing coefficients of variation. *Syst Zool.* 26:214–217.
- Lattorff HMG, Moritz RFA, Crewe RM, Solignac M. 2007. Control of reproductive dominance by the *thelytoky* gene in honeybees. *Biol Lett.* 3:292–295.
- Lattorff HMG, Moritz RFA, Fuchs S. 2005. A single locus determines thelytokous parthenogenesis of laying honeybee workers (*Apis mellifera capensis*). *Heredity.* 94:533–537.
- Lee HY, Adler PN. 2004. The grainy head transcription factor is essential for the function of the frizzled pathway in the *Drosophila* wing. *Mech Dev.* 121:37–49.
- Lewontin RC. 1966. On the measurement of relative variability. *Syst Zool.* 15:141–142.

- Mackensen O. 1943. The occurrence of parthenogenetic females in some strains of honey-bees. *J Econ Entomol.* 36:465–467.
- Moritz RFA, Simon UE, Crewe RM. 2000. Pheromonal contest between honeybee workers (*Apis mellifera capensis*). *Naturwissenschaften.* 87:395–397.
- Moritz RFA, Southwick EE. 1992. Bees as superorganisms. Berlin (Germany): Springer-Verlag.
- Oldroyd BP, Rinderer TE, Buco S. 1991. Heritability of morphological characters used to distinguish European and Africanized honey bees. *Theor Appl Genet.* 82:499–504.
- Oldroyd BP, Wongsiri S. 2006. Asian honey bees. Biology, conservation and human interactions. Cambridge (MA): Harvard University Press.
- Ratnieks FLW. 1988. Reproductive harmony via mutual policing by workers in eusocial Hymenoptera. *Am Nat.* 132:217–236.
- Ruttner F. 1977. The problem of Cape bee (*Apis mellifera capensis* Escholtz): parthenogenesis—size of population—evolution. *Apidologie.* 8:281–294.
- Ruttner F. 1988. Biogeography and taxonomy of honeybees. Berlin (Germany): Springer-Verlag.
- Tucker KW. 1958. Automictic parthenogenesis in the honey bee. *Genetics.* 43:299–316.
- Verma LR, Ruttner F. 1983. Cytological analysis of the thelytokous parthenogenesis in the Cape honeybee (*Apis mellifera capensis* Escholtz). *Apidologie.* 14:41–57.
- Wang Y, Jorda M, Jones PL, Maleszka R, Ling X, Robertson HM, Mizzen CA, Peinado MA, Robinson GE. 2006. Functional CpG methylation system in a social insect. *Science.* 314:645–647.
- Winston ML. 1987. The biology of the honey bee. Cambridge (MA): Harvard University Press.
- Wossler TC. 2002. Pheromone mimicry by *Apis mellifera capensis* social parasites leads to reproductive anarchy in host *Apis mellifera scutellata* colonies. *Apidologie.* 33:139–163.

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