

Pupal developmental temperature and behavioral specialization of honeybee workers (*Apis mellifera* L.)

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Received: 12 November 2008 / Revised: 6 April 2009 / Accepted: 9 April 2009 / Published online: 24 April 2009
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Abstract Honeybees (*Apis mellifera*) are able to regulate the brood nest temperatures within a narrow range between 32 and 36°C. Yet this small variation in brood temperature is sufficient to cause significant differences in the behavior of adult bees. To study the consequences of variation in pupal developmental temperature we raised honeybee brood under controlled temperature conditions (32, 34.5, 36°C) and individually marked more than 4,400 bees, after emergence. We analyzed dancing, undertaking behavior, the age of first foraging flight, and forager task specialization of these workers. Animals raised under higher temperatures showed an increased probability to dance, foraged earlier in life, and were more often engaged in undertaking. Since the temperature profile in the brood nest may be an emergent property of the whole colony, we discuss how pupal developmental temperature can affect the overall organization of division of labor among the individuals in a self-organized process.

Keywords Honeybee · Brood temperature · Foraging · Division of labor · Self-organization

Abbreviations

AFF Age of first foraging flight
JH Juvenile hormone

Introduction

Mechanisms regulating division of labor are decisive for the effective functioning of social insect colonies. The caste system (sensu Wilson 1971), typical for all eusocial insects, results in the most obvious division of labor with the queen specializing in reproduction and sterile workers performing other tasks to maintain the colony. However, there is usually also specialization within the worker caste. In honeybees *Apis mellifera*, division of labor among workers is realized as a temporal polyethism, which has been studied in detail since the pioneering work of Rösch (1925). The individual development of a honeybee worker starts with in-hive tasks, including cell cleaning, comb building, and brood tending, and ends with the outside tasks, such as guarding and foraging (Winston 1987). These changes of behavioral patterns go along with fundamental changes in the physiology of workers including gland activity, juvenile hormone (JH) titers, and levels of biogenic amines (Huang et al. 1994; Robinson and Vargo 1997; Schulz and Robinson 1999; Wagener-Hulme et al. 1999; Sullivan et al. 2000; Schulz et al. 2002; Deseyn and Billen 2005). In addition to a large suite of social interactions among individuals and reactions to intracolony, as well as environmental conditions (Robinson 1992), also genetic variance has been identified to be an important factor for task specialization in honeybees. Workers of specific subfamilies preferentially participate in specific tasks, such as foraging for nectar, pollen (Robinson and Page 1989; Fewell and Page 2000), and water (Kryger et al. 2000). The genetic mechanisms regulating pollen and nectar foraging have been analyzed down to the gene level, suggesting that a few major loci can have strong effects on the forager's specialization (Page et al. 2000; Hunt et al. 2007). As the environment of a social insect is largely determined by the behavior of its

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nestmates, also indirect genetic effects will play a role in the behavioral differentiation of the individuals. This means, that the phenotype of an individual is influenced by the expression of genes in a conspecific individual (Wolf et al. 1998; Linksvayer 2006).

One example for genetically controlled threshold variance has recently been shown for temperature regulation in honeybees (Jones et al. 2004). Honeybee colonies maintain an accurate thermal homeostasis in the brood nest of the colony. Worker bees are able to regulate the brood nest temperature between 33 and 36°C (Seeley 1995) by either heating through flight muscle activity, or cooling through ventilation and evaporation of water. The regulation of hive temperature primarily depends on the number of workers expressing heating behavior rather than the intensity of individual heating (Southwick 1982; Kronenberg and Heller 1982). The workers keep the temperature fluctuations in the brood nest sufficiently low to ensure appropriate conditions for larval and pupal development (Himmer 1927; Lindauer 1954; Harrison 1987). Nevertheless, there is temperature variation in the brood nest which varies within a range of 3°C. Though this small variance does not obviously affect morphological traits in wings, stinger, proboscis, or legs of workers (Himmer 1927; Groh et al. 2004), behavioral traits of adult workers, as well as the synaptic organization of their brains have recently been shown to be substantially affected by pupal developmental temperature variation (Tautz et al. 2003; Groh et al. 2004; Jones et al. 2005). If pupal developmental temperature affects behavior and brain function, this might also interfere with the overall division of labor among the workers, and thus be a central and far reaching mechanism to fine-tune colony organization.

In this study, we analyze the influence of the nest temperature during the pupal phase on the behavioral repertoire of the adult bees. We particularly focus on the transition from in-hive to foraging tasks as the most drastic, and best studied change in worker behavior (Beshers et al. 2001) to reveal the potential impact of nest temperature on worker specialization.

Methods

Honeybee workers

Experiments were conducted in the summers of 2004 and 2005 in Halle/Saale (Germany). In both years, three brood combs of *A. mellifera carnica* with capped worker cells were transferred from one colony to three different incubators set to 32, 34.5, and 36°C, respectively, each at 60% r.h. Larvae in uncapped cells left the comb after a few hours and were then removed from the rearing box. The pupae

were reared in the incubators until emergence of the adult bees. The duration of controlled temperature treatment for individual pupae thus lasted for a minimum of 6 days to a maximum of 12 days, depending on the initial age of the pupae. Pupae on the same comb emerged within a period of not more than 6 days. Once a day, newly emerged worker bees (aged 0–24 h) from the incubated combs were individually labeled each with a numbered color tag (Opalith-Plättchen) and immediately released into the observation hives. One week later, a second set of three brood combs from the same colony was transferred to the incubators under the same conditions.

In 2004 we released 2,374 individually labeled workers (965 workers raised at 32°C, 900 at 34.5°C, and 509 at 36°C) over a period of 12 days into three queenright foster colonies in observation hives with two combs and about 2,000 unlabeled worker bees. All observation hives contained equal numbers of target bees of all temperature treatment groups. The observation colonies were placed inside the laboratory and connected to the outside by a trap system, which allowed to easily catch arriving or departing bees. In a replicate experiment in the following year, we used the same setup, but introduced 753 workers raised at 32°C, 714 workers at 34.5°C, and 564 workers at 36°C into two observation hives. All brood combs originated from the same donor colony in both years.

Observations

We drew a grid (40 quadrants, 8 × 7.5 cm) on each side of the observation hives and consecutively scanned all quadrants from left to right and top to bottom. Three daily observation scans were performed in parallel in all colonies by two observers per colony, each scan lasting about half an hour. In the experiments in 2004, we recorded various activities of in-hive bees, including cell inspection, fanning, grooming, retinue behavior, trophallaxis, moving, and being inactive. Alas, since none of these traits yielded differences between the treatment groups we focused our subsequent studies in 2005 on the transition from in-hive to outdoor worker and on activities of foragers. We recorded the behavior of all sighted marked bees on the combs, and on their way out of and into the hive and focused our behavioral analyses on dancing, undertaking, and foraging behavior. Bees were classified as dancers, when they were observed in dancing at least 1 time anywhere on the combs. We defined those workers as undertakers which engaged in removing dead bees from the hive. Labeled bees returning from a flight were caught in the trap during periods of high foraging activity. Bees carrying pollen pellets were classified as pollen foragers. Nectar foragers were identified by gently squeezing the abdomen, until a droplet of crop content was disgorged. We used qualitative glucose test-strips

(Biophan G, Kallies Feinchemie AG, Germany) to assess the glucose concentration in the crop content on the basis of five reaction categories (0–4) to distinguish between water and nectar foragers. Foragers with a negative or the least positive reaction (0, 1) were classified as water foragers, all others (2–4) as nectar foragers. We continued these observations for 18 days.

Statistical analyses were conducted with analyses of variance and χ^2 -tests using the Statistica® and the R software packages. Normality of the data was tested with a Kolmogorov-Smirnow test.

Results

Proportion of dancers

There was no significant effect of the host colony environment on dancing ($\chi^2 = 5.9$, $P = 0.20$, $N = 1577$, $df = 4$) but we found a significant correlation between the proportion of dancers and the treatment temperature (Spearman $R = 0.64$, $P = 0.01$, $N = 15$) (Fig. 1). Higher developmental temperatures significantly increased the proportion of dancers observed in the five test colonies. Altogether, we found 14 dancing bees out of 549 bees observed in outdoor activities in the 32°C treatment group, 23 dancers out of 545 outdoor bees in the 34.5°C group, and 26 dancers out of 483 outdoor bees in the 36°C group.

Age of first foraging

In total, 1,577 workers were observed in outdoor activities (2004: 547, 2005: 1,030). 549 workers were from the 32°C temperature group (232 in 2004, 317 in 2005), 545 from the 34.5°C group (206 in 2004, 339 in 2005), and 483 from the 36°C group (109 in 2004, 374 in 2005). The age of first

foraging could be determined for 1,262 individuals. We assessed the influence of the host colonies on worker behavior with a factorial ANOVA using “age of first flight” as dependent variable and pupal developmental temperature, year and hive as categorical predictors.

We found significant differences for the age of first flight among the three temperature treatments ($F = 4.3$, $P = 0.0014$, $N = 1262$, $df = 2$), among the two test seasons in 2004 and 2005 ($F = 139.2$, $P < 0.001$, $N = 1262$, $df = 1$), and among the colonies within years (2004: $F = 11.1$, $P < 0.001$, $N = 241$, $df = 2$; 2005: $F = 83.0$, $P < 0.001$, $N = 1021$, $df = 1$). The interaction between hive and pupal developmental temperature was not significant (ANOVA: in 2004: $F = 1.2$, $P = 0.33$, $N = 241$, $df = 4$; in 2005: $F = 1.1$, $P = 0.33$, $N = 1021$, $df = 2$). Thus, the effect of pupal developmental temperature on the age of first foraging did not differ between the host colonies.

Focussing on the age of first foraging within the colonies, we found a highly significant negative correlation of brood temperature and the age of the first observed foraging flight for both colonies in 2005 and also in 2004 there was a trend for a negative correlation in two out of three colonies (Table 1). In all colonies, the bees reared at 36°C had the earliest onset of foraging, whereas the bees reared at 32°C were the latest to start foraging in four of five colonies (Table 1). In a pooled analysis of the data of 2004 and 2005 using the deviation from the mean age of first foraging in the corresponding year for each individual, we found a highly significant correlation between treatment temperature and age of first foraging (Fig. 2 Spearman $R = -0.12$, $P = 0.00002$, $N = 1262$).

Undertaking

A worker was classified as an undertaker if it was observed removing a dead bee from the colony. Undertaking was an extremely rare behavior in all three observation colonies (only recorded in 2004). Only 20 cases of undertaking out

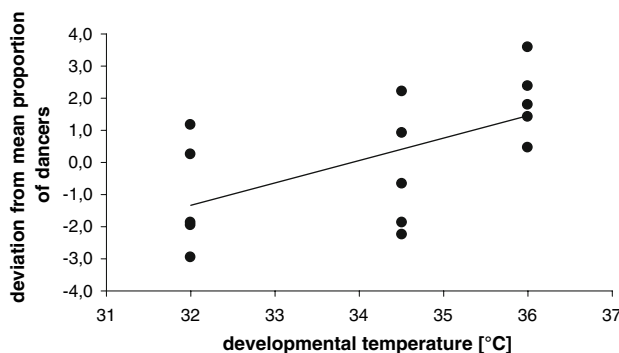


Fig. 1 Relationship between dancing and the developmental temperature. Shown on the y-axis is the deviation from the mean frequency of dancers in each hive. We found a significant, positive correlation (Spearman $R = 0.64$, $P = 0.01$, $N = 15$)

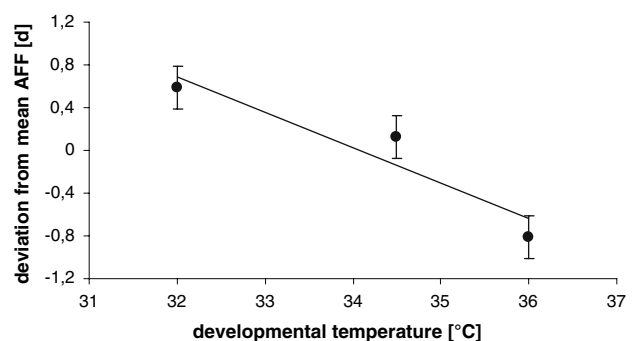


Fig. 2 The effect of the brood temperature on the age of first flight (AFF) shown as the deviation from mean age of the first flight in days \pm s.e

Table 1 Mean age in days of first observed foraging flight (AFF) for all five colonies and number of individuals for each treatment group and colony

Colony	AFF	<i>N</i>	AFF	<i>N</i>	AFF	<i>N</i>	<i>N</i> total	<i>P</i>
	32°C		34.5°C		36°C			
1	8.6	42	9.2	36	7.8	4	82	0.561
2	13.1	38	12.2	47	12.1	9	94	0.299
3	12.1	32	11.5	28	7.6	5	65	0.072
4	16.9	143	16.8	150	15.4	158	451	0.001
5	14.8	172	14.2	186	13.6	212	570	0.003

The *P* values correspond to a Spearman rank order correlation. Experiments for colonies 1–3 were conducted in 2004, for colonies four and five in 2005

of 7,006 individual behavioral observations were recorded. The proportion of undertaker bees was not significantly different among the three host colonies tested in 2004 ($\chi^2 = 4.4$, $P = 0.11$, $N = 547$, $df = 2$). Pooling the data from the three colonies, we found that the frequency of undertakers in the 36°C group was about 3–4 times higher than in the other two groups (Table 2) resulting in a significant difference ($\chi^2 = 8.5$, $P = 0.014$, $N = 547$, $df = 2$).

Forager task specialization

We observed a weak preference of 36°C reared bees for water foraging, a slightly raised frequency of 34.5°C reared bees for pollen collection and the 32°C reared bees showed higher frequencies for nectar collection or returning empty from foraging trips (Table 3). The developmental temperature was negatively correlated with the proportion of nectar foragers in the five colonies (Spearman $R = -0.55$, $P = 0.03$, $N = 15$). No correlations were detectable for water foraging, pollen foraging, and bees returning empty (water foragers: $R = 0.36$, $P = 0.19$, $N = 15$; pollen foragers: $R = -0.04$, $P = 0.89$, $N = 15$; empty bees: $R = 0.32$, $P = 0.24$, $N = 15$). To test, whether a non-monotonous relation between developmental temperature and foraging preference might exist, we used the χ^2 -statistics. An overall χ^2 -test yielded no significant differences ($\chi^2 = 6.2$, $df = 6$, $P = 0.40$, $N = 368$). Based on hypotheses pointed out in the discussion, we compared the 36°C water foragers against

Table 2 The effect of the pupal developmental temperature on the frequency of undertakers in all outdoor bees

Temperature (°C)	Undertakers <i>N</i>	Other outdoor bees <i>N</i>	Undertakers %
32.0	7	225	3.0
34.5	4	202	1.9
36.0	9	100	8.3
Total <i>N</i>	20	527	547

the 32 and 34.5°C water foragers and we compared the 34.5°C pollen foragers versus the 32 and 36°C pollen foragers without significant results. However, we found a significantly increased number of nectar foragers and foragers returning empty in the 32°C temperature group compared to those of the 34.5 and 36°C treatment group ($\chi^2 = 4.0$, $P < 0.05$, $N = 368$, $df = 1$).

Discussion

Active regulation of hive temperature by adult worker bees is supposed to provide optimal conditions for brood rearing (Himmer 1927). However, in spite of this high capability to maintain colonial homeostasis there is clearly no perfectly uniform and even temperature distribution in the colony's brood nest (Levin and Collison 1990; Kraus et al. 1998). The temperature in the colony is often characterized by steep gradients depending on the size of the colony and the ambient temperature (Southwick and Heldmaier 1987; Fahrenholz et al. 1989). Bees in the periphery can be much cooler than in the centre of the nest. Moreover, the temperature distribution can be highly patchy, as workers are able to generate heat in empty cells scattered over the brood nest and thereby efficiently warm the brood in the adjacent cells (Kleinhenz et al. 2003). Irrespective of how brood temperature varies, our findings suggest that even small deviations in the brood nest temperature may have global effects on the social organization of the colony.

Dance frequency

Worker bees reared at 32°C showed a reduced frequency of dancers compared to the other test-groups. These results support the findings of Tautz et al. (2003), who also regarded bees developed under 32°C as “bad dancers”, as they performed less dance circuits and had an increased variance in the duration of the waggle dance in comparison to the 36°C group.

Thus again, the specialization of adult workers depended on the temperature regime during their pupal phase. Variation in environmental conditions therefore caused variation of the behavioral performance and resulted in division of labor among the workers.

Age of first foraging

Brood rearing temperature also had significant effects on the transition from in-hive to outdoor workers. An increased pupal developmental temperature reduced the age of first foraging. Brood temperature therefore adds to the already known factors regulating the onset of foraging.

Table 3 Absolute numbers of water, nectar, pollen foragers, and empty bees in each treatment group and the expected values

	32°C (observed)	32°C (expected)	34.5°C (observed)	34.5°C (expected)	36°C (observed)	36°C (expected)
Water	11	15.2	14	13.6	18	14.3
Pollen	19	23.7	26	21.1	22	22.2
Nectar	51	45.6	35	40.7	43	42.8
Empty	49	45.6	41	40.7	39	42.8
<i>N</i> (individuals)	130		116		122	

Foraging loads correspond to foragers response thresholds for sucrose, which are influenced by the bees juvenile hormone titers. If developmental temperature interferes with the juvenile hormone metabolism, we expect a preference for the 36°C bees to collect water, for the 34.5°C bees to collect pollen and for the 32°C bees to collect nectar or to return empty from foraging trips. Testing the 36°C water foragers against the 32 and 34.5°C water foragers we find no significant difference ($\chi^2 = 1.2$, $P = 0.26$, $N = 368$, $df = 1$). Also the frequency of 34.5°C pollen foragers is not significantly different from the pooled 32 and 36°C pollen foragers ($\chi^2 = 1.6$, $P = 0.20$, $N = 368$, $df = 1$). However, there is a significant increase of nectar foragers and bees returning empty in the 32°C group compared to the 34.5 and 36°C group ($\chi^2 = 4.0$, $P < 0.05$; $N = 368$, $df = 1$)

Besides genotypic variability for age polyethism (Calderone and Page 1988; Giray and Robinson 1994; Pankiw and Page 2001), and behavioral plasticity due to social factors and colony age demography (Huang and Robinson 1992; Huang and Robinson 1996), also pheromonal effects have been suggested to influence the rate of behavioral development (Pankiw 2004). Workers start foraging at older ages if they are exposed to increased concentrations of queen mandibular gland pheromone (Pankiw et al. 1998) or to ethyl oleate, produced by foragers (Leoncini et al. 2004). Similar effects were observed when bees were treated with brood pheromone (LeConte et al. 2001). In general, the physiological interplay of vitellogenin and JH titers seems to be an essential element driving task specialization in honeybees (Amdam et al. 2004; Amdam et al. 2006).

Particularly the transition from in-hive worker bee to outdoor forager is well-understood and closely associated with increased JH titers (Huang et al. 1994). JH has been suggested to act as “behavioral pacemaker” (Robinson and Vargo 1997; Sullivan et al. 2000) and is supposed to be responsible for the onset of foraging. Similar to JH, levels of the biogenic amine octopamine influence the onset of foraging but probably acting on a shorter time-scale (Schulz et al. 2002). Treatment of young bees with the biogenic amine octopamine resulted in precocious foragers (Schulz and Robinson 2001) and it may well be that pupal developmental temperatures interfere with JH metabolism. Huang and Robinson (1995) were able to show a close relationship between temperature and JH biosynthesis by moving colonies into a cold-room. Foragers had significantly reduced JH titers after this treatment. Accordingly, low pupal developmental temperatures might lead to reduced JH biosynthesis rates of the adult bees, cause a delayed behavioral development and hence a later onset of foraging.

Foraging preferences

If pupal developmental temperature interferes with JH metabolism and/or octopamine levels in the bee’s brain, then foraging preferences of bees reared at higher temperatures should be similar to those treated with octopamine or the JH analogue methoprene. Pankiw and Page (2003) showed that treatment with octopamine and methoprene both reduced the sucrose response thresholds. They further examined the sucrose response threshold for the different forager groups and found that water foragers had the lowest thresholds, followed by pollen foragers. Nectar foragers had higher thresholds and workers returning empty had the highest response thresholds for sucrose (Pankiw and Page 2000). Thus, the response thresholds of foragers increase in the following order: water < pollen < nectar < empty foragers. In light of these findings it may well be that our temperature treatment interfered with octopamine or JH titers in the developing pupae pathways, causing the observed differences in task specialization. We would then expect the foragers with highest developmental temperatures to preferably collect water, bees with medium developmental temperatures to forage pollen, and workers developed under cool conditions to forage for nectar or return empty from foraging trips. These predictions match our data with a negative correlation of developmental temperature and the proportion of nectar foragers, and with the highest frequency of nectar foragers and empty bees in the 32°C treatment group. The number of pollen foragers in the 34.5°C group and the number of water foragers in the 36°C group were higher than expected, however, not significantly. Hence, the effect of pupal developmental temperature on foraging specialization was not very strong but significant for some aspects in our data set.

Other differentiation mechanisms including genetic variance within the colony due to the multiple matings of the

queen may have a stronger effect on behavioral thresholds and on what a forager is collecting than the developmental temperature. Nevertheless, we conclude that temperature during pupal development has quantifiable consequences for the behavior of adult workers and may influence task allocation on the colony level: Temperature dependent differences in the behavioral development from 1–2 days represent about 5–10% of an individual's total time spent for in-hive duties. Hence in a colony of 40,000 workers, this effect would result in enhancing the in-hive workforce by 2,000–4,000 individuals at the expense of the foraging tasks.

It may therefore well affect the global structure of division of labor in the colony and social regulation as a whole, because it interferes with the crucial transition from in-hive to outdoor activities. Together with age polyethism and genetic variance, the thermal profile in the brood nest may therefore be a possible parameter when studying the regulation of division of labor in insect societies. As the colony grows so will the brood nest, and the number of in-hive bees heating the brood could affect the pace of behavioral development in the next generation of workers. This sets the stage for reinforcing feedback loops influencing the proportion of in-hive workers. A low number of heating bees will result in lower brood nest temperatures and might induce a delay in the behavioral development in the forthcoming worker generation—more in-hive bees available for heating the brood would then be the consequence. Hence, brood temperature might be one factor to fine-tune division of labor via self-organized patterns among the honeybee workers.

Acknowledgments We thank the DFG for funding (RM) and K. Dahlke, T. Janik, C. Opitz, T. Schnelle, M. Ellis, C. Nossol, A. Schmidt and M. Thoss for helping with the behavioral observations and the anonymous reviewers for helpful comments on the manuscript. All our experiments complied with the current German laws and also with the “Principles of animal care”, publication No. 86-23, revised 1985 of the National Institute of Health.

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