

Cross-species correlation between queen mating numbers and worker ovary sizes suggests kin conflict may influence ovary size evolution in honeybees.

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Abstract:

During social evolution, the ovary size of reproductively specialized honey bee queens has dramatically increased while their workers have evolved much smaller ovaries. However, worker division of labor and reproductive competition under queenless conditions are influenced by worker ovary size. Little comparative information on ovary size exists in the different honey bee species. Here, we report ovariole numbers of freshly dissected workers from six *Apis* species from two locations in Southeast Asia. The average number of worker ovarioles differs significantly among species. It is strongly correlated with the average mating number of queens, irrespective of body size. *Apis dorsata*, in particular, is characterized by numerous matings and very large worker ovaries. The relation between queen mating number and ovary size across the six species suggests that individual selection via reproductive competition plays a role in worker ovary size evolution. This indicates that genetic diversity, generated by multiple mating, may bear a fitness cost at the colony level.

Keywords: social evolution | levels of selection | worker reproduction | caste divergence | reproductive conflict | ovarioles | social insects | biology | *apis dorsata* | honey bees

Article:

Introduction

Lifetime egg production varies drastically among insect species, with maximum estimates in the reproductive queen caste of some eusocial insects. These specialized reproductives act as universal stem cells of their colonies, producing the colony workforce and new sexuals of the next generation. In several taxa of social insects, the reproductive specialization of queens has

increasingly led to enlarged ovaries with numerous ovariole filaments while workers have comparatively small or no ovaries (Bourke 1999).

In the Western honey bee, *Apis mellifera*, queen ovaries regularly exceed 140 ovarioles, while worker ovaries contain generally less than 12 ovarioles (Snodgrass 1956). However, the ovariole number of *A. mellifera* workers is highly variable, varying among (Ruttner and Hesse 1981) and within populations (Linksvayer et al. 2009; Phiancharoen et al. 2010). This variability may be due to selection: On the one hand, variation in worker ovary size is part of the pollen-hoarding syndrome, influencing multiple aspects of the division of labor among workers (Page and Amdam 2007). For example, workers with experimentally enlarged ovaries initiate foraging earlier (Wang et al. 2010). On the other hand, workers with relatively larger ovaries have a reproductive advantage when the queen is absent, and workers compete for individual reproduction because workers with more ovarioles activate their ovaries faster and produce more offspring (Makert et al. 2006). The reproductively active *A. mellifera capensis* workers also have more ovarioles than workers of other subspecies (Phiancharoen et al. 2010).

In queenright colonies, workers normally cooperate, and colony-level selection acts to increase the efficiency of the division of labor among all workers (Oster and Wilson 1978). Genetic variation in worker ovary size may be maintained by colony-level selection on the division of labor (Graham et al. 2011). In addition, selection at the colony level against large worker ovaries is expected because large ovaries are physiologically costly to build and sustain. Conversely, queenless workers compete for individual reproduction, and selection at the individual level is predicted to favor large worker ovaries that lead to a fast activation and more egg production (Makert et al. 2006). This individual-level selection becomes more intense with the presence of multiple patrines per colony because workers are three times more related to male offspring produced by their full sisters (within one patriline) than to male offspring produced by their half-sisters (from a different patriline).

Thus, we hypothesize that worker ovary size is subject to conflicting selection pressures at the colony and individual levels and that the evolutionary outcome is influenced by the amount of reproductive conflict among workers. Therefore, a positive correlation between worker ovary size and the number of patrines is predicted. We decided to test this prediction in the genus *Apis*, because all honeybees share a relatively similar biology (Oldroyd and Wongsiri 2006), reducing confounding factors that vary among taxa, such as trophic egg production by workers (Khila and Abouheif 2008). However, few data on worker ovaries exist except for *A. mellifera*: A limited study has indicated that *Apis cerana* workers have typically five to eight ovarioles per ovary and *Apis dorsata* have an average of 24 (Velthuis et al. 1971). Thus, we have determined worker ovary sizes for all accessible six *Apis* species from two biogeographic areas and correlated it to the average queen mating number of these species.

Methods and materials

Worker honeybees from multiple colonies of six species (*Apis florea*, *Apis andreniformis*, *A. cerana*, *A. mellifera*, *Apis koschevnikovi*, and *A. dorsata*) were collected from the vicinity of the Agricultural Research Station Tenom (Sabah, Malaysia: 5.4° N/115.6° E) or the Ratchaburi (13.4° N/99.4° E), Tak (16.9° N/99.1° E), and Chiang Mai (18.9° N/99.1° E) provinces in Thailand (see Electronic Supplementary Material). Adult worker bees were randomly sampled from colonies, except for the "Tak" samples of *A. florea* and *A. dorsata*, which consisted of newly emerged workers. Bees were euthanized by cooling before both ovaries were dissected out of the detached abdomen and mounted on a microscope slide for counting of the ovarioles under a compound microscope. Ovary size was computed as the average of the ovariole counts from the left and right ovary. If ovariole number could only be determined on one side, this value was used. Asymmetry (Table 1) was calculated as the difference between the two ovaries divided by their sum.

Ovary size was also evaluated relative to worker body size, estimated as the species' average forewing length given by Oldroyd and Wongsiri (2006). For *A. mellifera*, the average forewing length of European races (Daly and Balling 1978) was used because European populations are presumably the source of Thai *A. mellifera* (Suppasata et al. 2007). We compared original and body-size-adjusted ovary size to the average observed mating number of the respective species (Tarpay et al. 2004). The small number of taxa precluded a meaningful adjustment of these comparisons for a potential phylogenetic signal (Blomberg et al. 2003). Non-parametric statistics were employed, using the computer program PASW 18.0, and uncorrected p values are presented throughout.

Results

The ovariole number of 596 worker honey bees from 37 colonies of six species was determined, with values ranging across species from one to 44 per ovary. No significant difference between the number of ovarioles of the left and right ovary was detected in *A. florea* (sign test, N = 35, p = 0.719), *A. andreniformis* (N = 32, p = 0.571), *A. cerana* (N = 90, p = 0.368), *A. koschevnikovi* (N = 33, p = 0.281), and *A. dorsata* (N = 20, p = 1.0), indicating the absence of directional asymmetry. No data were available to test directional asymmetry in *A. mellifera*.

Table 1
Species means of ovary size and asymmetry with standard errors

Species	Number of workers assessed	Ovary size	Ovary asymmetry
<i>A. florea</i>	47	3.5 ± 0.2	0.17 ± 0.02

Species	Number of workers assessed	Ovary size	Ovary asymmetry
<i>A. andreniformis</i>	77	4.8 ± 0.2	0.18 ± 0.02
<i>A. cerana</i>	161	5.2 ± 0.2	0.21 ± 0.01
<i>A. mellifera</i>	60	4.3 ± 0.2	0.21 ± 0.02
<i>A. koschevnikovi</i>	59	6.7 ± 0.3	0.17 ± 0.02
<i>A. dorsata</i>	192	22.6 ± 0.4	0.09 ± 0.01

Species differences in ovary size were significant at both locales (Malaysia, $H = 257.4$, $df = 3$, $N = 350$, $p < 0.001$; Thailand, $H = 150.1$, $df = 3$, $N = 246$, $p < 0.001$). In Malaysia, post hoc tests indicated that all species are significantly different from each other, except the *A. cerana* and *A. andreniformis* pairing. In Thailand, all pairwise comparisons indicated significant species differences except for the differences between *A. florea* and *A. mellifera* and between *A. mellifera* and *A. cerana* (see Electronic Supplementary Material). Within species, differences between the two locales were significant in *A. dorsata* ($U = 2,456$, $N = 192$, $p < 0.001$) but not in *A. cerana* ($U = 3,622$, $N = 161$, $p = 0.194$).

Across species, the average queen mating number was significantly correlated with the average ovariole number (Fig. 1; Spearman's $\rho = 0.94$, $N = 6$, one-sided $p = 0.0025$). The data also indicated a tentative correlation between body size and ovary size, although this was not significant ($\rho = 0.66$, $N = 6$, $p = 0.156$). Body size adjustment weakened the correlation between worker ovary size and queen mating number ($\rho = 0.77$, $N = 6$, one-sided $p = 0.036$). In both locales, significant species differences in relative ovary size were found (Malaysia, $H = 251.9$, $df = 3$, $N = 350$, $p < 0.001$; Thailand, $H = 144.5$, $df = 3$, $N = 246$, $p < 0.001$) which was due to the significantly larger values of *A. dorsata* relative to all other species (all pairwise comparisons, $p < 0.001$): The body-size-adjusted ovary size of *A. dorsata* workers was on average 2.7–3.8 times larger than that of other species at both locations.

Figure 1 is omitted from this formatted document.

Discussion

The results confirm the predicted positive correlation between the average number of ovarioles in workers and the average mating frequency of queens across six honey bee species, independent of worker body size. This finding supports the hypothesis that worker ovary size may be influenced by individual-level selection due to the positive associations between ovary

size, ovary activation, and individual reproduction of workers under queenless conditions (Makert et al. 2006). Individual selection for reproduction predicts an increase of ovary size with the number of patrines per colony due to the increasing number of competitors that are half-sisters. In contrast, we cannot explain our result based on colony-level selection. The exceptionally large worker ovaries of *A. dorsata* strengthen our interpretation further because very few workers assume a reproductive role under queenless conditions in this species (Velthuis et al. 1971) which intensifies the individual reproductive competition. The strength of the correlation between worker ovary size and queen mating number, the specific comparisons discussed below, and the sampling of all three major clades of the genus bolster our main conclusion, although a meaningful correction for a potential phylogenetic bias in the overall correlation is precluded by the small number of honey bee taxa (Blomberg et al. 2003). In addition, ovary size did not strictly correlate with phylogeny or with the fact that some species nest in the open while others nest in cavities.

The exceptionally large worker ovaries of *A. dorsata* cannot be explained by body size or a general increase in ovary size in this species because queen ovaries are relatively small (Velthuis et al. 1971). However, *A. dorsata* has an exceptionally high queen mating number compared with other *Apis* species (Wattanachaiyingcharoen et al. 2003), which could select for larger ovaries via worker reproductive conflict. A general reproductive role of these large ovarioles can be excluded because there is no genetic evidence for *A. dorsata* worker reproduction under queenright conditions (Wattanachaiyingcharoen et al. 2002), and accordingly, none of our investigated worker ovaries showed any sign of reproductive activation. An alternative explanation that cannot be ruled out might be the role of the large worker ovaries in nutrient storage (Amdam and Omholt 2002; Martins et al. 2008) to facilitate the species' migratory behavior (Itioka et al. 2001).

Among the tree-cavity-nesting species, *A. koschevnikovi* had the largest worker ovaries followed by *A. cerana*, and then *A. mellifera*. No information on worker reproduction is available in *A. koschevnikovi*, but *A. cerana* workers are reported to activate their ovaries more readily than *A. mellifera* under queenright and queenless conditions (Oldroyd et al. 2001; Tan et al. 2009), which may be the reason for their larger worker ovary size. Alternatively, the relatively small worker ovaries in *A. mellifera* could also result from artificial selection for increased honey yield during domestication due to the association between small ovaries and nectar collection in honeybee workers (Amdam et al. 2006; Rueppell et al. 2008).

The smallest ovaries were found in the dwarf honey bee, *A. florea*. This species is similar in body size and biology to *A. andreniformis* (Hepburn et al. 2005; Higgs et al. 2010), but *A. andreniformis* has relatively large ovaries, compared to its body size (see Electronic Supplementary Material). We were unable to compare the two species in the same environment, but between the two locations, *A. florea* had significantly smaller ovaries than *A. andreniformis* with or without body size correction. The significance of this difference is unclear because worker reproduction in mixed species colonies was not biased towards *A. andreniformis*

(Wongvilas et al. 2010), and natural rates of worker reproduction in both species are unknown. Yet, the larger worker ovaries coincide with a higher number of matings in *A. andreniformis* relative to *A. florea*, supporting our main conclusion.

For each species, only a limited number of colonies could be sampled. However, colony differences were only significant in *A. dorsata* (see Electronic Supplementary Material). Although this colony effect was significant, changes of its magnitude of ± 4 ovarioles would not affect any of our conclusions. Population differences were also indicated only in *A. dorsata*. Therefore, we combined the data from colonies and treated worker ovary size as a species-specific trait, irrespective of sampling location. Our study was most severely limited by the number of species available because none of the remaining three to four *Apis* species was available from our study sites and has a known queen mating frequency. This resulted in a limited power of our analysis which allowed us to only detect the very strong correlation between ovary size and mating frequency, while the correlation between body size and ovary size remained suggestive. Therefore, we analyzed both body-size corrected and uncorrected ovary sizes.

Directional asymmetry for ovary size has been reported in *A. mellifera* (Chaud-Netto and Bueno 1979), but we could not confirm this result in five other *Apis* species. The degree of asymmetry differed significantly between *A. dorsata* and all other species, but the direction depended on the specific asymmetry metric used (see Electronic Supplementary Material). Thus, the differences largely result from differences in ovary size, and therefore, we conclude in general that there is little evidence for size-independent evolutionary divergence in ovary asymmetry among the six investigated *Apis* species.

In sum, we found a strong, positive correlation between worker ovary size and queen mating number that we had predicted based on individual-level selection for reproduction under queenless conditions. Together with previous studies (Makert et al. 2006; Page and Amdam 2007), this suggests that the honeybee worker ovary is under contrasting, multi-level selection. Thus, worker ovary sizes that are individually selected for may be sub-optimal at the colony level. This suggests a possible fitness cost of multiple mating at the colony level because the impact of the individual level of selection increases with multiple mating.

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Wongvilas S, Higgs JS, Beekman M, Wattanachaiyingcharoen W, Deowanish S, Oldroyd BP (2010) Lack of interspecific parasitism between the dwarf honeybees *Apis andreniformis* and *Apis florea*. *Behav Ecol Sociobiol* 64:1165–1170

Electronic Supplementary Material

Single colony data are presented in Table S1. However, colony identity (Figure S1) showed only a significant effect on worker ovary size in *Apis dorsata* in Malaysia ($H = 32.6$, $df = 2$, $N = 132$, $p < 0.001$) and Thailand ($H = 18.4$, $df = 2$, $N = 60$, $p < 0.001$). No significant colony effect was detected in *A. cerana* in Malaysia ($H = 9.2$, $df = 6$, $N = 82$, $p = 0.163$), *A. koshevnikovi* ($H = 4.8$, $df = 5$, $N = 59$, $p = 0.441$) and *A. florea* ($H = 9.1$, $df = 4$, $N = 82$, $p = 0.059$) before Bonferroni correction, and in Thai *A. cerana* ($H = 9.6$, $df = 3$, $N = 79$, $p = 0.022$), *A. andreniformis* ($H = 14.5$, $df = 5$, $N = 77$, $p = 0.012$), and *A. mellifera* ($H = 6.8$, $df = 2$, $N = 60$, $p = 0.033$) after Bonferroni correction. Therefore, we conducted all species comparisons with non-parametric tests without taking the colony level into account. However, a parametric, generalized linear model, nesting “locale” within “species”, and “colony” within “locale”, confirmed the results of significant species differences ($p < 0.001$) and colony level effects in *A. dorsata* ($p < 0.001$).

The two dwarf honeybees could only be compared between locales: *A. andreniformis* workers had significantly more ovarioles per ovary than workers of *A. florea* ($U = 980$, $N = 124$, $p < 0.001$). This difference was not affected by body size correction ($U = 1131$, $N = 124$, $p < 0.001$). The other pair of closely related species that could only be compared between the two different locales consisted of the cavity breeders *A. koshevnikovi* and *A. mellifera*. In this case, *A. koshevnikovi* workers displayed larger absolute ($U = 2795$, $N = 119$, $p < 0.001$) and relative ($U = 2883$, $N = 119$, $p < 0.001$) ovary sizes (Figure S2). Relative to forewing length, *A. mellifera*

workers had the smallest ovaries of all species (Figure S3), which is not due to exceptionally long forewings in this species (compare data in S1)

Species also significantly differed in the asymmetry between the ovaries on both sides of the workers, measured as the ratio ($H = 81.6$, $df = 5$, $N = 579$, $p < 0.001$), the absolute difference ($H = 91.9$, $df = 5$, $N = 579$, $p < 0.001$), or the relative difference ($H = 81.6$, $df = 5$, $N = 579$, $p < 0.001$) of the two sides. Post-hoc tests indicated that only *A. dorsata* was significant ($p \leq 0.002$) from all other species, but the direction of this difference depended on the asymmetry measurement (*A. dorsata* showed the largest absolute difference but smallest ratio and relative difference).

Supplement References:

S1: Oldroyd B.P., Wongsiri S. 2006 Asian Honey Bees: Biology, Conservation and Human Interactions. Cambridge: Harvard University Press.

Figure S1: Ovary size distributions across all 37 colonies from the six investigated *Apis* species

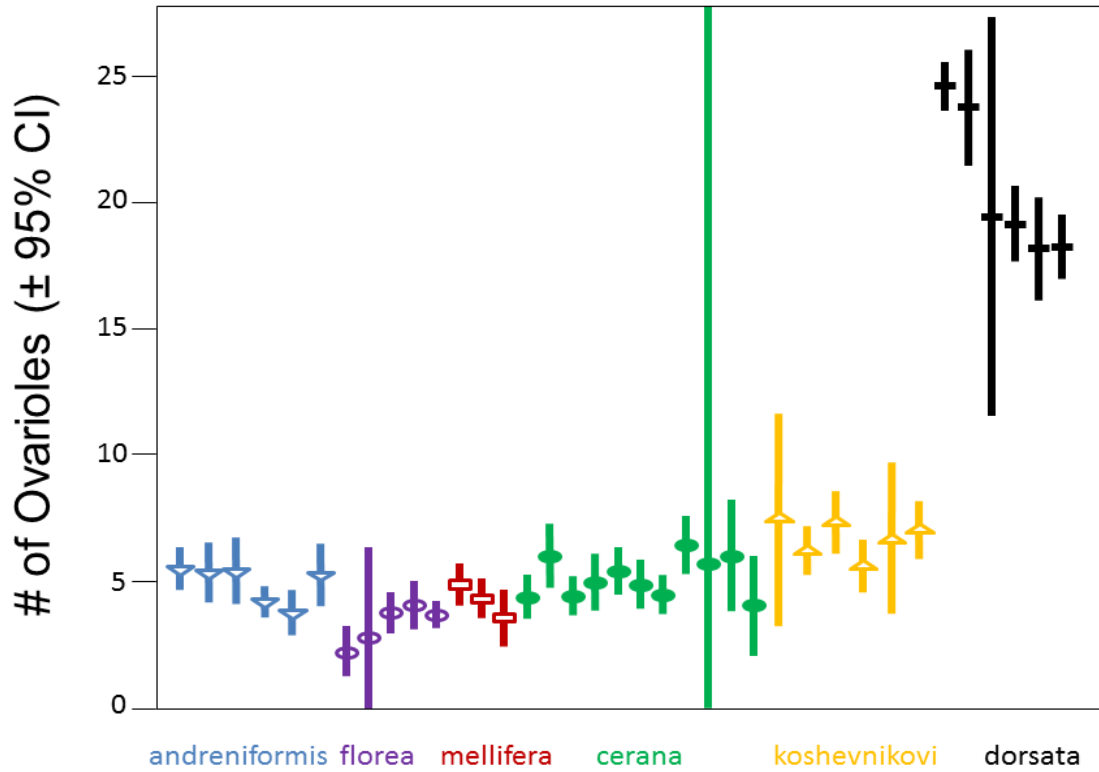


Figure S2: Species averages of worker ovary size in the locales “Thailand” and “Malaysia”

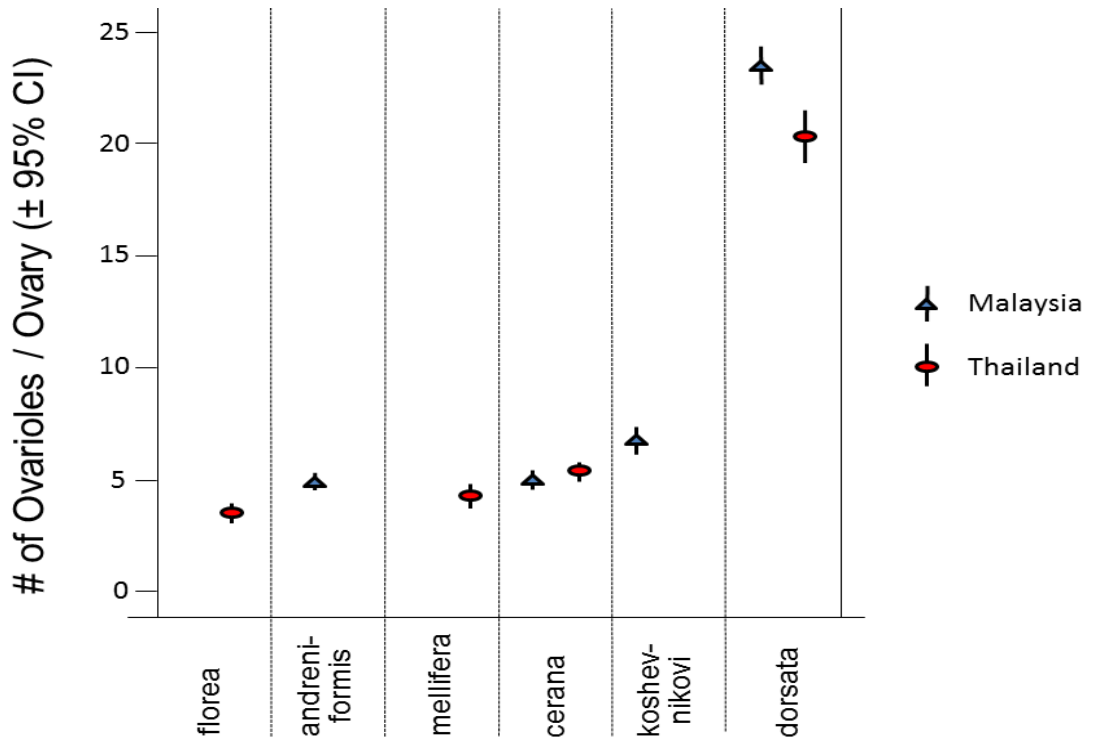


Figure S3: Species averages of worker ovary size relative to their forewing length.

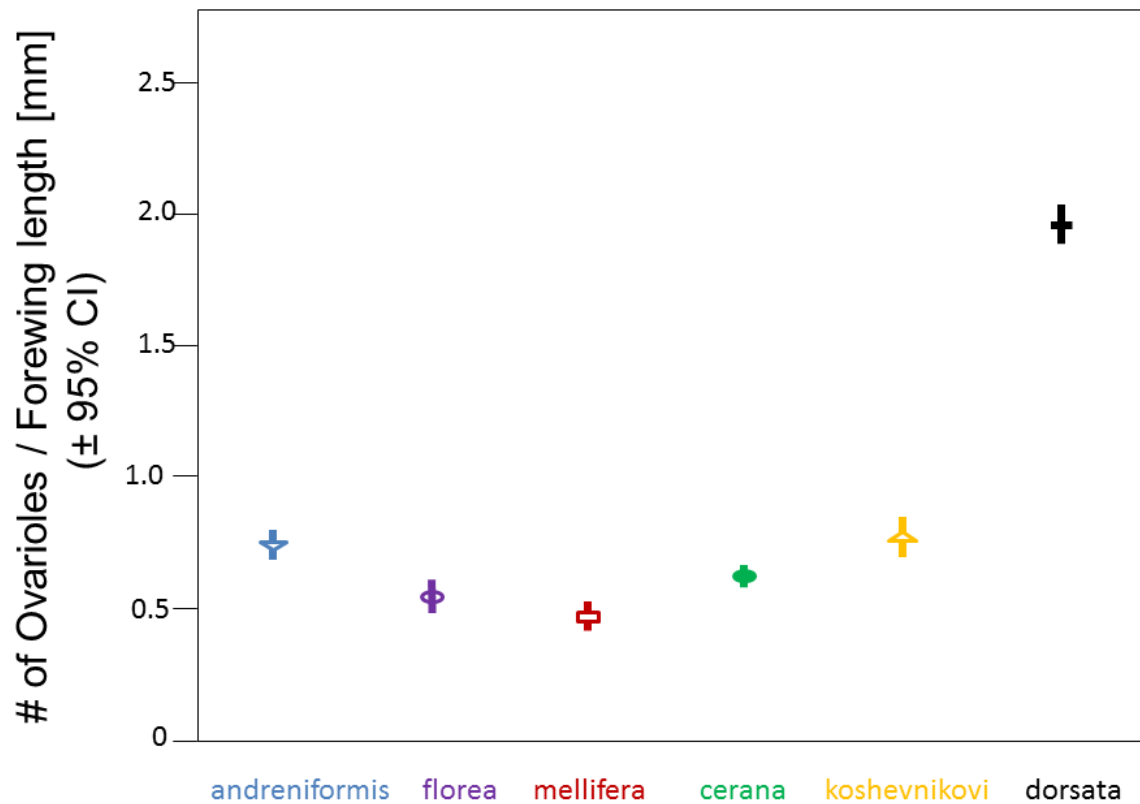


Table S1: Sampling information of the workers included in the study and basic results

Location	Species	Colony	Worker number	Average # of Ovarioles per Ovary*	Ovary asymmetry (relative)*
Malaysia (Tenom)	<i>A. cerana</i>	1	34	4.5 (3.8 – 5.3)	0.25 (0.18 – 0.31)
		2	2	5.8 (-22.8 – 34.3)	0.20 (-0.48 – 0.88)
		3	10	6.1 (3.9 – 8.2)	0.09 (0.05 – 0.13)
		4	4	4.1 (2.1 – 6.1)	0.16 (0.04 – 0.28)
		5	10	4.5 (3.6 – 5.3)	0.13 (0.08 – 0.18)

Malaysia (Tenom) continued		6	12	6.1 (4.9 – 7.3)	0.14 (0.11 – 0.18)	
		7	10	5.1 (4.0 – 6.1)	0.15 (0.08 – 0.21)	
	<i>A. andreniformis</i>	1	26	4.3 (3.7 – 4.8)	0.20 (0.13 – 0.27)	
		2	9	3.8 (3.0 – 4.7)	0.19 (0.09 – 0.28)	
		3	10	5.3 (4.1 – 6.5)	0.25 (0.12 – 0.37)	
		4	11	5.5 (4.8 – 6.3)	0.11 (0.05 – 0.16)	
		5	10	5.4 (4.2 – 6.6)	0.15 (0.09 – 0.21)	
		6	11	5.5 (4.2 – 6.7)	0.16 (0.12 – 0.20)	
	<i>A. koshevnikovi</i>	1	7	7.5 (3.2 – 11.8)	0.29 (0.05 – 0.53)	
		2	12	7.4 (6.1 – 8.7)	0.18 (0.09 – 0.26)	
		3	7	6.8 (3.8 – 9.8)	0.19 (-0.02 – 0.40)	
		4	11	6.3 (5.3 – 7.3)	0.13 (0.09 – 0.18)	
		5	11	5.7 (4.6 – 6.8)	0.11 (0.07 – 0.16)	
		6	11	7.1 (6.0 – 8.2)	0.15 (0.10 – 0.19)	
	<i>A. dorsata</i>	1	4	19.5 (11.6 – 27.4)	0.06 (0.02 – 0.10)	
		2	108	24.7 (23.7 – 25.6)	0.09 (0.07 – 0.10)	
		3	20	18.3 (17.1 – 19.5)	0.10 (0.08 – 0.12)	
	Thailand (Tak)	<i>A. florea</i>	1	8	2.3 (1.3 – 3.3)	0.56 (n=1)
			2	4	2.9 (-0.7 – 6.5)	0.09 (n=1)
<i>A. dorsata</i>		1	20	19.2 (17.8 – 20.6)	0.11 (0.07 – 0.14)	
		2	20	18.2 (16.2 – 20.3)	0.10 (0.08 – 0.13)	
		3	20	23.9 (21.6 – 26.1)	0.09 (0.05 – 0.13)	
Thailand (Chiang Mai)	<i>A. cerana</i>	1	19	4.5 (3.7 – 5.2)	0.29 (0.19 – 0.39)	
		2	20	5.0 (4.0 – 5.9)	0.26 (0.18 – 0.34)	
		3	20	5.5 (4.6 – 6.3)	0.17 (0.09 – 0.26)	
		4	20	6.5 (5.4 – 7.6)	0.25 (0.17 – 0.34)	
	<i>A. mellifera</i>	1	20	4.9 (4.1 – 5.7)	0.24 (0.14 – 0.33)	
		2	20	4.4 (3.6 – 5.1)	0.15 (0.09 – 0.21)	

		3	20	3.6 (2.6 – 4.7)	0.24 (0.19 – 0.29)
Thailand	<i>A. florea</i>	1	11	3.8 (3.0 – 4.6)	0.17 (0.11 – 0.23)
(Ratcha-		2	12	4.1 (3.2 – 5.1)	0.17 (0.11 – 0.23)
Buri)		3	12	3.8 (3.2 – 4.3)	0.15 (0.09 – 0.21)

* Averages are reported with 95% confidence intervals.