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**Original article**

# Flight performance of artificially reared honeybees (*Apis mellifera*)*

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**Abstract** – Artificially reared larvae are an ideal model for experiments involving brood diseases or testing pesticides. Because conditions during larval development can influence the general performance of adult honeybees, we created an evaluation method for the viability of artificially reared honeybees. We compared the flight performance of honeybees artificially reared in the laboratory with that of their sisters naturally reared in the colony. Fresh and dry weight, wing surface area, flight speed, flight duration, and distance covered by honeybee workers after feeding defined amounts of different sugar solutions were measured during tethered flight in a roundabout. Our results demonstrate that after artificial rearing, adult honeybees at the natural age of flight exhibit similar flight performances to their naturally reared sisters. The naturally reared honeybees, however, attained higher maximum flight speeds when fed energy-rich 2molar glucose solution.

***Apis mellifera* / flight / in vitro rearing / larval nutrition / worker quality**

## 1. INTRODUCTION

All social insects practice intensive brood care, which is especially true in honeybees because the larvae need a homeostatic environment (temperature and humidity) and a stable nutritional supply (Haydak, 1970; Tautz et al., 2003; Tautz, 2007). A larva-containing cell is carefully inspected as many as 2785 times, and food is added if required (Lindauer, 1952; Schmickl and Crailsheim, 2002), so the larvae are supplied with a constant flow of protein-rich brood food mixed with honey and pollen. Honeybee larvae grow very fast (Wang, 1965) and the number of feedings and the food applied is adjusted to the age of larvae (Haydak, 1970; Schmickl and Crailsheim, 2002). Feedings are administered by middle-aged nurse bees (Haydak, 1970), which are able to digest pollen (Moritz and Crailsheim, 1987) and synthesize brood food, whilst other adult bees are involved in thermoregulation or capping cells. Brood food is derived from pollen, the colony's sole protein source. Pollen income is regulated according to the colony's need by foragers (Al-Tikrity et al., 1972; Hellmich and Rothenbuhler, 1986; Pankiw et al., 1998), the number of larvae is regulated according to the colony's supply of protein (Schmickl and Crailsheim, 2001, 2002), and there is a positive correlation between the amount of stored pollen and the amount of brood reared (Allen and Jeffree, 1956; Imdorf et al., 1998). Even during pollen shortages, a colony tends to maintain the quality of its brood, rather than feeding the brood insufficiently (Imdorf et al., 1998). Therefore, investigating the effect of deficiencies in larval nutrition is difficult, because malnourished larvae do not reach the pupal stage but rather are cannibalized by adult bees (Schmickl and Crailsheim, 2001). Starvation of honeybee larvae leads to

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developmental failures (Jay, 1964), and experimental pollen shortage slightly affects protein content (Schmickl and Crailsheim, 2001) or leads to lowered dry weights in adults but not in pupae (Imdorf et al., 1998). The worker/larvae ratio is positively correlated with dry weight (Eischen et al., 1982) and wing size in offspring (Daly et al., 1995). Seasonal times of low pollen stores can lead to variation in protein content and fresh and dry weight of emerging bees (Kunert and Crailsheim, 1988) and the nutritional state of the colony produces behavioural and physiological consequences including longevity in adult bees (Mattilla and Otis, 2006).

While the physical conditions of the inner honeybee nest can be easily regulated in the laboratory (and brood combs can be replaced by plastic cups), the brood care provided by nurse bees cannot be easily reproduced. Feeding protocols have been developed to rear honeybees in vitro (Rembold and Lackner, 1981; Vandenberg and Shimanuki, 1987; Aupinel et al., 2005; and citations therein), mainly to investigate the effects of plant protection products (Wittmann and Engels, 1981; OEPP/EPPO, 2001, 2003) or parasites (e.g. Brødsgaard et al., 2000) on larval mortality or rearing success (survival to eclosion).

For such studies, it is important to evaluate the in vitro rearing methods themselves by developing standard measures of the viability of the adults produced. A good method will produce individuals with levels of robustness, tolerance, or susceptibility very close to nature. Herbert et al. (1988) found morphometric differences of laboratory reared honeybees, but they did not use adult bees of defined age but rather used bees that may have died shortly after metamorphosis. Riessberger-Gallé et al. (2008b) showed that artificially reared larvae have a delayed gain in weight but compensate by the time of eclosion.

In this study we focused on flight, which is indispensable for a honeybee's successful foraging. The adult bee's body is aerodynamically and morphologically adapted for flight with high wingbeat frequencies (Harrison et al., 2005). During flight, honeybees increase their metabolic rate to high values (Gmeinbauer and Crailsheim, 1993; Nachtigall et al., 1995; Harrison and Fewell, 2002) and foraging honeybees cover on average 59 km per day (Neukirch, 1982). Two of the bees' special adaptations for flight are a high density of myofibrils and mitochondria in flight muscles, and dense packing of enzymes for carbohydrate catabolism (Suarez, 2000). Other adaptations enable a honeybee to heat its thorax before flight and to control body temperature during flight (Heinrich, 1980).

When honeybee pupae are reared in the laboratory in an unnatural vertical position, they develop into adults with humpbacks and wing deformations that fail to fly fast and steadily. We therefore instituted the practice of rearing honeybees horizontally in the pupal stage (Riessberger-Gallé et al., 2008a).

A key question of our study was whether the feeding protocol of Aupinel et al. (2005) supplies larvae with adequate amounts of protein for them to develop flight musculature and the flight-muscle oxidative capacity needed for high-metabolic rates during flight. To evaluate the flight performance of artificially reared honeybees, we compared them to their half- or supersister of the same age that were reared under natural conditions with adequate food. Both groups of bees were treated the same after adult eclosion and housed in the same colony at the same time until flight experiments in a roundabout were carried out. After flight experiments, the wing surface area and fresh and dry weights of the bees were determined. Any observed differences in these or other flight performance measures would be attributable to the feeding received during the first 6 days of larval life.

## 2. MATERIALS AND METHODS

### 2.1. Rearing of bees

The rearing of worker larvae was mainly carried out according to Aupinel et al. (2005). The queen was caged on an empty comb inside a 10-frame colony (*Apis mellifera carnica* Pollmann) on 23 August 2007. We grafted 386 first instar larvae on 27 August 2007.

Plastic queen cups were disinfected for 15 minutes in a solution of Milton Sterilising Tablets (Milton) at room temperature; no Methyl Benzethonium

Chloride was used. Queen cups were set into 48-well tissue culture plates. Each well was half filled with a piece of dental roll, wetted with 15.5% glycerol.

The larvae were fed with a diet consisting of 50% royal jelly (obtained from the Styrian school of beekeepers) and 50% an aqueous solution (w/w). On the first day 20 μL diet per larvae was given, the aqueous solution contained yeast extract (Sigma; 2% w/w), glucose (12%) and fructose (12%). Larvae were not fed at all on the second day. On the third day, 20 μL diet per larvae was given, and the aqueous solution contained 3% yeast extract, 15% glucose, and 15% fructose. On the fourth and fifth days, 40 μL was provided, and on the sixth day 50 μL of diet per larvae was given, both with the aqueous solution containing 4% yeast extract, 18% glucose, and 18% fructose. The diet was warmed to 34.5 °C prior to each feeding. The plates with the larvae were placed in an incubator for the first 7 days at 34.5 °C and a relative humidity of at least 90%. Later, the dental rolls were removed and the humidity was reduced below 80%.

To prevent the bees from developing humpbacks or wing deformations due to the abnormal vertical position of larvae or pupae in oversized plastic queen cups, the plates were sealed with a very thin, almost transparent, perforated wax layer and put in a vertical position on the 11th day, and the larvae were repositioned horizontally (Riessberger-Gallé et al., 2008a). The wax layer was prepared by melting 2.5 g wax foundation between two sheets of baking paper and then shaping the molten wax with a rolling pin. Mortality during larval development was 5.4%, and the total mortality until emerging (eclosion) was 16.3%.

After the grafting of the first instar larvae, individual larvae remaining in the comb were put back into their original colony and raised naturally by their sisters. A surplus of pollen and honey was present in the colony to ensure optimal brood care. Shortly before emergence, the combs were put in an incubator under the same conditions as the artificially reared bees. The bees that eclosed were used as controls to minimize genetic differences. After adult eclosion (14–16 September 2007), all artificially reared and a similar number of control bees were marked on the abdomen with different colours and added daily to the same colony (unrelated to their parent colony) housed in an observation hive. The entrance of the hive was checked regularly for marked bees making exit flights.

## 2.2. Flight experiments

For the flight experiments, marked bees were collected randomly from the observation hive on the morning before testing. All bees used were at least 20 days old, an age when worker bees are typically flying (Neukirch, 1982). During our experiments, the number of artificially reared honeybees diminished drastically, and eventually the last honeybee used for flight experiments was 31 days old. In a blind testing, each bee was treated exactly the same by the experimenter, who did not know to which group a given bee belonged. Each honeybee was attached to the 14 cm long arm of a flight mill by affixing a small tube on the thorax, so one revolution of the mill covered 87.96 cm. Flight was stimulated by removing a small ball of paper that the bee held with her legs. Because ambient temperature affects flight metabolic rate and speed (Hrassnigg and Crailsheim, 1999), the temperature in the roundabout was measured every two minutes and controlled at 25.4 ± 1.1 °C (n = 472). Ambient air temperature did not differ between the two experimental groups ($P > 0.2$, Student's $t$-test).

First, a test bee was stimulated to fly in the roundabout without prior feeding (an "emptying flight") to deplete the sugar reserves in her honey stomach. After a bee stopped actively flying in the apparatus, she was stimulated again; this was repeated several times until the movements of wings were very weak and the bee could no longer move the arm of the roundabout. After this first flight, the bee was fed a defined amount of sugar solution and again stimulated to fly. The number of revolutions flown by the bee every minute was recorded, and the overall flight time was clocked, so that only the active flight period was considered in further calculations.

After the emptying flight, each bee was fed with 10 μL of 1M glucose solution. Then, after a resting period of exactly five minutes, she was stimulated to another flight (=first flight, 1M) during which she metabolized the fed sugar. This procedure was repeated with a feeding of 10 μL of 2M glucose solution (=second flight, 2M). Before and after each feeding and after flights, each bee was weighed on an analytical balance to the nearest 0.1 mg. This confirmed that the fed solution was ingested entirely and measured each bee's empty body weight. Flight time, distance covered, maximum speed and average speed were calculated according to Gmeinbauer and Crailsheim (1993).

### 2.3. Fresh and dry weight of body sections and wing surface area

After the two flights with defined feedings, the test bee was dissected into head, thorax (including legs and wings), and abdomen, and each part was weighed to the nearest 0.1 mg. The sections were dried to constant weight and dry weights were measured to the nearest 0.1 mg.

Left and right forewing and hindwing were dissected from the thorax, placed between slides, and scanned using a Minolta Dimage Scan Dual III AF-2840. The surface area of each wing was measured using UTHSCSA ImageTool Version 3.0 and also the number of hamuli on each hindwing was counted.

## 3. RESULTS

The artificially reared honeybees eclosed from the rearing plates autonomously by biting through the wax layer. None exhibited obvious morphological deformities. For both the natural controls and the artificially reared bees, the first orientation or defecation flights were observed at the age of 6 days.

The mean age of bees used for flight experiments was 23.9 ± 4.7 d (n = 11) for natural controls and 25.8 ± 3.4 d (n = 11) for artificially reared bees (mean and SD, $P > 0.2$, Student's *t*-test). Bees that did not start flying within 20 minutes were rejected. In total, 52.4% (11 out of 21) of the artificially reared bees and 68.8% (11 out of 16) of the control bees did fly successfully in the roundabout (chi-square 1.0094, n = 37, $P > 0.05$). One flight result of an artificially reared honeybee with 10 µL of 2M glucose feeding was discarded because she interrupted her flight multiple times rather than flying continuously.

The flight data for both feedings are shown in Figures 1a and b. Artificially reared bees flew significantly longer than naturally reared control bees in both the 1M flight and the 2M flight (Tab. I). The two groups of bees did not differ significantly in the mean distance flown in the roundabout for either flight (Tab. I) or in their average speed for the entire period of flight, including the early period of acceleration and the final deceleration phase near fatigue and exhaustion (Tab. I). The two groups

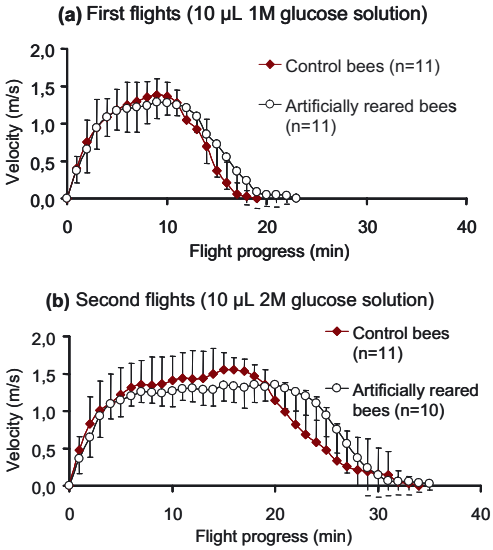


**Figure 1.** Mean flight speed of artificially reared and control bees after being fed (a) 10 µL of 1M glucose solution and (b) 10 µL of 2M glucose solution. Means and SD are shown. Means were calculated for each minute of flight from all individuals, including those which had already stopped flying due to exhaustion.

did not differ in the maximum speed reached during flight after the 1M feeding, but control bees attained significantly higher speeds compared to artificially reared bees after the 2M feeding (Tab. I). For control bees, both average speed ($P = 0.0088$, Student's *t*-test) and maximum speed ($P = 0.0255$) were higher in 2M flights than in 1M flights (Tab. I), but these differences were not significant for the artificially reared bees (average speed, $P = 0.0692$; maximum speed, $P = 0.6416$).

Knowing the amount of consumed glucose and flight duration, we calculated oxygen consumption and the energy turnover rate according to Gmeinbauer and Crailsheim (1993). Mean metabolic power for flights with 1M glucose solution was 30.1 ± 2.9 mW (n = 11) for control bees and 26.3 ± 3.8 mW (n = 11) for artificially reared bees. For 2M glucose solution, mean metabolic power was 37.3 ± 5.0 mW (n = 11) for controls and 32.1 ± 1.9 mW (n = 10) for artificially reared bees.

Artificially reared bees (76.6 ± 11.6 mg) and control bees (83.1 ± 9.7 mg) showed no

**Table I.** Flight parameters for artificially reared bees and control bees in two feeding regimes (10 μL of 1M Glucose solution, 10 μL of 2M Glucose solution). Means and standard deviations, sample sizes, and *P*-values (Student's *t*-test) are given. For comparisons between 1M and 2M flights, see Results.

| Feeding regime | | Control | Artificially reared | *P* |
|---|---|---|---|---|
| 10 μL 1M glucose | n | 11 | 11 | |
| | Flight time (s) | 948.4 ± 92.9 | 1097.9 ± 163.0 | 0.0156 |
| | Distance (m) | 936.0 ± 100.3 | 1018.5 ± 157.6 | 0.1587 |
| | Average speed (m/s) | 1.00 ± 0.15 | 0.94 ± 0.16 | 0.4022 |
| | Maximum speed (m/s) | 1.44 ± 0.16 | 1.38 ± 0.19 | 0.4170 |
| 10 μL 2M glucose | n | 11 | 10 | |
| | Flight time (s) | 1542.5 ± 226.1 | 1767.8 ± 115.1 | 0.0107 |
| | Distance (m) | 1844.9 ± 315.7 | 1902.4 ± 305.1 | 0.6764 |
| | Average speed (m/s) | 1.20 ± 0.18 | 1.08 ± 0.16 | 0.1074 |
| | Maximum speed (m/s) | 1.61 ± 0.17 | 1.42 ± 0.19 | 0.0253 |

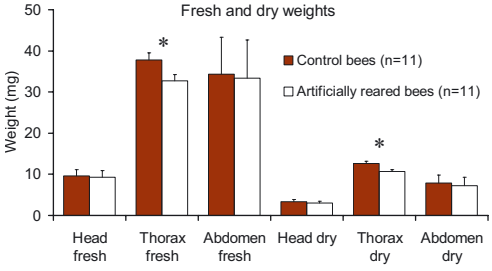


**Figure 2.** Fresh weight and dry weight of the head, thorax, and abdomen of artificially reared and control bees used in flight experiments. Means and SD are shown. * indicates $P < 0.05$, Student's *t*-test.

differences in fresh weight (n = 22, $P > 0.1$, *t*-test). The artificially reared bees had lower mean dry weight (21.0 ± 2.7 mg) than the controls (23.9 ± 2.8 mg, n = 22, $P < 0.05$, *t*-test). The two groups did not differ in fresh or dry weights of either their heads or abdomens ($P > 0.2$, *t*-test), but both fresh and dry weights of the thorax were significantly lower in artificially reared honeybees (n = 22, $P < 0.05$, *t*-test, Fig. 2).

The average forewing surface area of control bees (17.42 ± 0.51 $mm^2$, n = 22) and the average hindwing surface area (8.43 ± 0.50 $mm^2$, n = 20) was larger than in artificially reared bees (forewing: 16.16 ± 0.47 $mm^2$, n = 22, $P < 0.01$ and hindwing: 7.99 ± 0.37 $mm^2$, n = 21, $P < 0.01$, *t*-test, respectively). The number of hamuli on each hindwing of artificially (21.40 ± 1.88, n = 20) and naturally reared (21.55 ± 1.73, n = 20) bees did not differ ($P > 0.7$, *t*-test).

## 4. DISCUSSION

The honeybees we reared in the laboratory according to the protocol of Aupinel et al. (2005) satisfied the challenging demands of insect flight despite the restricted food support: only 5 feedings of an artificial diet in vitro compared to the nearly continuous nursing provided by the numerous bees in a colony (Lindauer, 1952; Schmickl and Crailsheim, 2002). By rotating the pupae into horizontal positions, we succeeded in producing adult bees with no developmental deformities. The artificially reared bees took their first orientation or defecation flights at the age of six days, just as our control bees did.

The weight of an adult worker is an accurate indication of nutritional investment during the larval stage (Eischen et al., 1982). In accordance with Riessberger-Gallé et al. (2008b), who investigated the fresh weights of newly emerged bees, the two groups of bees in our experiments did not differ in fresh weight at the age of foragers, but we found an effect of our artificial larval diet on dry weights. By investigating separate body sections, we could attribute this to lower fresh weight and also dry weight of the thorax (Fig. 2), where the flight musculature is housed.

Flight muscles are formed mainly during pupal development, but several changes

concerning the flight machinery also occur gradually during adult life (Harrison, 1986), like the increase in sarcosome volumes (Herold, 1965) or the post-pupal maturation of several glycolytic enzymes (Hersch et al., 1978; Roberts and Elekonich, 2005). It also has been shown that endothermic capacities develop during the first days after emerging as adults (Vollmann et al., 2004). Since both groups of bees in our experiments shared the same environment as adults, all observed differences in flight performance must be due to larval nutrition, and these deficiencies may only be partially or not at all compensated by young adult bees feeding pollen. Another option is that poorer nutrition of larvae causes deficiencies in post-pupal development, such as delayed maturation of enzymes of carbohydrate metabolism.

Forewing and hindwing surface areas of artificially reared bees were only slightly smaller than in controls: this expands upon the findings of Herbert et al. (1988).

After the 1M glucose feeding, artificially reared honeybees flew for a longer time, on average, but not significantly further, than the controls given the same food (Tab. I). The two groups did not differ in maximum or average speed. In general, the artificially reared honeybees' flight performance was similar to that of the control bees when 1M glucose solution was fed (Tab. I). The longer flight duration of artificially reared honeybees may have been due to differences in the acceleration and deceleration phases of flight (Fig. 1). Hrassnigg et al. (2005) showed that dwarf drones — only 63% as heavy as normal drones — flew longer in a roundabout than the normal drones.

Honeybees fly faster when more concentrated glucose solutions are fed (Gmeinbauer and Crailsheim, 1993) or when a nectar source is more profitable (v. Frisch and Lindauer, 1955). In our experiments, feeding 2M glucose solution led to higher average and maximum flight speeds compared to 1M glucose feeding in control bees, but not in artificially reared honeybees. Maximum flight speed was lower for artificially reared than for control bees when fed the 2M glucose solution. These differences may be attributable to less densely packed flight muscles or less muscle mass, as indicated by slightly lower thoracic weights of artificially reared bees, or slightly smaller wing surfaces (Hepburn et al., 1999).

Because the same amount of glucose (10 µL of 1M or 2M, respectively) was consumed by artificially reared and by control bees in each flight, the longer flight durations of artificially reared honeybees result in lower calculated metabolic power compared to that of control bees in both feeding regimes. However, metabolic power and glucose consumption were similar to values found in other studies (Gmeinbauer and Crailsheim, 1993; Nachtigall et al., 1995; Harrison and Fewell, 2002).

The viability of honeybees reared in vitro is important for toxicity testing (OEPP/EPPO, 2001, 2003). In contrast to oral $LD_{50}$, contact $LD_{50}$, field tests, or cage tests, our approach makes it possible to investigate the effects of substances applied to larvae not simply by measuring mortality of larvae but also by evaluating the viability of adult insects. We focused on flight performance because of the obvious significance of larval dietary protein on the development of flight musculature. Tautz et al. (2003) demonstrated that another method, conditioning of the proboscis extension reflex, is precise enough to evaluate differences in learning and memory consolidation in honeybees reared at different temperatures as pupae. Larval nutrition may also affect other parameters of viability of adult bees, such as disease susceptibility, development of hypopharyngeal glands (Moritz and Crailsheim, 1987), age at onset of foraging (Neukirch, 1982), behaviour, complex social interactions, and longevity (Kunert and Crailsheim, 1988; Mattila and Otis, 2006). We did not gather detailed data on the longevity of artificially reared honeybees, but in agreement with Tautz et al. (2003), who raised pupae at lowered temperatures, we found that the number of artificially reared bees present in the hive decreased with aging stronger than the number of control bees. This could be because artificially reared honeybees were more likely than other bees to fail to return from flights. However, our bees reached adult maturity and at least one of the artificially reared bees used for flight experiments attained the age of 31 days.

Our study has demonstrated that honeybee larvae can be well nourished in vitro and develop into healthy adults without morphologic deformations. The diet we provided was sufficient to produce working flight musculature, enabling the artificially reared bees to achieve similar flight performance to naturally reared bees. High-performance flight speed, thorax weight, and wing surface may still be slightly reduced, as described by authors for honeybees reared under difficult nutritional conditions (Eischen et al., 1982; Kunert and Crailsheim, 1988; Daly et al., 1995; Mattila and Otis, 2006). However, our findings definitively support the use of the described method in toxicity testings, although other parameters of viability remain unexplored.

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**Les performances de vol d'abeilles (*Apis mellifera*) élevées arificiellement.**

***Apis mellifera* / vol / élevage in vitro / nutrition larvaire / nourrissement / qualité des ouvrières / aile**

**Zusammenfassung – Flugleistung von künstlich aufgezogenen Honigbienen (*Apis mellifera*).** Honigbienen können im Labor künstlich aufgezogen werden. Dazu werden Larven aus Brutwaben in Plastikschälchen übersiedelt und die umfassende Brutpflege der Ammenbienen im Stock durch wenige definierte Fütterungen ersetzt sowie die Temperatur und die Luftfeuchtigkeit genau reguliert. Diese Methode ermöglicht es nun standardisierte Untersuchungen über die Auswirkungen von Chemikalien wie Pflanzenschutzmitteln oder Infektionen mit Krankheitserregern durchzuführen, ohne gesamte Völker in Kontakt mit den Schadstoffen oder Erregern zu bringen. Wir haben erstmals die Qualität von Arbeiterinnen die mit Hilfe dieser Technik aufgezogen wurden anhand ihrer Flugleistung in einem Karussell analysiert und mit der Leistung ihrer natürlich aufgezogener Schwestern verglichen (Abb. 1). Die durchschnittliche Fluggeschwindigkeit im Karussell betrug etwa 1 m/s und die maximale Fluggeschwindigkeit 1,4 m/s (Tab. I). Diese unterschieden sich bei künstlich und natürlich aufgezogenen Bienen nicht, wenn 10 µL einer 1-Molaren Glukoselösung gefüttert werden. Bei Fütterung von hochenergetischer 2-Molarer Glukoselösung flogen die Kontrollbienen, nicht aber die künstlich aufgezogenen, schneller als mit 1-Molarer Glukoselösung nämlich mit 1,2 m/sec Durchschnittsgeschwindigkeit. Die Maximalgeschwindigkeit (1,6 m/s) der Kontrollbienen war bei dieser Fütterung auch höher als die der künstlich aufgezogenen (1,4 m/s, Tab. I).
Ein guter Indikator für die Qualität der Larvalernährung ist das Gewicht der Arbeiterinnen, und wir fanden, dass das Trockengewicht bei den von uns künstlich aufgezogenen Bienen niedriger als bei den Kontrollbienen war. Wir konnten auch zeigen, dass dieser Unterschied vor allem in einem leichteren Thorax, in dem sich die Flugmuskulatur befindet, der künstlich aufgezogenen Bienen begründet ist (Abb. 2). Außerdem fanden wir schwache Unterschiede in den Flächen der Vorder- und Hinterflügel: wiederum waren die der künstlich aufgezogenen etwas kleiner als die der Kontrollbienen.
Unsere Ergebnisse zeigen, dass künstlich aufgezogene Bienen das Alter von Sammlerinnen (über 20 Tage) erreichen können. Trotz eines etwas leichteren Thoraxgewichtes, in dem sich die Flugmuskulatur befindet, und etwas kleinerer Flügel zeigten sie annähernd ähnliche Flugleistungen wie natürlich aufgezogene Honigbienen.

***Apis mellifera* / Flug / in vitro Aufzucht / Larvalernährung / Qualität der Arbeiterinnen**

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