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ARTICLE *in* JOURNAL OF COMPARATIVE PHYSIOLOGY · JANUARY 2006

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DOI 10.1007/s00359-005-0035-z

ORIGINAL PAPER

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The effects of rearing temperature on developmental stability and learning and memory in the honey bee, *Apis mellifera*

Received: 9 May 2005 / Revised: 27 June 2005 / Accepted: 2 July 2005 / Published online: 28 July 2005

Abstract Honey bee workers maintain the brood nest of their colony within a narrow temperature range of 34.5 ± 1.5°C, implying that there are significant fitness costs if brood is reared outside the normal range. However, the effects of abnormal incubation temperatures are subtle and not well documented. Here we show that short-term learning and memory abilities of adult workers are affected by the temperature they experienced during pupal development. In contrast, long-term learning and memory is not significantly affected by rearing temperature. Furthermore, we could detect no effects of incubation temperature on fluctuating asymmetry, as a measure of developmental stability, in workers, queens or drones. We conclude that the most important consequence of abnormal rearing temperatures are subtle neural deficiencies affecting short-term memory rather than physical abnormalities.

Keywords Temperature · Developmental stability · Learning and memory · Honey bee

Abbreviations FA: Fluctuating asymmetry · FDR: False discovery rate · LT: Long-term · PER: Proboscis extension reflex · ST: Short-term

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Introduction

Many social insects maintain precise environmental conditions, such as relative humidity, temperature and carbon dioxide levels, within their nests. These conditions are thought to be those that are optimal for normal development of the brood (Simpson 1961; Seeley and Heinrich 1981; Kronenberg and Heller 1982; Korb and Linsenmair 1998, 1999; McMullan and Brown 2005). Honey bee colonies need to maintain their brood nest temperature between 32 and 36°C (Kleinhenz et al. 2003) so that their brood develops normally (Seeley and Heinrich 1981). However, colonies expend much energy on maintaining brood nest temperatures in a much more narrow range (~34 to 35°C) (Kronenberg and Heller 1982; Jones et al. 2004). This suggests that optimal development of the brood requires an extremely stable brood nest temperature.

Tautz et al. (2003) studied the effects of brood incubation temperature on the foraging performance of adult honey bees. They examined two important aspects of foraging behaviour: the ability to perform communication dances, and the ability to associate a floral odour with a reward.

For their first experiments Tautz et al. (2003) trained forager bees to visit a feeder and analysed three characteristics of the dance communication (Von Frisch 1993) used by the trained foragers to recruit nest-mates to a profitable food source. Despite low sample sizes, their data strongly indicate that adult workers that pupate at 36°C are normal with respect to their performance of communication dances. However adult workers that pupated at 32°C are less likely to perform dances and perform shorter ones than bees that pupated at 36°C.

In their second experiment, Tautz et al. (2003) used the Proboscis Extension Reflex (PER) test (Menzel 1993) to determine the learning and memory abilities of bees pupated at different temperatures. The PER test is widely accepted as being a useful measure of learning and memory in the honey bee (Brandes et al. 1988; Gauthier

et al. 1992; Menzel 1993, 1999; Gerber et al. 1996; Morgan et al. 1998; Ray and Ferneyhough 1999; Maleszka et al. 2000; Laloi et al. 2001; Maleszka and Helliwell 2001; Gramacho and Spivak 2003). In the PER test, a scented sucrose solution is offered to a tethered bee. Some time later, the bee is again exposed to the odour, and is deemed to have remembered to associate it with a food reward if it extends its proboscis. Successful conditioning is achieved very quickly, and a large percentage of workers respond to an odour after a single entrainment event (Bitterman et al. 1983; Hammer and Menzel 1995). The ability of forager bees to rapidly learn to associate a particular floral odour with a nectar reward is undoubtedly a critical part of successful foraging. Thus it can be argued that the PER test evaluates an important component of an individual bee's foraging abilities.

Tautz et al. (2003) found that adult workers that pupated at 36°C show better performance in PER tests than workers that pupated at the normal hive temperature of 34.5°C or the lower bound of 32°C. Because the PER test was performed 1 or 10 min after entrainment, Tautz et al. tested the *short-term* rather than the *long-term* memories of the entrained bees. As with *Drosophila* (Zars et al. 2000), there is evidence that honey bees use different areas of their brains for short- (ST) and long-term (LT) memory (Hammer and Menzel 1995, 1998; Müller 1996; Menzel 1999; Maleszka et al. 2000; Zars et al. 2000; Maleszka and Helliwell 2001; Pascual and Preat 2001; Strausfeld 2002) and temperature effects on the brain are position and modality specific (Groh et al. 2004). A single conditioning trial induces a ST memory that lasts for hours, while multiple trials lead to LT memory. In contrast to ST memory, LT memory involves protein synthesis, and results in memory that can last for several days (Menzel 1999).

Tautz et al.'s results raise important questions about why honey bee colonies maintain brood nest temperatures within a very narrow range. Why does pupation at slightly higher than normal temperature result in adults that perform better in tests of ST memory? Does temperature during pupal development affect LT memory too? What, if any, deficiencies occur in workers that pupate at slightly atypical temperatures, such as 32°C? Do they exhibit increased developmental instability or are the deficiencies solely neurological? To answer these questions, we required both a measure of general developmental stress, and measures of neurological impairment.

Fluctuating asymmetry (FA) is widely used as a measure of developmental stability (Palmer 1994; Palmer and Strobeck 2003). Fluctuating asymmetry refers to random deviations between sides in a bilaterally symmetrical organism (Palmer 1994; Palmer and Strobeck 2003). The assumption behind FA analysis is that the development of both sides of a bilaterally symmetrical organism is influenced by identical genes, and that therefore any deviations between the two sides of such organisms results from developmental perturbations (Clarke 1998). Thus an organism showing higher FA than is typical for its species is thought to have suffered from environmental or genomic stress during its development.

A variety of factors such as chemical exposure (Mpho et al. 2001), unusually high or low temperatures (Imasheva et al. 1997) and inbreeding (Leary and Allendorf 1989) have been shown to affect levels of FA in insects. Temperature during pupation has been repeatedly shown to affect FA in holometabolous insects like the blowfly *Lucilia cuprina* (Clarke and McKenzie 1992; McKenzie and Yen 1995) and *Drosophila* (Imasheva et al. 1997). We therefore considered that FA is likely to provide a useful measure of temperature-induced developmental stress in honey bees.

Here we explore the effects of rearing pupae at different constant temperatures within, and on the margins of the normal range experienced in the brood nest of a honey bee colony. We used two different measures to determine the effect of rearing temperature on adult bees: fluctuating asymmetry and learning and memory ability as measured by PER. We conducted FA analyses on all castes: workers, queens and drones. We predicted that drones and queens might be more tolerant of suboptimal rearing temperatures than workers because they are usually reared on the periphery of the comb where temperatures fluctuate more, whereas worker brood tends to be highly concentrated towards the centre of the brood nest where the temperature is usually higher and more precisely regulated. The crucial component of foraging behaviour, the ability to associate a particular odour with a lucrative reward, was tested on workers only using the PER, both immediately after entrainment, and after 24 h.

Materials and methods

Rearing of workers, queens and drones

All individuals were offspring of the same queen and were produced during summer in Sydney, Australia. In honey bees, the pre-pupal phase occurs just after a brood cell has been sealed by workers (Winston 1987). We transferred pre-pupal brood, on the same day or 1 day after capping, from the study colony into seven different incubators (Thermoline refrigerated incubators Model-RI 250 SG). Individuals were then incubated at seven different constant temperatures: 31, 32, 33, 34, 35, 36, and 37 ± 0.5°C and an average of 52% RH for the duration of pupal development (workers ~13 days, queens ~10 days, drones ~15 days). We confirmed that the incubators had maintained the temperatures that we had specified by using temperature recording data loggers (Dallas Semiconductor, Texas) that had been calibrated against a mercury thermometer. The difference in emergence time between individuals reared at the lowest and highest temperatures was approximately 2 days.

Workers

To obtain worker brood of known age we caged our experimental queen on two frames of empty comb within her colony for 2 days. After the brood was sealed (~10 days after oviposition) we cut the combs containing brood into seven sections using a scalpel, and transferred the sections into wire cages (8.5×11.5×6.5 cm). We then randomly allocated each of the pieces of brood comb (containing at least 60 pre-pupal workers) to one of the seven incubators. After all individuals incubated at a particular temperature had emerged, we transferred the cage containing the adult workers to a 33°C incubator for maturation. We fed the caged workers ground pollen and sugar syrup (~2 M). After the learning and memory experiments had been completed we stored the workers in 70% ethanol for FA analysis.

Queens

We raised queen pupae using a standard commercial beekeeping protocol (Matheson 1984; Harbo 1986). We grafted 1-day old larvae (collected from the same colony as the workers) into individual artificial queen cells. Batches of approximately 30 queen cells were reared in each of ten host colonies. Cells were removed 5 days after grafting (development time of queens is a lot shorter than in the other castes) and 48 randomly selected sealed queen cells were placed in each of the incubators. After the queens emerged, we transferred them into separate cages containing five workers and a ball of queen candy as a food source. We then transferred the queens to the 33°C incubator. After 2 days, when their wings had hardened, the queens were stored in ethanol for FA analysis.

Drones

We raised drones in the same way as the workers, except that we provided comb containing the larger drone-rearing cells for the queen to lay in. In addition, we fed the source colony extra pollen to stimulate drone production. Individual sections of the drone comb (containing at least 60 pre-pupal drones) were moved into the different incubators approximately 10 days after oviposition. After all individuals at a particular temperature had emerged we transferred the cage containing the adult drones to a 33°C incubator for 2 days, along with 15 workers to help with feeding. We provided queen candy for feeding. After 2 days we stored the drones in ethanol for FA analysis.

Learning and memory tests

We tested the workers for their ST and LT memory abilities (see below) 7 days after they had emerged from their brood comb following Laloi et al. (2001), Maleszka et al. (2000) and Maleszka and Helliwell (2001). The total number of bees tested in the LT memory trials was 378 and the total tested for their ST memory was 546.

The workers to be tested were first placed on ice until they became immobilised. We then secured each individual in a separate thin-walled aluminium tube (7 mm in diameter) using a thin strip of adhesive tape. After we had placed the workers in the tubes, we fed each bee to satiation with a 1 M sucrose solution provided via a syringe fitted with a 23-gauge needle. After we had fed the workers, we placed them in an incubator overnight at ~24°C. The following day we trained the workers to associate an odour (limonene: Sigma, St Louis, Missouri, 4 µl/ml) with a reward stimulus (the 1 M sucrose containing the limonene). To train a worker, we extruded a drop of the scented sucrose onto the tip of a syringe needle. We exposed the worker to the odour for 6 s and then touched both antennae with the reward. Following this stimulus the worker would extend it's proboscis whereupon we allowed it to taste the sucrose. This conditioning procedure was either completed once (ST trial) or three times at 6 minute intervals (LT trial). A small exhaust fan positioned behind the bee prevented build up of odour during all trials. To test a worker, we again exposed the worker to the odour for 6 s, but did not touch the antennae. If the worker extended its proboscis it was recorded as having learnt successfully, if the worker did not respond it was recorded as a non-learner.

Short-term memory tests were conducted 1 h after the training. LT memory tests were performed 24 h after training, during which time bees were fed with sucrose and placed in a 24°C incubator overnight. These time intervals, between training and testing, have been successfully used in other studies to compare ST and LT memory retention (Maleszka et al. 2000; Maleszka and Helliwell 2001) and to test LT memory (Ray and Ferneyhough 1999).

We conducted ST tests on workers reared at 32, 33, 35 and 36°C, and LT tests on workers reared at 31, 32, 33, 34, 35, 36 and 37°C. Both experiment types were repeated once each using different cohorts of bees. For the second LT trial, workers from the 31°C treatment could not be tested because not enough individuals survived for testing.

Chi-squared tests were used to determine if there was a significant difference in the proportion of bees that learned to associate the odour of limonene among the cohorts that had pupated at the different temperatures. Chi-squared tests of homogeneity were used to determine if the data from the two replicate experiments could be combined. To correct for making multiple comparisons we used the false discovery rate (FDR) bootstrap procedure in the QVALUE program (Storey and Tibshirani 2003). The FDR is the expected proportion of type 1 errors among all significant results (Garcia 2003, 2004; Verhoeven et al. 2005). The

resulting Q-values are similar to P-values and provide a measure of each test's significance taking into account that multiple tests were performed (Storey and Tibshirani 2003).

Wing measurements

We removed the four wings from each individual and then mounted the wings on glass slides using Euparal. We then photographed all the wings using a digital camera and microscope (Leica IM300 with IM1000 software, Photomakroscope M 400). Six morphometric characters (wing veins) and one meristic character (number of hamuli) were measured as per Clarke et al. (1992) with one additional wing vein character. All wings were measured using the computer program Object-ImagePre2.11j, at a scale of 150:1.

Asymmetry measures for each trait were combined for each individual. This overall measure of asymmetry gives a better estimate of the developmental instability of an individual and provides additional statistical power (Palmer 1994; Leung et al. 2000; Palmer and Strobeck 2003). The average asymmetry index was used to compare individuals raised at different temperatures. The index for each individual was calculated as the average relative FA for all traits. ANOVA was used to determine if there was a significant difference between individuals that pupated at different temperatures. Comparisons were done within and between castes.

Imperfections in drone wing veins

As with Phillips (1929) we observed a number of imperfections in the venation patterns of the drone wings, such as extra and absent veins. We categorised these imperfections into 27 different classes. We used the number of individuals exhibiting vein imperfections as an additional measure of developmental stability. Chi-squared analysis was used to determine if individuals reared at different temperatures differed in the number of vein imperfections.

Results

Learning and memory tests

For both the LT and ST memory tests there was no significant difference between the two replicate trials (Heterogeneity Chi-square, LT: $\chi^2_5 = 5.199$, $P = 0.392$, ST: $\chi^2_3 = 0.404$, $P = 0.939$, see Table 1). Therefore the two replicate trials were combined for each temperature for both ST and LT tests.

Rearing temperature had a significant effect on ST learning and memory abilities of workers, after correcting for multiple comparisons (Table 2 and Fig. 1a). To determine which temperatures caused significantly different learning and memory abilities we compared each temperature pair. There was no difference between the highest temperature treatments (35 and 36°C). However, there was a significant difference between the 33°C treatment and the 32°C treatment. The effect of the two lowest rearing temperatures on learning and

Table 1 χ^2 analysis of the effect of rearing temperature on long and short-term memory abilities in honey bees

	χ^2	df	P
Short-term memory			
Trial 1	9.636	3	0.02
Trial 2	38.163	3	< 0.001
Total of χ^2	47.799	6	< 0.001
χ^2 of total	47.395	3	< 0.001
Heterogeneity	0.404	3	0.94
Long-term memory			
Trial 1	5.048	5	0.41
Trial 2	9.612	5	0.09
Total of χ^2	14.660	10	0.14
χ^2 of total	9.461	5	0.09
Heterogeneity	5.198	5	0.39

Table 2 Pairwise comparisons (chi-square) of the effect of temperature on short-term and long-term learning and memory in honey bees

Temperature (°C)	χ^2	P	Q
A			
32 vs 33	28.079	< 0.001	< 0.001
32 vs 35	28.228	< 0.001	< 0.001
32 vs 36	26.561	< 0.001	< 0.001
33 vs 35	9.983	0.001	0.0004
33 vs 36	7.184	0.007	0.0015
35 vs 36	0.381	0.537	0.0942
B			
31 vs 32	1.546	0.214	0.397
31 vs 33	0.445	0.505	0.428
31 vs 34	0.225	0.635	0.475
31 vs 35	0.603	0.437	0.397
31vs 36	0.239	0.625	0.475
31vs 37	1.546	0.214	0.397
32 vs 33	0.696	0.404	0.397
32 vs 34	1.173	0.279	0.397
32 vs 35	6.331	0.012	0.102
32 vs 36	0.777	0.378	0.397
32 vs 37	< 0.001	1	0.606
33 vs 34	0.083	0.773	0.547
33 vs 35	4.087	0.043	0.182
33 vs 36	0.026	0.872	0.584
33 vs 37	0.696	0.404	0.397
34 vs 35	3.184	0.074	0.235
34 vs 36	0.006	0.937	0.596
34 vs 37	1.173	0.279	0.397
35 vs 36	2.470	0.116	0.295
35 vs 37	6.331	0.012	0.102
36 vs 37	0.777	0.378	0.397

Data from both replicate experiments, for each experiment type, were combined for the analysis. 'A' shows individual temperature comparisons for ST learning and memory tests and the Q-value (Storey and Tibshirani 2003) after correcting for multiple comparisons. 'B' shows individual temperature comparisons for LT learning and memory tests and the Q-value after correcting for multiple comparisons

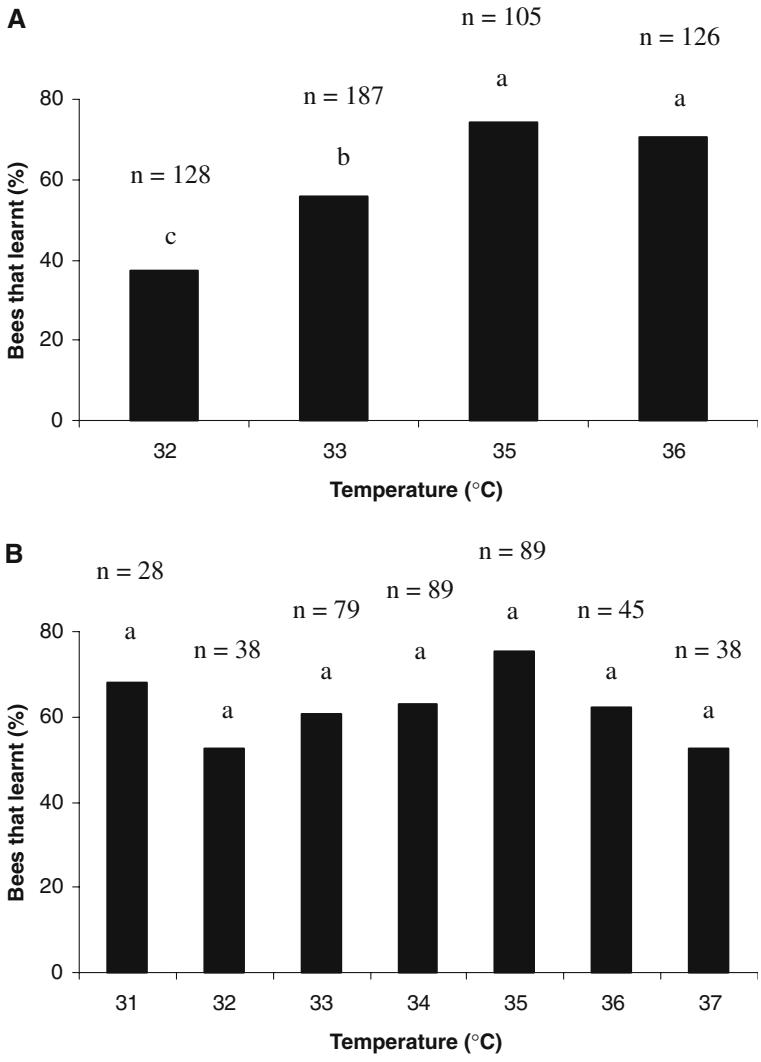

Fig. 1 Learning and memory abilities in workers reared at different temperatures. **A** Short-term memory, **B** long-term memory. *Bars* with a different letter are significantly different at the 5% level after correcting for multiple comparisons (Q-value)

memory were consistently significantly different from the effect of the two highest rearing temperatures (Table 2).

Overall, rearing temperature had no significant effect on the LT learning and memory abilities of workers (Fig. 1B, Table 1). This was also the case when we excluded the data of 31, 34 and 37°C; those temperatures that were not used in the ST test. There was also no overall difference when the single replicate of 31°C was included in the analysis ($\chi^2_6 = 9.720$, $P = 0.137$). When individual temperature treatments were compared, there were no significant differences after correcting for multiple comparisons (Table 2).

Fluctuating asymmetry preliminary analyses

Fluctuating asymmetry (FA) analysis, using the difference between left and right side measurements, was considered justifiable because there was no consistent evidence of directional asymmetry (Palmer 1994; Palmer and Strobeck 2003). Antisymmetry was measured by a Kolmogorov–Smirnov test for departures from normality (Palmer 1994). The small number of instances of antisymmetry (pattern of bilateral variation in a sample of individuals, where a significant difference exists between sides, but where the side that is larger varies randomly among individuals) were considered to be a result of type 2 errors. There was also a significant level of FA relative to measurement error for all traits, thus indicating that measurement error was not swamping any underlying FA. Relative FA was used for all comparisons. A relative measure of FA is the absolute difference between sides divided by the mean of the two sides (Palmer 1994).

Fluctuating asymmetry results

Rearing temperature had no significant effect on average asymmetry across traits for any of the castes (one way ANOVA with temperature as a factor; workers: $F_{6,264} = 0.942$, $P = 0.466$, queens: $F_{6,184} = 1.395$, $P = 0.219$ and drones: $F_{5,158} = 1.189$, $P = 0.317$) (Fig. 2). There was no significant effect of 'caste', on FA when all data were

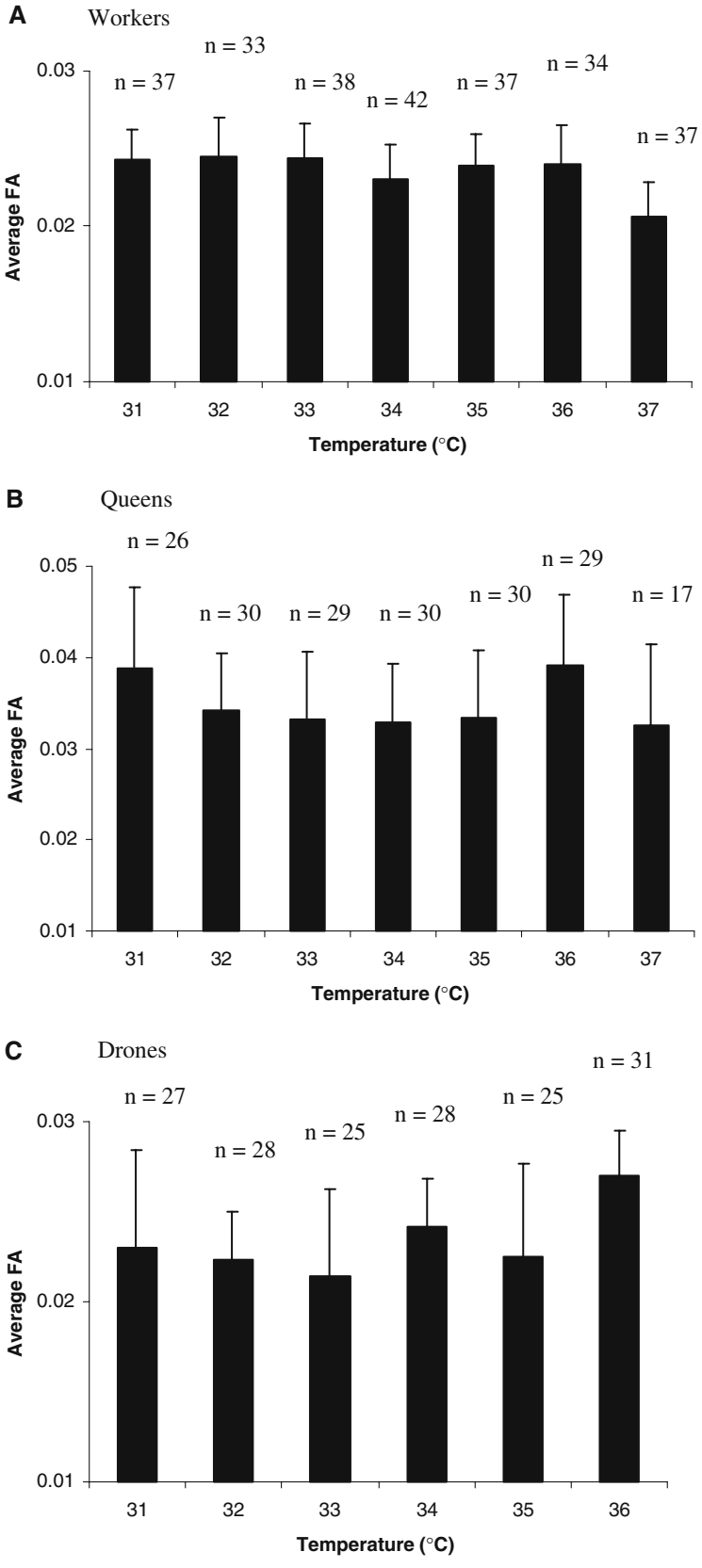

Fig. 2 Wing fluctuating asymmetry in honey bees reared at different temperatures. *Columns* represent mean asymmetry scores across six traits (Clarke et al. 1992). *Bars* are standard errors of the mean. There are no significant differences between fluctuating asymmetry scores at any temperature for any caste or overall

pooled for analysis (two way ANOVA of caste and temperature). Rearing temperature also had no effect on average FA when castes were combined (temperatures 31–36°C). There was no effect of rearing temperature on the number of individuals exhibiting venation imperfections in the drones ($\chi^2_{10} = 9.652$, $P = 0.471$).

Discussion

Our study shows that bees reared at 35 and 36°C have a better ST memory (as measured in a PER assay, 1 hour after a single learning trial) than bees reared at 32 and 33°C. These results are consistent with the results obtained by Tautz et al. (2003), despite the differences in time between training and testing in the two studies (Tautz et al. (2003) tested workers after 1 and 10 min). In addition, our study shows that bees reared at 33°C have a significantly better ST memory than bees reared at 32°C. Thus the difference between the rearing temperatures 32 and 33°C may be an important boundary in the normal development of the honey bee brain.

In contrast to the effects on ST memory, our study also shows that rearing honey bee pupae at different constant temperatures within, and on the margins of, the normal rearing range has no effect on LT memory in workers, or wing FA in adult workers, queens or drones. The FA results suggest that the temperature range used is either not stressful, or that wing FA is a poor indicator of temperature stress on development in *A. mellifera*.

The fact that rearing temperature has a considerable effect on a worker's ability to associate olfactory cues with a reward, but no effect on FA, strongly implies that the most important consequence of abnormal rearing temperatures are neural deficiencies rather than physical abnormalities. In particular, preserving effective ST learning and memory abilities may be one of the reasons for the constancy of the incubation temperatures maintained by honey bee workers in their brood nest.

Caste differences

Typically brood cells used for rearing queens and drones are located on the periphery of the nest, whereas workers are reared at the centre. Therefore we expect queens and drones to experience greater fluctuations and lower absolute temperatures during pupation than do workers. Queens and drones are fed and groomed by workers and do not forage. Thus they may not require the same learning and memory capacities as workers. Almost all workers will forage for pollen and nectar and perform dances indicating the distance, direction and quality of the resources at some time during their lives. As workers reared at higher temperatures have greater learning and memory capacities, it is tempting to speculate that worker brood is generally reared in the central brood nest area, where the temperature is higher and thus results in greater learning and memory abilities. Queens and drones are reared on the periphery of the nest where the temperature is lower and, as the reproductive castes do not forage, learning and memory in the form of associating an odour with a reward is not as essential in these castes.

Why does temperature not affect FA?

Failing to detect a relationship between rearing temperature and FA in honey bees is surprising. We suggest that there are four potential reasons why there seems to be no association between FA and rearing temperature in honey bees.

First, it is possible that the temperature range used in our study was not stressful enough to cause increases in FA. This is unlikely because our range incorporated temperatures well outside the normal rearing range for workers, queens and drones, and some individuals failed to emerge. We could not use these individuals in the FA study, as most failed to develop normal wings at all. Thus our inability to detect FA may be explained by the fact that in our analysis we only used individuals that emerged successfully.

Second, temperature variation, rather than the absolute temperature maintained, may be the more important element for optimal development of the brood. If so, this may explain why different constant rearing temperatures had no effect on the level of wing FA detected. Development, both neurological and physiological, may be adjusted such that there is a decrease in the impact of a continuous temperature stress. However, Bjorksten et al. (2001) found that there was a similar lack of FA in stalk-eyed flies exposed to transient heat shocks as those exposed to continuous food stresses.

Third, a lack of effect of incubation temperature may have arisen because of an inability to separate the effects of our temperature manipulation from pre-existing levels of FA caused by developmental stresses while individuals were larvae (Hogg et al. 2001). This seems unlikely as all colonies used for rearing larvae were strong, disease free, outbred, and well fed, thus providing optimal conditions for the rearing of larvae.

Finally, the traits chosen may not be effective indicators of FA. Similar wing traits have been used in a variety of FA studies on honey bees with mixed results (Clarke et al. 1992; Clarke 1997; Smith et al. 1997; Schneider et al. 2003). We suggest, as did Woods et al. (1999), that only traits which show consistent and increasing levels of FA under a particular stress (or combination of stresses) should be used as a measure of developmental stability. Our findings suggest that wing FA is a poor indicator of temperature stress during development in honey bees.

Short-term memory versus long-term memory

As our study shows no overall effect of temperature on LT memory in *A. mellifera*, but does show an effect on ST memory, it provides support for the hypothesis that short and LT memory are controlled by different areas of the brain (Hammer and Menzel 1995, 1998; Müller 1996; Maleszka et al. 2000; Zars et al. 2000; Maleszka and Helliwell 2001; Pascual and Preat 2001; Strausfeld

2002). In line with this dichotomy, Maleszka and Helliwell (2001) showed that individual bees treated with Juvenile Hormone demonstrate a significant improvement in ST memory whereas LT memory is unchanged. In the honey bee the mushroom bodies become relatively large and complex by the middle of the pupal stage (Farris et al. 1999) suggesting that stress during that time could affect their function. However, one of the areas of the brain known to be involved in memory, the antennal lobe, continues to develop for the first few days after emergence. Thus it is also possible that there was no overall effect of temperature on LT memory because all bees experienced the same conditions after emerging.

Conclusion

We conclude that an important reason why honey bees maintain strict temperature boundaries during brood development is to preserve greater ST learning and memory abilities in workers. However, this study and that of Tautz et al. (2003), both indicate that workers that pupate at 36°C, at least 1°C higher than normal brood nest temperature (Jones et al. 2004), perform better in ST memory tests, than workers reared at lower temperatures. Why then, do workers maintain the brood nest temperature at 34.5°C if temperatures slightly higher than normal are beneficial for behaviours associated with foraging task performance? One possibility is that incubation at higher temperatures is too energetically expensive for colonies. Alternatively, there may be some costs to higher hive temperatures. For example, it is possible that high temperatures, although beneficial in learning and memory skills, are detrimental to other important physiological or neuronal factors not tested for in this study, or higher temperatures may promote the growth of pathogens. Thus it is still possible that maintaining the temperature at ~34.5°C is, overall, the best temperature for optimal brood maturation.

Acknowledgements We thank Michael Duncan for his help with bee-keeping. This research was funded by an ARC grant to B. P. Oldroyd and R. H. Crozier. Our experiments comply with the "Principles of animal care", publication No. 86–23, revised 1985 of the National Institute of Health, and also with the current laws of Australia where the experiments were performed.

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