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Review article

Reproductive isolation among species of the genus *Apis*

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(Invited paper)

Abstract – In the 1960s, research on reproductive isolation in honeybees started with the pioneering work on *Apis cerana* and *A. mellifera* of F. Ruttner. Since then, the number of recognised *Apis* species increased from four to nine, and data on reproductive isolation played a key role in this development. In this paper, we discuss the behavioural mating barriers (mating season, mating place, sexual signals, daily mating periods), copulatory barriers (size, genitalia, mating sign) and physiological barriers (sperm transfer, sperm storage) and postzygotic barriers (fertilisation, development, hybrids). Allopatric *A. mellifera* and allopatric populations of the other species had a uniform mating period during the afternoon hours. Sympatric honeybee species were separated mainly by different daily mating periods. The mating period differed between populations of the same species from different regions. The sequence of the mating periods, however, described from Sri Lanka, Thailand and Sabah (Borneo) followed the same pattern and showed a taxonomic and size correlation: the dwarf bees (*A. andreniformis* and/or *Apis florea*) occupied the first position shortly after noon. The next mating period was occupied by cavity-dwelling bees and at sunset, *A. dorsata* drones flew out for mating. In addition, in the honeybee species that have been studied, various non behavioural mating barriers have been demonstrated.

reproductive isolation / *Apis* / mating behaviour / genitalia / hybrid

1. INTRODUCTION

Among evolutionary biologists, definitions of 'what a species is' are highly diverse. Furthermore, different species concepts have had a considerable influence on systematics [8, 23, 50]. In spite of these differences, however, traditional definitions acknowledge the central importance of reproductive isolation. Effective barriers to gene flow between populations prevent any possibility of subsequent reintegration. Thus

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This work is dedicated to the memory of the late Friedl Ruttner, whose unfailing enthusiasm for research on honeybee reproduction has been an inspiration to us.

achieving reproductive isolation becomes an evolutionary 'key event' which marks a point of independent genetic development and divergence.

In honeybees, research into reproductive isolation has had a relatively recent start. The Western honeybee species *Apis mellifera* L. with its wide, natural distribution in Europe, in the Middle East and Africa, and its successful introduction into the Americas and Australia has dominated scientific interest. Since the start of modern beekeeping in Europe [10] about 150 years ago, numerous honeybee colonies and queens have been imported from various places (Africa, the Middle East, the Mediterranean islands). These exotic bees, crossed with the local population, resulted in multiple hybrids, all of them fertile between one another without any apparent limitation [74]. This 'absence' of reproductive isolation lasted until 1965, when *A. cerana* Fabricius colonies from China were imported into Germany, and the first pioneering research on reproductive isolation among honeybees was initiated by F. Ruttner.

Traditionally, isolation mechanisms are categorised according to their temporal relation to fertilisation as prezygotic – or postzygotic barriers [50]. To accommodate the special mating biology of honeybees and the complicated process of multiple mating in the genus *Apis* [27], we divided the prezygotic phase into three subgroups of isolating factors. The following definitions apply to this paper (Tab. I): i) behavioural barriers operate early, and prevent the physical contact between queen and drone; ii) copulatory barriers operate during the process of copulation (from the first contact to the separation of the queen and the drone). These mechanisms prevent sperm transfer to the queen; iii) physiological barriers operate after copulation. These barriers block the path of the sperm from the queen's oviduct into the spermatheca and further to the fertilisation of the egg; iv) postzygotic barriers disturb normal development, resulting in the death or in the infertility of hybrids.

2. BEHAVIOURAL BARRIERS

2.1. Reproductive isolation due to seasonally different mating periods

Monogyny is one of the basic features of the honeybee colony. This links the rearing of new queens to the process of colony multiplication [69]. In other words, the mating season in honeybees is inevitably linked to the swarming season. Reproductive swarming depends on favourable environmental conditions. Specifically, ample pollen and nectar must be available for two reasons: to produce enough bees before colony fission, and to support the swarms which do not have combs or any honey storage at the beginning [84]. For survival, a new swarm needs more or less immediate access to nectar and pollen for comb building and brood rearing. Otherwise the natural mortality of

Table I. Categories of mating barriers and 'factors' which may cause reproductive isolation in the genus *Apis*.

Behavioural barriers (prezygotic)	Copulatory barriers (prezygotic)	Physiological barriers (prezygotic)	Postzygotic barriers
Mating season	Size	Sperm transfer	Fertilisation
Mating places	Genitalia	Sperm storage	Hybrids
Sexual signals	Mating sign		
Daily mating periods			

workers cannot be compensated and later the swarm (new colony) is reduced to beyond the critical threshold. Therefore, the mating season in honeybee populations depends on seasonal blooming cycles. This holds true for allopatric *A. mellifera* in Africa and Europe [74], and for populations of sympatric Asian species. Accordingly, in Sri Lanka [39], in Thailand [67] and in Borneo [44] all sympatric *Apis* species produced drones simultaneously. We assume, because of the uniform mode of colony multiplication by swarming within the genus, that there is not much 'evolutionary flexibility' to change the reproductive season between sympatric honeybee species.

2.2. Reproductive isolation due to different mating places

Among the Apoidea we find an impressive variability of locations where mating occurs. Several solitary species mate at the nesting sites. Often the males emerge earlier and assemble (and compete) at the nesting sites where they mate with freshly emerging females. In the case of species-specific nesting sites, such behaviour would serve as a mating barrier. Other Apoidea mate on flowers which, in the case of oligolectic bees, may lead to the 'rendezvous' of conspecific mates [1]. Another example of spatial differentiation of mating places comes from sympatric *Bombus* species. The males build an odour track by deposition of pheromones which attract virgin queens. The altitude (height) of these odour tracks is different, ranging from the ground to the canopy [20]. Thus spatial separation seems to work in favour of reproductive isolation.

In discussing the genus *Apis*, we shall start with a short description and review of the drone congregation areas of honeybee species. This is intended to lay the basis for the question of whether or not a spatial separation of mating locations can function as a behavioural barrier between honeybee populations.

During mating flights, *A, mellifera* drones congregate in the open air above their drone congregation area [106] where they remain, flying in wide loops until they return to the colony to feed [75]. Congregation areas usually have a diameter of 30–200 m. More recently, the area above which the drones flew was measured by radar as 1 600 m^2 [48]. Nonetheless, a congregation area has a limited spatial extension and *A. mellifera* drones are not attracted by a queen flying outside the area [75]. Depending on weather conditions, *A. mellifera* drone populations fly at a preferential height above the ground that varies from 5–40 m above the ground. Within a single drone congregation area, *A. mellifera ligustica* drones and *A. mellifera carnica* drones showed a distinct difference in vertical distribution: *A. m. ligustica* drones were more frequently caught at lower altitudes (4 m above ground), while *A. m. carnica* drones preferred to fly at higher altitudes [29]. Virgin queens that mated with that mixed drone population subsequently produced significantly more 'pure' worker offspring: Thus, *A. m. ligustica* queens mated more often with *A. m. ligustica* drones and *A. m. carnica* queens mated preferably with *A. m. carnica* drones. The above differences between cruising altitudes of *A. m. ligustica* and *A. m. carnica* drones were discussed as a mechanism of assortative mating (i.e., incomplete reproductive isolation) [29]. Assortative mating has been reported to occur in a mixed population of *A. m. ligustica* and *A. m. scutellata* in South America [25].

Since virgin queens commence their mating flights significantly later than drones, the congregation of drones is formed irrespective of the presence of a queen [29, 79]. The same drone congregation area was visited by *A. mellifera* drones each season for more than 30 years ([29]; Pechhacker, unpubl. data). Near Selbourne (England) a drone congregation area has been recorded for 197 years [95]. In *A. mellifera*, several drone congregations have been found within the flight range of a colony [77, 78, 106].

Drones of *A. mellifera* were found visiting a drone congregation area at a distance of up to 8 km from their colonies, and drones from a considerable number of different colonies and apiaries were found at drone congregation areas [75]. Calculations of the relatedness of drones captured in a congregation area in Germany revealed that these drones originated from about 240 different colonies, and *A. mellifera* probably represents one of the most elaborate panmictic systems possible among terrestrial animals [3]. The physiographical structure of *A. mellifera* drone congregation areas seems to vary greatly [77]. In plains and less structured areas, however, drones have been reported to be distributed more uniformly, and were attracted to queens wherever they were placed [4, 47, 95]. The local conditions that make the *A. mellifera* drones stay or return to a drone congregation area remain unknown [43, 83].

In contrast to *A. mellifera* drones which congregate in the open air, *A. cerana indica* drones in Sri Lanka and in Borneo gather in close proximity to the trees. These drones restrict their flight to an open space within or near the canopies of the trees. They do not follow a (caged) queen far into the open space above or at the side of the canopy [46, 64]. The distance between the drone congregation area and the drone colonies is clearly less than in *A. mellifera* and ranges up to 2 km (Tingek, unpubl. data). In Japan, however, drones of *A. c. japonica* congregate in the open air high above prominent trees [13, 104]. In Germany, *A. c. indica* drones originating from Northern Pakistan visited a drone congregation area in an open valley far away from the trees [71, 80]. Taken together, drone congregation areas of *A. cerana* show a high degree of variability. Since these differences can occur within the same subspecies (*A. c. indica*), the specific features of these drone congregation areas may be mainly the result of local adaptations to environmental factors. For example, avoiding predators such as birds (*Merops* sp., etc.) by flying near or within a tree canopy might have a higher selective advantage under tropical conditions than in the mountains of Northern Pakistan [63]. Considering the limited data available (in comparison to *A. mellifera)*, we expect that an even wider range of differences among the drone congregation areas of *A. cerana* may become apparent with further research on Asian honeybees.

Drone congregations of *A. koschevnikovi* were regularly observed to occur under a thick cover of vegetation, and the height above the ground of different drone congregation areas varied between 1.5 to 12 m [46]. At present, there is no information available on the drone congregation areas of the other two cavity-dwelling honeybees, *A. nuluensis* and *A. nigrocincta*.

In Borneo, drones of *A. dorsata* congregate under the canopy of tall emergent trees. The eminent, tall tree tops seem to serve as a visual landmark, and applying this criterion, several 'new' *A. dorsata* drone congregation areas have been located [43]. Recently, several *A, dorsata* drone congregation areas have been detected under tall trees in Sri Lanka (Punchihewa, unpubl. data). The drones of *A. dorsata* assemble under the umbrella of the canopy, and do not follow the queens which move into the open air. Further, drone attraction showed a maximum of 3–5 m below the canopy. The height above the ground ranged between 10 and 35 m depending on the size of the tree [43]. The drone congregation area of the other giant honeybee species, *A. laboriosa*, remains undiscovered. Also the drone congregation areas of the dwarf honeybees, *A. andreniformis* and *A. florea*, have not yet been found.

It is not surprising that among allopatric species, drone congregation areas show similarities. Some convincing evidence for these similarities came from Ruttner [71]. In Germany, drones from imported *A. c. indica* colonies (which originated from Pakistan) were caught together with simultaneously flying *A. m. carnica* at the same drone

congregation area. An estimation of drone numbers in the nearby colonies (*A. cerana* and *A. mellifera*) showed that the ratio of *A. cerana* drones at that drone congregation area was similar to that of *A. mellifera* drones. A second case of heterospecific drone mixing, although to a lesser extent, was recorded from drone congregation areas in Japan. In that study, a small number of heterospecific drones was caught at each congregation area [104].

At present, the only comparative observations and experiments on drone congregation areas of sympatric Asian honeybees have been carried out in Borneo (Tenom). So we must restrict the following section to the situation in Tenom. Further, we caution that the conclusions remain more or less preliminary until confirmed by investigations from other places. During our experiments, fairly large numbers of honeybee colonies of four species were found. *A. cerana indica* and *A. koschevnikovi* colonies were kept in modern hives, and both species supplemented a larger natural population of feral colonies. About 50 to 100 *A. dorsata* colonies were found nesting in several bee trees near (within a radius of 5 km) the experimental area. *A. andreniformis* foragers were frequently observed on various flowers. During our 10-year study period, we located 5–10 colonies of this species each season. Because of the small size and the hidden nest sites, we assume that this was only a rather small proportion of a sizeable population of *A. andreniformis* in the area. However, drone congregation areas of *A. andreniformis* were not detected.

A general scheme is presented (Fig. 1), which is mainly based on observations from three different drone congregation areas [46]. At these places the major landmark was an outstanding tree top which clearly protruded from the horizon line. Drones of *A. c. indica* had their maximal flight frequency measured by attraction to our standard dummies (Fig. 2) slightly outside the canopy of the trees and larger shrubs, about 10 to 12 m above the ground. When disturbed by birds or by the insect net, *A. cerana* drones escaped rapidly into the cover of the branches. Drones of *A. koschevnikovi* remained under the dense cover of the canopy, and flew in a space 6 to 8 m above the ground. *A. dorsata* drones flew under the first layer of branches at a height of 20 to 25 m. So the distribution of drones resulted in a clear spatial separation without any overlap between the three species.

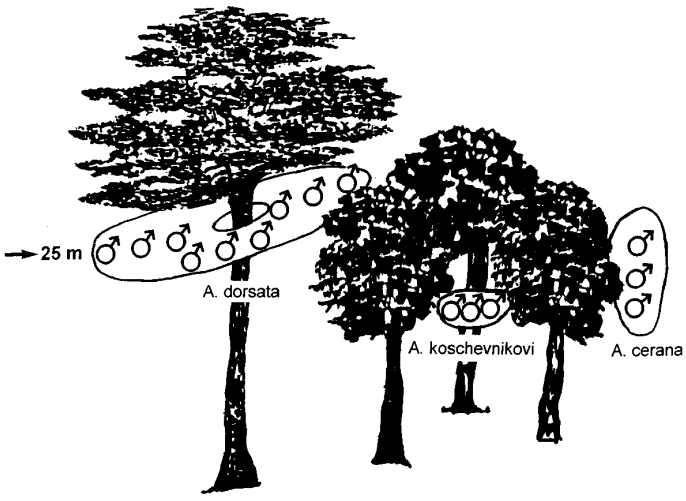

Figure 1. Drone congregation areas in Sabah (Borneo). *A. dorsata* drones congregated directly under the canopy of high emergent trees. *A. koschevnikovi* drones congregated under the thick cover of branches and trees. *A. cerana* drones remained near the branches of neighbouring vegetation. All three drone congregation areas were within a distance of 30 m. ♂ Spaces occupied by drone flight.

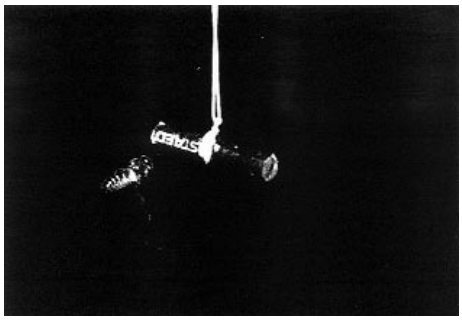

Figure 2. An *A. koschevnikovi* drone attracted to the standard dummy. A section of black pencil (length 34 mm, diameter 8 mm) was tied to a thread and impregnated with 1 mg 9-ODA.

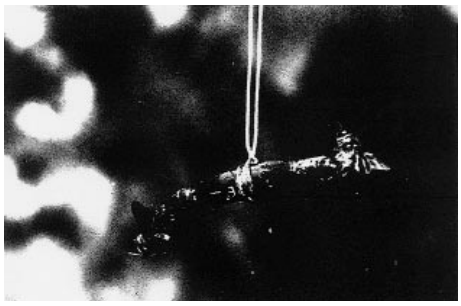

Figure 3. Copulation dummy with two *A. koschevnikovi* drones. At both ends of the standard dummy (1 mg 9-ODA) the hollowed adomen of an *Apis* queen was glued. The *A. koschevnikovi* drones were attracted, copulated and became stuck in the abdomen.

But was this separation sufficient to ensure a complete reproductive isolation among the species? Initially, we carried out some unintentional experiments: Lifting the standard dummy (Fig. 2) to a height of 25 m, we observed a few *A. koschevnikovi* drones pursuing the dummy at a height of 1.5 to 20 m above the ground, and single drones of *A. koschevnikovi* were attracted even to a height of 25 m (optimal for *A. dorsata* drones). Later, we used copulation dummies (Fig. 3) with an opening from which the copulating drone could not extricate himself, and was pulled down for careful examination [43]. A few *A. koschevnikovi* drones were caught at the optimum of the *A. cerana* drone congregation area and at the *A. dorsata* drone congregation area. *A. cerana* drones were caught at the *A. dorsata* drone congregation area, but not at the *A. koschevnikovi* drone congregation area. *Apis dorsata* drones came to the drone congregation area of *A. cerana*, but not to the densely covered drone congregation area of *A. koschevnikovi*. As a result of these experiments, single drones were attracted and copulated at a drone congregation area of another species (with the exception of the *A. koschevnikovi* drone congregation area).

A critical argument in this discussion must address the standard dummy (Fig. 2), which was impregnated with 1 mg 9-ODA. This quantity is about 5 to10 times higher than the amount of pheromone of a young *A. mellifera* queen [86]. In two cases, however, we had the opportunity of comparing the dummy to a natural free-flying *A. cerana* queen: the queen was found to be far more attractive – the drones left the dummy and chased the queen.

So, the above observations indicate that the spatial separation between drone congregation areas does not function as a complete reproductive isolation mechanism between sympatric honeybee species in Borneo.

2.3. Different sexual signals as means of reproductive isolation

The highly developed social life of honeybees has affected mating behaviour in several ways. The sex ratio is strongly male-biased; colonies produce some hundred times more drones than queens [69]. Taking into account the fact that *Apis* drones perform multiple nuptial flights while a queen flies only once or twice for mating, the effective male bias at a drone congregation area is further increased. As a consequence, male-male competition [93] must

have a major impact on drone behaviour. In pursuit of a flying virgin queen, the speed (time) for a drone to reach his target has the highest priority. Under these conditions, drones depend on simple and rapidly detectable stimuli for queen recognition. Further, in order to obtain their goal, an immediate reaction to a first, albeit uncertain signal of a virgin queen is the better choice, because the probability of a drone encountering a second queen is close to zero [19].

The first reaction of drones to visual stimuli seems to depend on 'unspecific' movements. At a congregation area, drones of *A. mellifera* react to various moving objects by a fast, short turning reaction. Flying birds, butterflies and even stones thrown into the air area will momentarily attract some drones [24]. Without the presence of pheromones, however, these objects will never initiate any pursuit or consistent attraction. According to Strang [90], the optimal surface of a queen dummy (impregnated with pheromone: 5 mg 9-ODA) was slightly larger than that of a queen (about 3 cm^2). With further increase in size, the attraction to drones decreased. Black (and red) was the optimal colour. In other words, the visual signal attracting *A. mellifera* drones seems to be rather unspecific: fast moving, dark objects of a size slightly larger than a queen seem to be nearly optimal.

The major active chemical component of the *A. mellifera* queen's mandibular gland was identified as (*E*)-9-oxo-2-decenoic acid (9-ODA) by Callow and Johnston [6]. Among several other important biological functions, 9-ODA was found to be the main component of the *A. mellifera* queen's sex attractant [15, 59]. Later, it was demonstrated that extracts of queens of three other *Apis* species (*A. florea*, *A. cerana* and *A. dorsata*) attract *A. mellifera* drones, and that these extracts contained 9-ODA. *A. dorsata* and *A. cerana* queens had a quantity (150 to 300 μg) of 9-ODA similar to that of the *A. mellifera* queen [5, 87].

Consequently, dead, extracted queens or black dummies of similar size (Fig. 2) impregnated with 1 mg of 9-ODA successfully attracted drones of *A. cerana* [64, 71, 104] *A. dorsata* [43] and *A. koschevnikovi* [46]. Underlining the uniform mechanism of drone attraction further, Gries [18] attracted drones of *A. dorsata, A. mellifera, A. cerana* and *A. koschevnikovi* to the same dummy. Drones were not only attracted but also started to grasp the dummy and to initiate copulation. However, for successful copulation an opening at the end of the dummy (like an open sting chamber) is required (Fig. 3) [16].

Plettner et al. [62] found specific differences in the mandibular gland signals between queens of *A. mellifera, A. dorsata, A. florea* and *A. andreniformis*. The question must be addressed whether or not the results of the behavioural experiments explore the natural situation. We cannot exclude the fact that the demonstrated interspecific drone attraction was caused by our experimental techniques: for example, excessive use of 9-ODA or any other super stimuli of the dummies.

Direct observations and tests of interspecific reactions were carried out with *A. mellifera* and imported *A. cerana* in Germany by Ruttner and Kaissling [76]: electrophysiological responses of receptors (sensilla placodea) on the antenna of the *A. cerana* and *A. mellifera* drone showed no differences between extracts of mandibular glands of *A. cerana* and *A. mellifera* queens. However, at a drone congregation area in Germany, significantly more *A. mellifera* drones were attracted to conspecific (caged) queens than to caged *A. cerana* queens. These results indicate that the species-specific differences in queen pheromones between *A. mellifera* and *A. cerana* (as described by Plettner et al. [62]) might be of functional significance. However, the preference for conspecific queens does not prevent the mating of a heterospecific queen. Ruttner and Maul [79] reported a young *A. cerana* queen which had her bursa copulatrix blocked by a

mating sign of an *A. mellifera* drone (easily recognised by its chitinous plate). This is – as far as we know – the only direct evidence of heterospecific mating of a free-flying *Apis* queen!

Apparently, the drastic male bias at the drone congregation area and the resulting competition among drones has led to a fast, simple and uniform mechanism of queen recognition. The main olfactory signal and essential sex pheromone seems to be 9-ODA in all *Apis* species. The specific differences in the queen's pheromone spectrum among the species [62] may result in a reduced attraction of the heterospecific queen. It will, however, not prevent interspecific copulations as Ruttner and Maul [81] demonstrated. This leads to the conclusion that the differences in sexual signals do not play a major role as a behavioural barrier between sympatric honeybee species.

2.4. Reproductive isolation due to different daily mating periods

2.4.1. Apis mellifera: the 'allopatric situation'

As discussed before, the natural distribution of *A. mellifera* generally does not overlap with the distribution of other *Apis* species [73]. Thus for *A. mellifera,* we can assume that an evolution "under the condition of being the only honeybee species at a place" has shaped the mating behaviour and the daily mating period. Drones of European races of *A. mellifera* start flying shortly after the sun passes the zenith (12.15), and stop in the late afternoon (17.00) [70]. In Germany, a comparison of drone flight times between *A. m. ligustica* and *A. mellifera carnica* showed no significant differences [9, 29]. Observations in Africa, near Pretoria, with *A. m. scutellata* showed a period from 12.45 to 16.45 [95], and more recently Lahner [47] reported *A. m. monticola* drone flight activity in Malawi to occur from 11.20 to 16.00.

A. mellifera queens perform their mating flights during the peak period of drone flight. Successful mating flights of queens (returning with a mating sign!) occurred in Austria between 14.20 and 16.10 [29]. In Africa, *A. m. monticola* mated queens returned between 13.00 and 15.30 [47].

Overall, the mating flight period of *A. mellifera* invariably starts shortly after noon and covers a period of 4 to 5 h. The differences reported so far have been within a short range and do not exceed ± 1 hour. The period of actual mating (as documented by queens returning with a mating sign) is considerably shorter (2 to 3 h). Queens fly later and stop their flight activity earlier than drones. Compared to other behavioural characters (defence, swarming etc.), the daily mating period of *A. mellifera* seems to be rather uniform in Africa and Europe.

2.4.2. Apis cerana: the 'allopatric situation'

Among the Asian honeybee species, *A. cerana* has the most extensive natural distribution. In consequence, it overlaps many of the other Asian *Apis* species [73]. Regionally, there are, however, large areas where *A. cerana* is the only honeybee. Within the Asian continent these areas are mainly in the northern part of their range, in mountain ranges and in the Japanese islands (with the exception of Hokkaido).

A daily *A. cerana* mating period from 12.30 to 16.00 has been reported from Bihar in North India [85]. Drones of *A. c. indica* (originating from the mountains of the North West Frontier Province of Pakistan) flew between 12.00 and 15.30 in Germany [81]. Verma [98] observed mating flights of *A. c. indica* queens in the Shimla Hills (North India) between 12.30 and 15.30. In Japan, drones of *A. c. japonica* flew from 13.15 to 17.00, and successful mating flights of *A. c. japonica* queens occurred between 14.35 and 16.35 [105].

The mating period of *A. mellifera* and the observations from regions where

A. cerana occurs as the only *Apis* species shows a striking degree of similarity. The overall duration of drones' and queens' flights and the timing during the early afternoon seems to be nearly identical in *A. mellifera* and in 'allopatric' populations of *A. cerana*.

2.4.3. The 'sympatric situation'

The following table (Tab. II) focusses on observations and research which present data on mating flights of sympatric species at one location.

A. andreniformis drones (Tab. II) had a uniform short flight period after 12.00. The period of *A. andreniformis* queen flights was between 12.33 and 12.50 in Sabah, Borneo [38].

A. florea drones (Tab. II) show remarkable differences. In Sri Lanka, drones flew earlier than in South East Thailand. According to Koeniger et al. [44], the drone flight period of *A. florea* in Bangkok was between 13.45 and 15.30, and ended more than 1 hour earlier compared to the following data (Tab. II). Also, successful mating flights of *A. florea* queens in Bangkok were observed between 14.04 and 14.25 [42]. Apparently, some variability of *A. florea* mating periods occurs in Thailand. Perhaps the earlier mating period (in comparison to Tab. II) is typical for regions (like Bangkok) where *A. florea* is the only dwarf bee species.

A. cerana exhibits more variability in drone flight period than other *Apis* species. The drone flight period of *A. c. indica* drones (Tab. II) in Sri Lanka was confirmed by Punchihewa et al. [64]. Accordingly, queens successfully mated between 16.15 and 16.55 [63, 65]. The mating period of *A. c. indica* in Sri Lanka is the latest so far recorded for this species.

A. koschevnikovi drones fly during a long period of nearly 2 hours. Queens flew between 17.00 and 18.15 [33]. *A. dorsata* fly consistently at sunset. The flight period of *A. dorsata* drones is very short. Drones of this species perform daily only a single flight [43]. In Borneo, a slight overlap with *A. koschevnikovi* drones occurred. This was, however, too slight to affect the reproductive isolation.

Koeniger and Wijayagunasekera [39] observed that *Apis florea* in Sri Lanka occupies the time window which is nearest to the 'allopatric' mating period. Therefore, they argued that *A. florea* was the original *Apis* species to arrive at Sri Lanka. Consequently, the species with later periods would have established colonies in Sri Lanka some time later. With the evidence (Tab. II) available today, however, a general and uniform pattern related to taxonomy becomes apparent: the first position directly after noon is held by a dwarf bee species (*A. andreniformis* and/or *A. florea*). The next time window seems to belong to one or even two cavity-dwelling species (*A. cerana* and *A. koschevnikovi*); and at the very end of the day, just around sunset, *A. dorsata* holds

Table II. Drone flight periods of sympatric Asian honeybee species.

1st author (year) Locality	Koeniger [39] Sri Lanka	Rinderer [67] Thailand	Koeniger [45] Sabah, Borneo
A. andreniformis		12.15–13.45	12.00–13.45
A. florea	12.00–14.30	14.00–16.45	
A. cerana	16.15–17.15	15.15–17.30	14.00–16.15
A. koschevnikovi			16.45–18.30
A. dorsata	18.00–18.45	18.15–18.45	18.15–19.05

its mating time. It seems to be unlikely that the similarity in this sequence of mating periods originated by chance in three different locations (Sri Lanka, Thailand and Sabah). Another possibility, that this sequence evolved once in South East Asia and spread unchanged to the above places, seems to be equally unlikely. Therefore we hypothesise that this pattern originated from a (yet unknown) mechanism of interspecific reproductive competition, which causes predictable results independently of place and environment. Without exception, the temporal sequence of mating periods is strictly correlated with the size; it starts with the smallest *Apis* species (*A. andreniformis*) and ends with the largest sympatric species (*A. dorsata*)!

It is rather tempting to speculate on how drone behaviour can result in this sequence: The basic reaction of *Apis* drones is directed to queens which are larger than fellow drones. So smaller drones may try to copulate unidirectionally with larger drones, excluding them from access to queens. However, these speculations are premature, and the question of whether the size had a direct effect, or rather size-correlated factors caused the above sequence, remains unsolved.

Our above hypothesis, however, has come to a large-scale test. Recently, *A. florea* was involuntarily introduced into Africa [53]. Fairly large populations established in the region of Khartoum (where no feral *A. mellifera* exist) and *A. florea* colonies are spreading to the South, along the Nile [52]. Eventually *A. florea* will reach the habitat of *A. m. scutellata*. In the likely event of a sympatric co-existence, we predict that as a result of a rapid natural selection process *A. florea* drones will fly prior to *A. mellifera* drones!

Whenever the sequence of separated mating periods was established, it facilitated sympatric co-existence, and *Apis* species could spread simultaneously, sharing their habitats. However, in the case where they spread into a 'new' territory alone, each species will shift towards the 'allopatric' mating period. Several 'mountain' bee populations may serve as an example for the latter phenomenon: the allopatric population of *A. cerana* from Northern India and Pakistan has already been discussed above. Further, the open-nesting giant honeybee species *A. laboriosa*, adapted to the high altitudes of the Himalayas, has a drone flight period between 12.20 and 14.20 [97]. Likewise, *A. nuluensis* is the only honeybee species in the mountains of Borneo above 1 700 m, and its drone flight period is between 10.45 and 13.15 [44]. No observations on queen flights are yet available, and as the drone flight was recorded from one colony only (this applies to *A. laboriosa* too), there is need of additional confirmation.

Arguably, the time-sharing mating pattern of sympatric *Apis* species has evolved to form a nearly perfect behavioural barrier. Regarding the temporal pattern of *Apis* mating behaviour, a separate daily mating period becomes operational earlier than several complicated and 'risky' events in a behavioural sequence. After this successful 'a priori' reproductive isolation has been established, previously functional mechanisms which operate on later steps in mating behaviour become less meaningful. In other words, flying during their daily mating period, drones and queens of any *Apis* species do not gain further by maintaining different sexual signals or species-specific mating places.

What is involved in the timing of mating flights? Taber [91] confined an *A. mellifera* colony in a cool dark room for 12 hours, inducing earlier drone flight on the following day. Yoshida and Yamazaki [104] were able to shift the flight period of *A. mellifera* drones by changing the photoperiod. The above results suggest that the drone flight period seems to depend on an internal clock. Koeniger et al. [34] used cross-foster

techniques. They introduced drones of *A. koschevnikovi* and *A. cerana* into alien colonies. As a result, *A. koschevnikovi* drones flew during their species-specific mating period independently of their *A. cerana* host colony. Similarly, *A. cerana* drones followed their own mating period and not the *A. koschevnikovi* colony's timetable. Later, virgin *A. koschevnikovi* queens were introduced into *A. cerana* colonies and flew at their own species' mating period [44]. Drones and queens seem to decide on the right time for mating on the basis of an 'inherited timetable'. Consequently, a direct evolutionary impact on the individual drone or queen and selection for changes in mating period becomes operational and may act faster than any effect via the colony (workers). Thus, fast adaptations to predatory pressure, to other environmental alterations or even to 'new' honeybee species are facilitated.

3. COPULATORY BARRIERS

Many cases are known in which different sizes and shapes of genitalia do not totally prevent copulation. But then interspecific copulation often results in injury or even death of the participants [8]. The importance of size and shape of the genital organs in *Apis* shall be discussed below.

3.1. Reproductive isolation due to different size

A well-known example for size differences as a copulatory barrier comes from domestic animals. For example, females and males of different dog breeds are attracted to each other, but because of differences in size are unable to copulate.

In *Apis* species, worker bees vary considerably in size [84]. The weight relation of workers of the dwarf honeybee species and the Asian cavity-dwelling species is about 1 to 2.5; to the giant honeybees it is about 1 to 5 [32]. But the difference in weight in drones in these species is much smaller. The relation between the dwarf and the Asian hive-dwelling bees increases gradually to 1.5, and compared to the giant honeybees it is about 1 to 2 (Tab. III). Only the drone of the allopatric *A. mellifera* has a considerably higher weight. The weights of queens have about the same relation as in drones.

As previously mentioned, drones of all species will grasp the same queen dummy and try to copulate. Considering the small size differences (especially within the same taxonomic group), we conclude that body size 'per se' does not play a major role as an isolation mechanism in honeybees. However, it might be an important factor for the positioning of a specific mating period within the afternoon (see § 2.4.3).

Table III. Absolute weight of queens and drones in mg (*n* individuals measured).

Species	Drone	Queen (*virgin)
A. andreniformis	70.8 ± 3.0 (25)	112 (3)
A. florea	77.6 ± 2.6 (25)	86* (5)
A. cerana	83.4 ± 8.9 (38)	122 ± 13* (74)
A. koschevnikovi	105.5 ± 5.6 (45)	170 (2)
A. nuluensis	107.0 ± 6.7 (5)	–
A. nigrocincta	–	–
A. mellifera	211.1 ± 11.8(25)	202* (5)
A. dorsata	155.7 ± 8.5 (34)	290 (2)
A. laboriosa	–	–

3.2. Reproductive isolation due to different genitalia

The queen's genital tracts within the genus *Apis* are similar and simple in principle. The genital chamber opens at the base of the sting. Its outer part is the bursa copulatrix, the inner part the vagina with the valvula vaginalis. Small differences concerning the valvula vaginalis have been reported by Camargo [7].

In comparison, differences in male genitalia between honeybee species are very impressive. Generally, in *Apis* the copulative organ is a membranous endophallus which is differentiated into a broad vestibulum with cornua, a slender cervix and a thick bulb with its lobe. In situ they look quite similar. The characteristic marks to distinguish them seem to be limited to differences in the hairy fields, in the form of the cornua and the lobe [31, 61]. During copulation the endophallus is everted successively and introduced into the queen. The differences in form and size of the everted endophalli become striking (Fig. 4). For example, the cornua bend either dorsally or ventrally in the everted endophallus; the same is true for the bulbus [31]. These differences are expected to have major functional consequences in the case of copulation between species belonging to different taxonomic groups (hive-dwelling, giant and dwarf honeybees).

However, within these groups there are many similarities. In all cavity-dwelling

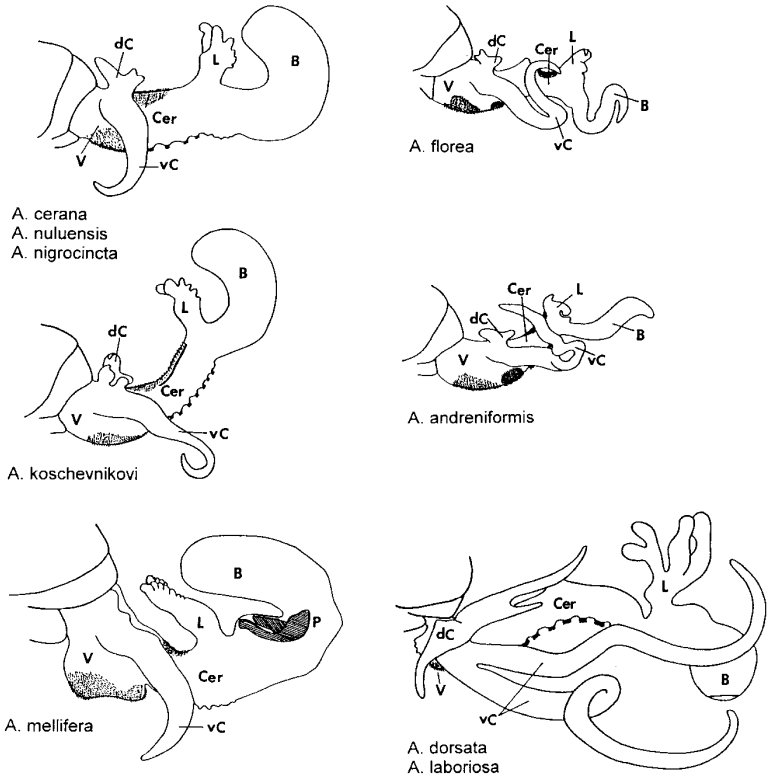

Figure 4. Everted endophalli of nine *Apis* species (lateral view). Symbols for all figures: B: bulbus; Cer: cervix; P: chitinous plates of bulbus; dC: dorsal cornus; L: lobe; V: vestibulum; vC: ventral cornua. ▨: hairy fields.

species it seems to be mainly the large endophallus which connects the drone to the flying queen. Filled under high muscular pressure with mucus from the male accessory glands and air, it seems to guarantee a sufficiently strong connection between the flying queen and the paralysed drone until sperm transfer into the oviducts is completed [42]. The short thick cornua with its 'orange coloured' sticky and greasy secretion may contribute to strengthen the attachment and later, after separation of the pair, it sticks to the mating sign and keeps it in place.

The giant honeybees have a more elongated endophallus with four long curled cornua also covered by an 'orange coloured' sticky secretion. The elongation of the endophallus is mainly caused by the extended cervix (Fig. 4). The drones also become paralysed during copulation. In these bees the broadened metatarsi seem to reinforce the attachment to the queen [72].

The everted endophallus of the dwarf honeybees shows many differences to the previous endophalli. The so-called bulbus is a thin tube (Fig. 4), and the mucus glands are tiny. They cannot produce enough mucus to strengthen the connection between the copulating pair. Instead they have a forceps-like appendix at the metatarsus of the hind leg, which is a bit shorter in *A. andreniformis* than in *A. florea*. With these 'thumbs' the drone locks himself to the hind legs of the queen [73], supported again by the sticky cornua pressed into the sting chamber. Thus the pair stays connected until the queen turns her legs in such a way that the drone is released. This different mode of attachment seems to have evolved together with the changed form of the bulbus: it ends in a fine tip, which enables the drone to deposit the sperm into the thin spermaduct instead of the wide oviducts.

Between these three taxonomic groups – dwarf, giant and cavity-dwelling honeybees – sperm transfer very likely is prevented by the morphology of the endophalli. This probably does not hold true within these groups. No difference could be found between the endophalli of *A. dorsata* and *A. laboriosa* [51], thus this will not function as a copulatory barrier.

There are some morphological differences in the tip of the bulb, the fimbriate lobe and the pattern of hairs between the dwarf species *A. andreniformis* and *A. florea* (Fig. 4) [31]. Whether or not these differences are sufficient to prevent mating and sperm transfer is questionable. The same holds true for the cavity-nesting species.

3. 3. Mating sign as a possible barrier

After copulation, mating signs are left in the sting chamber of the *Apis* queen. However, the queens of all *Apis* species mate several times during a single mating flight. So the mating sign does not prevent further mating but it must be removed by the next drone. In the case of interspecific mating however, the endophallus of a heterospecific drone might not be equipped to remove the mating sign from the sting chamber.

Mating signs have been described in the cavity-dwelling species [33, 99, 101]. They consist of mucus from the male accessory glands, secretions from the bulbus gland and orange-coloured secretions from the cornual glands [29, 35]. In *A. mellifera* there are also chitin plates from the bulbus. In *A. andreniformis* only the secretion from the cornual gland was found in the sting chamber (Fig. 5) [38]. While mating signs appear similar in the Asian species *A. cerana* and *A. koschevnikovi*, they are clearly different in *A. mellifera* (Fig. 5).

In *A. mellifera* it could be observed that drones are able to remove the mating sign at the beginning of copulation. It is attached to the hairy field on the ventral side at the base of the endophallus. At the end of mating, each drone leaves its own sign [26, 96]. After the last copulation more than 70% mated queens return carrying the last mating

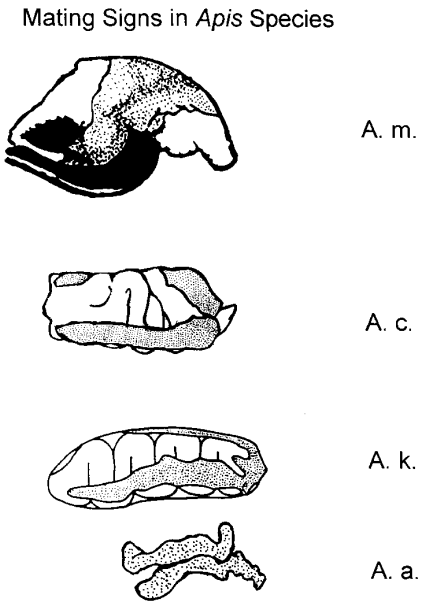

Figure 5. Mating signs in *Apis* species. A.m.: *Apis mellifera* ; A.c.: *Apis cerana*; A.k.: *Apis koschevnikovi;* A.a.: *Apis andreniformis.* ○ Mucus ; ◍ Cornual secretion ; ● Chitinous plates.

sign in their sting chamber. After returning from the mating flight, this is removed mostly by the queen rubbing her abdomen on the comb [69]. As queens of *A. cerana* and *A. koschevnikovi* also return from one mating flight with a mating sign and their oviducts filled with sperm of about five to 10 drones [33, 101], drones of these species, too, are able to remove the mating sign of their predecessors.

In *A. dorsata,* queens return from mating flights after sunset, during darkness! No mating signs were noticed protruding from the sting chamber, and the queen was permitted to re-enter the colony [92]. On the other hand, *A. dorsata* drones have well-developed mucus glands (own observations), and in a video film on mating with dummies by Gries (unpubl. results), the deposition of a white plug on the dummy was demonstrated.

A. florea and *A. andreniformis* have tiny mucus glands. While in *A. florea* no secretion of drones was found in the sting chamber of queens returning from mating flights [42], all three observed queens of *A. andreniformis* had a reddish yellow cornual secretion (Fig. 5) protruding from the tip of the abdomen [38].

The question of whether drones can remove heterospecific mating signs cannot be answered generally, but Ruttner and Maul [79] have observed that an *A. cerana* queen carried an *A. mellifera* mating sign for about 2 weeks. Dissection revealed that the chitinous plates had injured the membrane of the bursa copulatrix; the oviducts were filled with 1.7 μL sperm, which corresponds to one *A. mellifera* drone [99]. Therefore we conclude that the following *A. cerana* drones as well as workers (afterwards in the colony) were not able to remove the heterospecific (*A. mellifera*) mating sign.

4. PHYSIOLOGICAL BARRIERS

4.1. Sperm transfer

The percentage of drone spermatozoa stored in the spermatheca is quite different in different species, although DNA studies have revealed that the number of effective matings is similar among species [11, 54–58, 68]. The total number of spermatozoa in the queen's spermatheca divided by the effective number of matings indicates the amount of spermatozoa contributed by each drone (Tab. IV). For example, an *A. mellifera* drone produces about 12.7 million spermatozoa, but only about 370 000 reach the spermatheca. Although this is the highest number for all species, it corresponds to only about 3% of the spermatozoa of a single drone. This number is around 10% in *A. koschevnikovi*, *A. cerana* and *A. dorsata*. The other extreme occurs in *A. andreniformis*: one drone produces 0.13 million spermatheca, of which an average of 66% are present in the spermatheca [30]. These

Table IV. Number of spermatozoa of one drone reaching the spermatheca (million).

Species	Spermatozoa in spermatheca (*n* queens)	Spermatoz. in ves. sem.	Mean paternity	Effective paternity	*n* Spermat. per drone in spermatheca	Spermatoz. of one drone reaching the spermatheca (%)
A. a	0,78 (7) [38]	0.13 [30]	13.5 [57]	9.1 [57]	0.086	66
A. f	1.05 (15) [42]	0.43 [42]	8.0 [55]	5.6 [55]	0.187	44
A. c	1.35 (12) [63.101]	1.1 [63/101]	18 [58]	12 [58]	0.113	10
A. k	2.13 (4) [33]	1.7 [33]	16.3 [68]	10.5 [68]	0.203	12
A. m	4.73 (126) [100]	12.7 [99]	13.8 [11]	12.4 [11]	0.370	3
A. d	3.94 (8) [30, 92]	2.46 [30]	22.4 [54, 56]	22.8 [54, 56]	0.173	7

A. a: Apis andreniformis; A. f: Apis florea; A. c: Apis cerana; A. k: Apis koschevnikovi; A. m: Apis mellifera; A. d: Apis dorsata. ( ) *n* queens; [ ] reference.

calculations differ slightly to those of Oldroyd et al. [58] and Palmer and Oldroyd [60], but do not alter our conclusions.

These findings support the idea that the mode of sperm transfer (sperm injection into the oviducts versus sperm injection into the spermaduct) influences the filling process of the spermatheca. Injection into the oviducts results in a loss of more than 90% spermatozoa, whereas with injection into the spermaduct [38] only about 50% is rejected. Thus different sperm numbers together with the different modes of sperm transfer may act as a partial reproductive barrier.

4.2. Sperm storage

The technique of instrumental insemination has permitted the study of heterospecific sperm transfer and storage. The following combinations have been made (Tab. V).

In all cases some semen reached the spermatheca. In the interspecific and intraspecific inseminations of an *A. cerana* queen, "twice as many *mellifera* spermatozoa reached the spermatheca when injected into the oviducts than in the case of *cerana* spermatozoa. Thus heterospecific insemination is as efficient as homospecific." Up to 1.9 million living spermatozoa were counted in the spermatheca of *A. cerana* [79].

In the interspecific and intraspecific inseminations of *A. koschevnikovi* and *A. cerana*, about the same percentage of spermatozoa (8–9%) reached the spermatheca (Tab. VI), independent of hetero- or conspecific sperm. This percentage corresponds to that after natural mating (Tab. IV). In all cases, spermatozoa in the spermatheca were viable when queens were dissected 3 to 40 days after insemination. The amount of spermatozoa was below 1 million, except in *A. koschevnikovi* queens inseminated with conspecific sperm.

After insemination of *A. koschevnikovi* with *A. dorsata* sperm, the percentage of spermatozoa reaching the spermatheca was quite low. But with only two experiments and no reciprocal insemination, these results must be considered preliminary. Woyke [102] reports that after inseminating *A. florea* queens instrumentally with *A. mellifera* sperm, some spermatozoa entered the spermatheca, but he did not report the percentage. To a certain extent, a comparable migration of conspecific spermatozoa has been demonstrated in *A. mellifera* queens [17]. No reciprocal inseminations were performed. These data suggest that the physiology of the genital duct and its fluid is similar throughout all species.

Table V. Queens of 4 species instrumentally inseminated with sperm of various heterospecific species.

Sperm	*A. mellifera*	*A. cerana*	*A. koschev.*	*A. dorsata*
Queens				
A. mellifera		+ (1.2) [76, 103]		
A. cerana	+ (3) [76]		+ (4) [36]	
A. koschevnikovi		+ (5) [36]		+ (2) [36]
A. florea	+ (7) [102]			

( ) *n* queens; [ ] reference.

Table VI. Number of spermatozoa (million) in queens after instrumental insemination.

	cer × *cer* (15 queens) (%)	*cer* × *kosch* (10 queens) (%)	*kosch* × *kosch* (4 queens) (%)	*kosch* × *cer* (4 queens) (%)	*kosch* × *dors* (2 queens) (%)
n Drones	8 ± 1.8	7 ± 1.9	9 ± 1	7 ± 1	5
n Sperm in oviducts	7.3 ± 1.4	8.8 ± 2.4	11.7 ± 1.8	6.4 ± 1.9	9.0
n Sperm in spermatheca	0.7 ± 0.3	0.7 ± 0.3	1.1 ± 0.3	0.6 ± 0.2	0.2 / 0.5
% Sperm in spermatheca	9.2 ± 3.2	8.1 ± 4.4	9.3 ± 1.7	9.1 ± 2.2	2.2 / 5.6

5. POSTZYGOTIC BARRIER

5.1. Fertilisation and hybrids

Ruttner and Maul [79] collected eggs of cross-inseminated queens of both *A. cerana* and *A. mellifera* 1 hour after deposition. In 92% of the eggs "motile spermatozoa with rapidly undulation movements" could be detected close to the anterior pole. But no larvae developed. Squash preparations from late cleavage stages, fixed and stained, showed normal diploid chromosome sets at the same rates. Serial sections revealed that hybrid eggs of both types could form a 'preblastoderm', but during the blastula stage the initial cell walls disintegrated again and development ended with a complete breakdown. Apparently there is some variation in the degree of embryonic disintegration. Reciprocal hybrids with *A. cerana* from Pakistan were all highly disintegrated by the third day of development. Eggs of *A. mellifera* queens *fertilised* by *A. cerana* spermatozoa, however, showed patterns which at least were similar to the arrangement of an embryo [79]. With these results, complete reproductive isolation was proven between these two species.

In 1996, five *A. cerana* queens inseminated with *A. koschevnikovi* sperm produced progeny. Brood counts revealed altogether 338 drone and 454 worker cappings (Tab. VII). These queens had 0.6 ± 0.3 million spermatozoa in the spermatheca. In the brood of two queens, we found both drone heads and worker heads. Because of unfavourable circumstances, further analysis was not possible. In 1997, sealed brood combs of two *A. cerana* queens inseminated with *A. koschevnikovi* sperm were kept in the incubator and some hybrids with gynandromorph characters were reared (Tab. VIII). From one queen we collected 10 drones and 14 hybrids. In 13 worker-like hybrids, the distance between the complex eyes was more than 1.5 mm; in one bee, this measured only 0.15 mm. All had a long proboscis,

11 hybrids had no stings, and except for three bees which had 1 or 2 drone hind legs, all the others had worker legs. From the other queen we collected 24 offspring: 10 drones and 14 that appeared to be drone-like hybrids, which were recognised because of the dorsal distance between the complex eyes. The distances varied between 0.20 and 1.55 mm; in 11 hybrids, the distance was below 1.0 mm. The ocelli were situated either frontally or between the complex eyes. Six hybrids had short drone and eight had long worker proboscises. All but one had drone legs. In further experiments we will try to produce hybrid queens and test their ability to reproduce. No data on hybrids between *A. koschevnikovi* queens inseminated with *A. cerana* sperm are yet available.

It seems unlikely that the above hybrid bees could form a viable colony. Though we have not yet tried to breed hybrid queens, the results suggest complete reproductive isolation. According to the DNA and morphological results [2, 12], *A. cerana* is more related to *A. nuluensis* than to *A. koschevnikovi*. So cross-insemination between these species may result in 'better' hybrid bees.

6. CONCLUSION

Evidence of reproductive isolation among distinct honeybee populations has been used for confirmation and recognition of new species and for estimation of taxonomic relations. We now discuss the value and the limitations of this approach.

In the 1960s there were generally only four recognised species in the genus. Even the discussion about the taxonomic status of the two cavity-dwelling bees, *A. mellifera* and *A. cerana*, was not yet settled. In this context, Ruttner and co-workers started their first research project on reproductive isolation. As a result, a full array of post-mating barriers was presented, culminating in evidence of genetic incompatibility (lethal hybrids) between *A. cerana* and *A. mellifera* [79]. The question of the taxonomic status of *A. cerana* as a valid species was settled (Tab. IX).

The next step in research on reproductive isolation was the discovery by Koeniger and Wijayagunesekera [39] that different daily mating periods served as complete mating barriers among sympatric *A. florea*, *A. cerana* and *A. dorsata* in Sri Lanka. These results laid the basis for further developments. In 1988, Ruttner [73] discussed his research on *A. cerana* and *A. mellifera* and argued "that no premating barrier exists between these two species as is the case between the other species. Because of a not completely finished speciation, it appears totally unjustified to classify this taxon (*A. cerana*) as a subgenus, as was proposed by Skorikov [89] and Maa [49]. On the contrary, they (*A. mellifera* and *A. cerana*) have to be regarded as being in a late, but not yet finished stage of speciation" ([73] p. 148). So the 'missing premating barrier' between species was used as a basis for determination of a taxonomic position. We will postpone commenting on that argument, and first pursue the role of 'reproductive isolation research' in the recognition of further 'new' *Apis* species.

The rediscovery of *A. koschevnikovi* in Sabah and its recognition as a valid species was based on a combination of different results. The first argument was sympatric distribution with *A. cerana*, then morphometric differences between both species and distinct differences in the morphology of the endophallus were demonstrated [94]. After this, evidence for complete reproductive isolation by a separated daily mating period led to the 'final' acceptance of *A. koschevnikovi* as a 'good species [41]. Since then, determination of mating periods has become indispensable for the recognition of 'new' honeybee species.

Research on post-mating barriers (see § 5) between *A. cerana* and *A. koschevnikovi* revealed similarities. In all combinations

Table VII. 1996: Progeny of *A. cerana* queen inseminated with *A. koschevnikovi* sperm from one brood comb and numbers of spermatozoa in the spermatheca.

	Insem.	Eggs	Larvae	Dronecap	Workercap	*n* spermat. (million)
1.	*A. c.* × 8 *A. k.*	38	22	55	16	0.3
2	*A. c.* × 5 *A. k.*	86	43	33	64	1.2
3.	*A. c.* × 6 *A. k.*	68	60	94	6	0.3
4.	*A. c.* × 6 *A. k.*	?	?	39	121	0.3
5	*A. c.* × 8 *A. k.*	132	263	117	247	0.7

Queens 4 and 5 already had pupae; drone heads and worker heads could be distinguished. Insem.: insemination; spermat.: spermatozoa.

Table VIII. Offspring of two *A. cerana* queens inseminated with *A. koschevnikovi* spermatozoa.

Character	Workerlike		Mix		Drone like	
	Qu 1	Qu 2	Qu 1	Qu 2	Qu 1	Qu 2
Eye distance	11	3	0	9	1	2
Proboscis	12	7	0	1	0	6
Tomenta	6	6	0	0	6	8
Leg	9	0	1	1	2	13
Sternite	1	0	3	0	8	14

Qu: queen.

Table IX. Taxonomic distance and mode of reproductive isolation between cavity dwelling honeybee species.

Species to species	Taxonomic distance	Natural distribution	Premating barrier	Postmating barrier
A. cerana – A. nuluensis	Nearest	Sympatric, different habitat	Mating period	Not known
A. cerana – A. koschevnikovi	Second	Sympatric, same habitat	Mating period	Endophallus viable hybrids
A. cerana – A. mellifera	Third	Allopatric	None	Endophallus lethal hybrids

among the three cavity-dwelling species, heterospecific spermatozoa reached and stayed viable in the spermatheca. There were, however, some remarkable differences: while hybrids between *A. cerana* and *A. mellifera* died during blastogenesis, hybrids between *A. cerana* and *A. koschevnikovi* were viable and were reared to adult workers (with some gynandromorph patterns).

The next ‘case’ to be discussed was the discovery of *A. nuluensis*. As already mentioned, the habitat of *A. nuluensis* is restricted to the mountains of Borneo where

it is the only honeybee species. The main argument for the recognition of the new species was again evidence that the drone flight period of *A. nuluensis* was different from that of the other cavity-nesting species of Borneo (*A. cerana* and *A. koschevnikovi*). No data on post-mating barriers are yet available. Last, the recognition of *Apis nigrocincta* from Sulawesi as a separate species was mainly based on differences between mating periods to those of the sympatric *A. cerana* [22].

Collectively, the group of cavity-dwelling honeybees today comprises five species. This offers a new and promising base to explore the value of data on reproductive isolation for determination of the taxonomic relations among *Apis* species. For ease of discussion and because several comparable taxonomic papers do not include it, we have omitted the very recently recognised *A. nigrocincta* from the following considerations.

As a basis for the taxonomic positions of the species, we refer to the consistent results of several morphometric studies [12, 66, 82] which can be summarised in terms of a hierarchy of taxonomic distances: *A. cerana* (Borneo) / *A. nuluensis* < *A. cerana* / *A. koschevnikovi* < *A. cerana* / *A. mellifera*. Supporting genetic evidence for the above sequence comes from Arias et al. [2], and parts of it from Garnery et al. [14].

The nearest taxonomic distance within the above group and probably the most recent speciation separated *A. cerana* (Borneo) and *A. nuluensis*. The species are still (?) distributed in ecologically different habitats. The behavioural barrier might have an adaptive value in a small zone of overlap (1 500 to 1 700 m above sea level) with the other cavity-nesting species. However, between *A. cerana* and *A. koschevnikovi* the separated daily mating periods are of considerable adaptive significance because both species share the same habitat. So the three cavity-dwelling *Apis* species of Borneo (taxonomically close to each other) have perfect behavioural mating barriers.

A. mellifera is the sister taxon to the Asian cavity-dwelling species, and its separation must have occurred significantly earlier than the above branchings [87]. Therefore, the 'missing' premating barrier does not correlate to a recent speciation. It is more likely that because of the (still existing) allopatric situation of *A. mellifera*, there was no adaptive pressure for the development of any type of premating barrier. In other words, the evolution of a behavioural mating barrier in honeybees seems to be connected to a sympatric co-existence and does not depend on the taxonomic position. In contrast, non behavioural mating barriers seem to be a result of the general genetic divergence, which starts with the reduction and eventual elimination of gene flow between the two populations. Therefore the degree of differences in endophalli, in viability of hybrids etc. (see B and C) correlates well with the phylogenetic distance.

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Résumé – L'isolement reproductif parmi les espèces du genre *Apis*. Les études sur l'isolement reproductif des abeilles du genre *Apis* ont commencé relativement tard par rapport aux autres animaux ou aux plantes. Cela est dû principalement au fait que les colonies d'abeilles qui étaient importées d'Afrique ou du Bassin méditerranéen vers

l'Europe appartenaient à la même espèce (*Apis mellifera* L.), et se croisaient donc avec les abeilles déjà présentes. Ce n'est qu'en 1965 que F. Ruttner, après avoir importé de Chine des colonies d'*A. cerana* Fabricius, put ouvrir un nouveau chapitre de la recherche apidologique par des travaux expérimentaux sur les barrières reproductives entre races d'abeilles.

Traditionnellement les barrières reproductives ont été classées selon un ordre chronologique par rapport à la fécondation en obstacles prézygotiques et postzygotiques. Afin de mieux prendre en compte les particularités biologiques du processus compliqué de l'accouplement multiple chez le genre *Apis*, nous avons divisé la phase prézygotique en trois sous-groupes (Tab. I) et utilisé les définitions suivantes :

Barrières comportementales. – Les barrières comportementales empêchent le contact physique entre la reine et les mâles. Les barrières à la copulation interviennent pendant la copulation et empêchent le transfert du sperme. Les barrières physiologiques bloquent la voie au sperme entre les oviductes de la reine et la spermathèque, et plus tard la pénétration dans l'œuf. Les barrières postzygotiques perturbent le développement normal et conduisent à la mort ou à l'infertilité des hybrides.

Barrières reproductives liées au comportement. – La formation des essaims de multiplication et les accouplements n'ont lieu en règle générale qu'à la belle saison. En conséquence, les espèces sympatriques produisent des mâles et des reines toujours en même temps ; il n'y a donc pas de divergence dans la saison d'accouplement qui puisse agir comme barrière reproductive entre espèce d'*Apis*.

Un schéma général des lieux de rassemblements de mâles (LRM) de trois espèces (Fig. 1) a été déduit des observations faites en trois lieux différents à Bornéo. La question de savoir dans quelle mesure la séparation spatiale trouvée entre les LRM spécifiques à chaque espèce est valable comme barrière reproductive a été étudiée à l'aide de leurres (Fig. 2). On a pu montrer que les mâles pouvaient être attirés sur un LRM d'une espèce étrangère et y copuler avec des leurres spéciaux (Fig. 3). Les différents LRM ne peuvent donc pas jouer pleinement le rôle de barrière reproductive entre espèces du genre *Apis*. Les mâles réagissent aux extraits de reines d'espèces étrangères, et les mâles de différentes espèces copulent avec un même leurre, imprégné que d'une phéromone (9-ODA) (Fig. 3). On ne peut donc attribuer qu'une importance nulle ou faible au bouquet phéromonal des reines en tant que barrière reproductive.

Des périodes d'accouplement différentes au cours de la journée viennent en première position parmi les barrières reproductives liées au comportement. Le tableau II résume les résultats des recherches sur ce point et montre des différences dans les périodes d'accouplement au sein d'une même espèce. Néanmoins, la succession chronologique des périodes d'accouplement spécifiques à chaque espèce est dans l'ensemble semblable au Sri Lanka, en Thaïlande et à Bornéo. Les abeilles naines (*A. andreniformis* et/ou *A. florea*) arrivent en première position, au moment où le soleil est au zénith, ou juste après. La période suivante est occupée par les abeilles qui nidifient dans des cavités. Et peu avant le coucher du soleil, les mâles d'*A. dorsata* sortent pour effectuer leur bref vol nuptial. Il se dégage de l'ensemble un schéma uniforme, et la position de la période d'accouplement propre à une espèce semble n'être corrélée qu'avec la taille de l'insecte, que ce soit entre groupes taxonomiques ou au sein d'un même groupe. Il est encore prématuré de spéculer sur les mécanismes à l'origine de cette succession chronologique, mais une particularité du comportement d'accouplement des mâles pourrait jouer un rôle. La réaction des mâles est dirigée vers les reines qui, chez toutes les espèces du genre *Apis*, sont plus grosses que les mâles. Aussi les mâles des plus petits espèces pourraient essayer de copuler avec les mâles des plus grosses en les prenant

pour des reines et les empêcher ainsi d'avoir accès aux reines. Mais de telles considérations ne reposent pas encore sur des bases expérimentales, et il est tout autant possible que ce ne soit pas la taille corporelle elle-même qui soit décisive, mais des facteurs physiologiques liés à la taille, pour que les espèces les plus petites volent en premier.

Notre hypothèse en est arrivée au test à grande échelle, puisque *A. florea* a été récemment introduite accidentellement en Afrique, où ses populations s'étendent. Elles vont peut-être atteindre l'aire de répartition naturelle d'*A. mellifera scutellata* et parvenir à une coexistence sympatrique. Dans ce cas, nous prédisons que les mâles d'*A. florea* accompliront leurs vols de fécondation avant ceux d'*A. mellifera*.

Barrières reproductives liées à la copulation. – Des différences de taille aussi bien que des différences de structure des organes copulateurs peuvent empêcher la copulation. Les deux situations se rencontrent chez les abeilles naines et les abeilles géantes. Les abeilles qui nidifient dans les cavités se distinguent elles aussi nettement des espèces sus-mentionnées. Mais au sein des groupes ce type de barrières n'est pas total. Les organes de copulation des abeilles géantes *A. laboriosa* et *A. dorsata* ont la même structure et les rapports de taille sont semblables. Chez les deux espèces d'abeilles naines (*A. florea, A. andreniformis*) les organes de copulation, bien qu'ayant des différences nettes dans la pilosité et la forme, sont bâtis sur le même principe, si bien qu'une copulation semblerait possible. Mais puisque les sexués volent à des heures différentes, il n'y a aucune possibilité de le tester. Cela vaut également pour toutes les espèces sympatriques. L'importation d'*A. cerana* en Europe a permis d'étudier les interactions interspécifiques lors de l'accouplement. Au début de nombreuses reines d'*A. cerana* ont été perdues. Une reine d'*A. cerana*, qui était rentrée du vol de fécondation mais n'avait pas pondu d'œufs, a été mutilée par le signe de fécondation d'un mâle d'*A. mellifera* qui était resté enfoncé dans l'oviducte. Visiblement le signe de fécondation de l'espèce étrangère n'avait pu être ôté. Cela pourrait être valable pour des accouplements entre autres espèces car les signes de fécondation sont construits différemment (Fig. 5).

Barrières reproductives liées à la physiologie. – La variation selon l'espèce du nombre de spermatozoïdes des mâles semble empêcher une insémination suffisante. Ainsi une reine d'*A. mellifera* devrait s'accoupler avec 120 mâles d'*A. cerana* pour avoir une quantité de sperme équivalent à celui transféré par 12 mâles d'*A. mellifera*. Le tableau IV donne le nombre de spermatozoïdes transféré par un mâle de chaque espèce. Le pourcentage de spermatozoïdes qui atteint la spermathèque varie également d'une espèce à l'autre (Tab. IV).

La physiologie du tractus génital et de la spermathèque semble par contre semblable. Après insémination artificielle, les spermatozoïdes sont parvenus jusqu'à présent dans la spermathèque dans tous les cas et sont restés viables durant la période testée (de trois jours à cinq semaines) (Tabs. VI et VII).

Barrières postzygotiques. – Des essais de croisement entre espèces n'ont été menés à ce jour que sur une échelle réduite. Lors de croisements dans les deux sens entre *A. cerana* et *A. mellifera*, des œufs ont bien été fécondés, mais l'embryon est mort 48 h plus tard pendant la formation de la blastula. Lors de croisements entre deux reines d'*A. cerana* avec des mâles d'*A. koschevnikovi*, quelques hybrides se sont développés et ont donné des ouvrières. Une reine a donné naissance à des gynandromophes ayant un aspect de mâles, que l'on pouvait reconnaître principalement au grand écartement de leurs yeux (Tab. VIII). Les autres hybrides possédaient de nombreuses caractéristiques des ouvrières.

En conclusion, nous discutons le rôle qu'a joué la recherche sur les barrières reproductives dans la reconnaissance de nouvelles espèces d'abeilles et dans la détermination

des distances taxonomiques. La démonstration des différentes périodes de vol des mâles a été d'une importance décisive dans la reconnaissance d'*A. koschevnikovi*, d'*A. nuluensis* et d'*A. nigrocincta*. Par contre, les « barrières post-accouplement », tels que la forme de l'endophallus, le transfert de sperme et la viabilité des hybrides, conviennent mieux pour estimer la distance taxonomique.

***Apis* / isolement reproducteur / comportement d'accouplement / genitalia / hybride**

Zusammenfassung – Kreuzungsbarrieren zwischen Arten der Gattung *Apis*. Forschungen über reproduktive Isolation von Honigbienen begannen im Vergleich zu anderen Tieren oder Pflanzen relativ spät. Das lag vor allem daran, daß alle Bienenvölker, die aus Afrika oder dem Mittelmeerraum nach Europa importiert wurden, zur gleichen Art (*Apis mellifera* L.) gehörten und daher mit den bereits vorhandenen Bienen bastardierten. Erst 1965 konnte F. Ruttner nach der Einfuhr von *A. cerana* Bienenvölkern aus China mit experimentellen Arbeiten über Kreuzungsbarrieren zwischen Honigbienenarten dieses neue Kapitel der Bienenforschung aufschlagen.

Traditionell werden Kreuzungsbarrieren nach ihrer zeitlichen Zuordnung zur Befruchtung in präzygotische oder postzygotische Barrieren eingeteilt. Um die Besonderheiten der komplizierten Paarungsbiologie der *Apis*arten besser zu berücksichtigen, haben wir die präzygotische Phase noch einmal in drei Untergruppen geteilt (Tab. I). Für diese Arbeit gelten folgende Definitionen:

Verhaltensbedingte Kreuzungsbarrieren verhindern den Kontakt zwischen Königin und Drohnen. Kopulationsschranken greifen während der Kopulation ein und verhindern die Übertragung von Spermien. Physiologische Barrieren blockieren den Weg der Spermien von den Ovidukten der Königin in die Spermatheka und weiter bis zum Eindringen ins Ei. Postzygotische Barrieren stören die normale Entwicklung und führen zum Tod bzw. zur Unfruchtbarkeit der Hybriden.

Verhaltensbedingte Kreuzungsbarrieren. – Die Bildung von Vermehrungsschwärmen und auch die Paarungen finden in der Regel nur in der günstigsten Jahreszeit statt. Entsprechend erzeugen sympatrische Arten stets zur gleichen Jahreszeit Drohnen und Königinnen und eine unterschiedliche Paarungssaison als Kreuzungsbarriere zwischen *Apis*arten scheidet weitgehend aus.

Ein allgemeines Schema der Drohnensammelplätze von drei Arten (Abb. 1) wurde von Ergebnissen an drei unterschiedlichen Plätzen in Borneo abgeleitet. Die Frage inwieweit die gefundene räumliche Trennung zwischen den artspezifischen Drohnensammelplätzen als Paarungsschranke gelten kann, wurde mit Attrappen (Abb. 2) untersucht. Es konnte gezeigt werden, daß Drohnen auf artfremden Drohnensammelplätzen angelockt werden konnten und dort mit speziellen Attrappen (Abb. 3) kopulierten. Demnach können die verschiedenen Paarungsplätze nicht als vollständige Kreuzungsbarriere zwischen den Honigbienenarten gelten. Weiter wird diskutiert, inwieweit unterschiedliche Königinnenpheromone als Kreuzungsbarriere dienen können. Drohnen reagieren auf Extrakte von artfremden Königinnen und – soweit bisher getestet – kopulieren die Drohnen verschiedener Arten mit einer identischen Attrappe, die nur mit einem Pheromon (9-ODA) kontaminiert war (Abb. 3). Demnach scheint den unterschiedlichen Pheromonmustern der Königinnen keine oder eine nur geringe Bedeutung als Kreuzungsbarriere zu zukommen.

Als verhaltensbedingte Kreuzungsbarriere kommen in erster Linie tageszeitlich unterschiedliche Paarungsperioden in Frage. In Tabelle II sind Ergebnisse von Untersuchungen zusammengefaßt. Dabei ergaben sich Unterschiede zwischen den Paarungszeiten innerhalb einer Art. Insgesamt war jedoch die zeitliche Folge der artspezifischen

Paarungsperioden in Sri Lanka, Thailand und Borneo gleich. Die Zwerghonigbienen (*A. andreniformis* und/oder *A. florea*) halten die erste Position nahe bzw. kurz nach dem Zenit der Sonne. Die nächste Paarungsperiode wird von höhlenbrütenden Arten besetzt. Kurz vor Sonnenuntergang starten die Drohnen von *A. dorsata* zu ihrem kurzen Paarungsflug. Insgesamt entsteht so ein einheitliches Muster und die Position der artlichen Paarungsperiode scheint zwischen und auch innerhalb der taxonomischen Gruppe allein mit der Größe zu korrelieren. Es ist noch voreilig zu spekulieren, auf Grund welcher Mechanismen diese zeitliche Reihenfolge entstanden ist. Allerdings könnte eine Besonderheit des Paarungsverhaltens der Drohnen dabei von Bedeutung sein. Die Reaktion der Drohnen ist auf Königinnen gerichtet, die bei allen Honigbienenarten grundsätzlich größer sind als die Drohnen. So könnten die Drohnen der größeren Art "für Königinnen gehalten werden" und gezielt von Drohnen der kleineren Art angeflogen und auf diese Weise verdrängt werden. Aber solche Überlegungen sind noch nicht experimentell gesichert und es ist gleichfalls möglich, dass nicht die Köpergröße direkt sondern physiologische Faktoren, die mit der Größe korrelieren. Entscheidend dafür sind, dass die kleineren Arten früher fliegen. Insgesamt steht der Hypothese, dass jeweils die kleineren Drohnen früher fliegen, ein großer Freilandtest bevor. Es ist zu erwarten, dass sich *A. florea* weiter in Afrika ausbreitet und im natürlichen Verbreitungsgebiet von *A. m. scutellata* kann es zu einer sympatrischen Koexistenz kommen. Wir sagen voraus, dass dann die *A. florea* vor den Drohnen von *A. mellifera* zur Paarung ausfliegen werden.

Kopulationsbarrieren. – Sowohl unterschiedliche Größe als auch unterschiedlich gebaute Begattungsorgane können Kopulationen verhindern. Bei den Bienen trifft beides für die Zwerg- und Riesenhonigbiene zu. Auch die höhlenbrütenden Arten unterscheiden sich deutlich von den vorgenannten Zwerg – bzw. Riesenhonigbienen. Aber innerhalb der Gruppen sind derartige Barrieren sicher nicht vollständig. Die Begattungsorgane von den Riesenhonigbienen *A. laboriosa* und *A. dorsata* sind gleich gebaut und auch die Größenverhältnisse sind ähnlich. Bei den beiden Arten der Zwerghonigbiene sind die Begattungsorgane trotz deutlicher Unterschiede in der Behaarung und Form nach dem gleichen Prinzip geformt, so daß eine Paarung möglich erschiene. Da die Geschlechtstiere zu unterschiedlichen Zeiten fliegen, gibt es keine Möglichkeiten, dies zu testen. Gleiches gilt für alle sympatrischen Arten. Der Import von *A. cerana* nach Europa ermöglichte Untersuchungen über zwischenartliche Interaktionen bei der Paarung. Zunächst waren die Verluste von *A. cerana* Königinnen hoch. Eine *A. cerana* Königin, die zwar zurückkehrte aber keine Eier legte, war durch das im Ovidukt steckengebliebene Begattungszeichen eines *A. mellifera* Drohns verletzt. Offensichtlich konnte das Begattungszeichen der anderen Art nicht entfernt werden. Das könnte auch für Paarungen zwischen anderen Arten zutreffen, denn die Begattungszeichen sind unterschiedlich gebaut (Abb. 5).

Physiologische Barrieren. – Die unterschiedlichen Spermazahlen der Drohnen sprechen für eine Behinderung einer ausreichenden Besamung bei verschiedenen Arten. So müßte eine *A. mellifera* Königin von 120 *A. cerana* Drohnen gepaart werden, um die gleiche Menge Spermien zu erhalten die 12 *A. mellifera* Drohnen übertragen. Die Unterschiede in den Spermazahlen sind in Tabelle IV angegeben. Unterschiedlich sind auch die Prozentsätze von Spermien, die bei den Arten in die Spermatheka gelangten (Tab. IV).

Die Physiologie des Genitaltraktes und der Spermatheka dagegen scheint ähnlich zu sein. Nach künstlicher Besamung gelangten in allen bis jetzt getesteten Fällen (Tab. VI und VII) Spermien in die Spermatheka und waren für die getestete Zeit (3 Tage bis 5 Wochen) lebensfähig.

Postzygotische Barrieren. – Versuche zu Hybridisierungen sind bisher nur in geringem Umfang durchgeführt worden. Bei Kreuzungen von *A. cerana* mal *A. mellifera* in beiden Richtungen wurden Eier zwar befruchtet, aber der Embryo starb nach 48 Stunden während der Bildung der Blastula. Bei der Kreuzung von zwei *A. cerana* Königinnen mit *A. koschevnikovi* Drohnen entwickelten sich einige Hybriden bis zu fertigen Arbeiterinnen. Bei einer Königin entstanden drohnenähnliche Gynandromorphe, die vor allem an ihrem groβen Augenabstand zu erkennen waren (Tab. VIII). Die anderen Hybriden hatten viele Eigenschaften von Arbeiterinnen.

Abschlieβend wird diskutiert, welche Rolle die Forschung über Kreuzungsbarrieren bei der Anerkennung neuer Honigbienenarten bzw. bei der Bestimmung von taxonomischen Distanzen gespielt hat. Dabei war vor allem der Nachweis von unterschiedlichen Drohnenflugzeiten für die Anerkennung von *A. koschevnikovi*, *A. nuluensis* und *A. nigrocincta* von entscheidender Bedeutung. Für die Abschätzung der systematischen Distanz dagegen sind offensichtlich "post mating barriers", w.z.B. Gestalt des Endophallus, Spermaübertragung und Lebensfähigkeit von Hybriden aussagekräftiger.

***Apis* / Kreuzungsbarriere / Paarungsverhalten / Paarungsorgan / Hybrid**

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