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RESEARCH ARTICLE

Effects of the juvenile hormone analogue methoprene on rate of behavioural development, foraging performance and navigation in honey bees (*Apis mellifera*)

Lun-Hsien Chang, Andrew B. Barron* and Ken Cheng

ABSTRACT

Worker honey bees change roles as they age as part of a hormonally regulated process of behavioural development that ends with a specialised foraging phase. The rate of behavioural development is highly plastic and responsive to changes in colony condition such that forager losses, disease or nutritional stresses accelerate behavioural development and cause an early onset of foraging in workers. It is not clear to what degree the behavioural development of workers can be accelerated without there being a cost in terms of reduced foraging performance. Here, we compared the foraging performance of bees induced to accelerate their behavioural development by treatment with the juvenile hormone analogue methoprene with that of controls that developed at a normal rate. Methoprene treatment accelerated the onset of both flight and foraging behaviour in workers, but it also reduced foraging span, the total time spent foraging and the number of completed foraging trips. Methoprene treatment did not alter performance in a short-range navigation task, however. These data indicate a limitation to the physiological plasticity of bees, and a trade off between forager performance and the speed at which bees begin foraging. Chronic stressors will be expected to reduce the mean age of the foraging force, and therefore also reduce the efficiency of the foraging force. This interaction may explain why honey bee colonies react to sustained stressors with non-linear population decline.

KEY WORDS: Precocious foraging, Radio frequency identification, Life span, Homing, Stressors

INTRODUCTION

Adult worker honey bees (*Apis mellifera* L.) change their behavioural roles in the colony in a predictable sequence as they age (Seeley, 1995; Winston, 1987). This is a genomically and hormonally regulated process of behavioural development (Amdam et al., 2006; Page and Amdam, 2007; Robinson, 1992; Robinson et al., 1994; Whitfield et al., 2006). Ultimately, the workers' behavioural development enhances the efficiency of the colony by having different individuals specialise in each of the diverse roles needed by the colony, and delaying the most dangerous and metabolically demanding task of foraging to later in the worker's life once she has already made a significant contribution to colony growth (Woyciechowski and Moron, 2009). This process of behavioural development is highly plastic and responsive to changes in colony needs. A loss of foragers, disease or nutritional stressors will cause bees to accelerate their behavioural development to commence foraging precociously and boost food influx to the colony (Higes et al., 2008; Huang and Robinson, 1999; Robinson, 1992; Schulz et al., 1998). Behavioural development is a complex integrated physiological process, and it is not clear to what extent the process can be accelerated without a performance cost. Our objective here was to compare the foraging performance of bees that completed a normal profile of behavioural development with that of bees that were induced to forage precociously as a consequence of treatment with the juvenile hormone analogue methoprene to explore the limits of developmental plasticity in honey bees.

The transition to foraging is a particularly significant change in worker behaviour and physiology. It is associated with major physiological changes in flight muscle (Correa-Fernandez and Cruz-Landim, 2010; Herold, 1965), a significant decrease in body mass (Harrison, 1986), glands (Winston, 1987) and fat stores (Toth and Robinson, 2005), and changes in metabolic rate (Harrison, 1986) and circadian rhythm (Bloch et al., 2001). It is preceded by a number of orientation flights in which a bee flies through the area around the hive in a systematic way, apparently learning a range of celestial and terrestrial navigational cues by which foragers can locate food sources and return home (Becker, 1958; Capaldi et al., 2000; Dyer and Gould, 1983; Srinivasan, 2011; Towne, 2008; Towne and Moscrip, 2008).

The rate of behavioural development, and the age at which a bee commences foraging, is influenced in part by juvenile hormone (JH) (Robinson, 1992; Robinson et al., 1989; Robinson and Ratnieks, 1987). JH titres in blood have been shown to increase with age (Elekonich et al., 2001; Jassim et al., 2000). Precocious foragers (age: 7–10 days), together with normally aged foragers (age: 21–24 days) have higher JH titres than do nurse bees (brood carers) of any age (Robinson et al., 1989). Bees that have reverted to nursing after foraging have lower JH titres than do bees that continue foraging (Robinson et al., 1992). JH controls the pace of the nurse-to-forager transition but is not required in the maturation of the foraging caste (Sullivan et al., 2000).

Plasticity in the rate of behavioural development is considered adaptive in that it allows a colony to rapidly respond to acute losses of foragers, but it is unclear whether bees with an accelerated behavioural development (precocious foragers) are able to forage as effectively as bees that have completed behavioural development at a normal pace. Precocious foragers have been found to differ in a number of important physiological and behavioural traits. Precocious foragers have a shorter life span (Robinson, 1985) and poorer performance in their maximal hovering flight capacity (Vance et al., 2009) than normal-aged foragers. They were also found to perform poorly in an olfactory reversal-learning test (Ben-Shahar et al., 2000).

Department of Biological Sciences, Macquarie University, Sydney, NSW 2109, Australia.

*Author for correspondence (Andrew.Barron@mq.edu.au)

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Here, we examined the consequences of accelerated behavioural development by treatment with the JH analogue methoprene on bee foraging performance. Methoprene topically applied to young bees reliably induces a premature onset of orientation flights (Robinson, 1987) and foraging flights (Robinson, 1987; Robinson and Ratnieks, 1987; Schulz et al., 2002), but it does not disrupt foraging behaviour. When applied to bees that had foraged for 6 days, methoprene did not influence preference for collecting pollen or nectar, nor did it change the foraging period or nectar foraging rate (Deng and Waddington, 1997). Therefore, methoprene provides a robust method to pharmacologically manipulate the rate of behavioural development of individual bees independent of the social environment.

To measure aspects of foraging performance accurately, we used radio frequency identification (RFID) tags to monitor the entry into and exit from a small nucleus hive by individual marked bees throughout their life. In addition, we displaced a group of methoprene-treated and control bees a short distance (~650 m) to determine the effects of accelerated behavioural development by methoprene treatment on short-range navigation.

RESULTS

Experiment 1: effect of methoprene treatment on onset of foraging and foraging performance

Adult bees were treated when they were 1 day old adults with either 200 µg methoprene in 5 µl of acetone to the dorsal abdomen or 5 µl of acetone alone (solvent control), or were not treated (untreated control), and were fitted with RFID tags and introduced into the nucleus hive. This experiment was replicated five times from 6 March 2012 to 26 May 2012. The RFID system recorded between 101 and 119 bees per group at least once in each of the five replicates. Fig. 1 shows cumulative flight activity for each of the treatment groups for the five experimental replicates. Analyses were performed on six aspects of flight behaviour and foraging performance, which are summarised in Table 1 and Figs 2–7.

Treatment with methoprene accelerated the onset of orientation (defined as the day on which a bee completed its first flight; Table 1, Fig. 2) in all of the five replicates. There was no difference between the acetone group and the untreated group in any of the five replicates (Fig. 2A–E).

The RFID system allowed us to determine when bees were inside the hive and when they were outside, but it gave no information on what bees were doing outside the hive. For this study, we estimated foraging to have begun once bees had completed at least one flight ≥14 min. This is significantly longer than the mean orientation flight duration estimated by Capaldi et al. (2000) (5.5 min). Methoprene accelerated the onset of foraging relative to the acetone group in four of the five replicates (Table 1, Fig. 3A,B,D,E), and relative to the untreated control group in four of the five replicates (Table 1, Fig. 3). When all the replicates were pooled, the methoprene group started foraging at significantly younger ages than both of the control groups (Fig. 3F).

We assumed that the age at which the bee was last recorded departing the hive by the RFID system was an indicator of an effective time of her death or disappearance. A shorter life span of the methoprene group than the acetone group was seen in four (Fig. 4B–E) of the five replicates. The acetone group had shorter life span than the untreated group in two replicates (Fig. 4A,E). When all the replicates were pooled, a significantly shorter life span was found for the methoprene group than the acetone group and the acetone group was also shorter in life span than the untreated group (Fig. 4F).

Flight span was calculated as the difference (in days) between age at first orientation flight and age at the last record. Bees in the

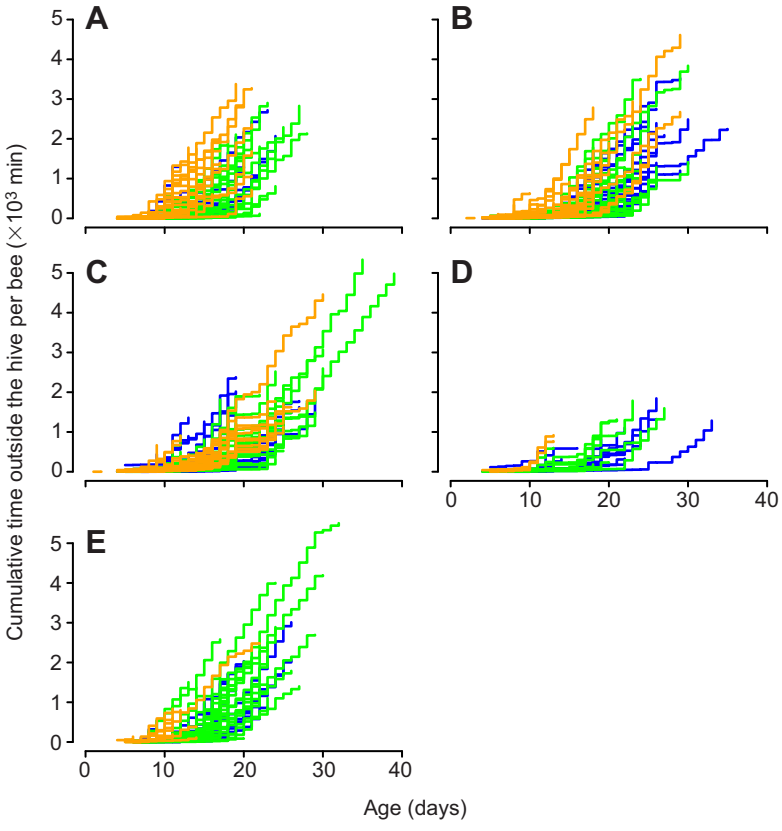

Fig. 1. Cumulative time outside the hive plotted against bee age for individual bees in the three treatment groups. Bees were treated with methoprene (orange), acetone (solvent control, blue) or nothing (untreated control, green). Data from five experimental replicates (A–E) are shown.

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Table 1. Comparison of six flight variables (experiment 1) between the methoprene (met), acetone (ace) or untreated (ctrl) groups for five different replicates, and all replicates pooled

Variable	Replicate	χ^2	*P*-value	Dunn's *post hoc*
Age at first orientation flight (Fig. 2)	1	47.87	<0.0001	ace=ctrl>met
	2	37.50	<0.0001	ace=ctrl>met
	3	25.08	<0.0001	ace=ctrl>met
	4	15.97	0.0003	ace=ctrl>met
	5	55.77	<0.0001	ace=ctrl>met
	Pooled	160.9	<0.0001	ace=ctrl>met
Age at first foraging flight (Fig. 3)	1	13.25	0.0013	ace=ctrl>met
	2	18.57	<0.0001	ace=ctrl>met
	3	9.48	0.0087	ace=ctrl, ctrl>met, ace=met
	4	6.45	0.0398	ace=ctrl, ctrl=met, ace>met
	5	15.50	0.0004	ace=ctrl>met
	Pooled	53.32	<0.0001	ace=ctrl>met
Age at last record leaving the hive (Fig. 4)	1	64.85	<0.0001	ctrl>ace=met
	2	39.65	<0.0001	ace=ctrl>met
	3	5.81	0.0548	
	4	56.87	<0.0001	ace=ctrl>met
	5	102.1	<0.0001	ctrl>ace>met
	Pooled	170.2	<0.0001	ctrl>ace>met
Flight span in days (Fig. 5)	1	6.21	0.0448	ace=ctrl=met
	2	13.90	0.0010	ace=ctrl>met
	3	4.51	0.1050	
	4	18.87	<0.0001	ace=ctrl>met
	5	51.62	<0.0001	ace>ctrl>met
	Pooled	52.90	<0.0001	ace=ctrl>met
Total time outside the hive (Fig. 6)	1	2.63	0.2683	
	2	7.46	0.0240	ace=ctrl, ctrl>met, ace=met
	3	1.05	0.5921	
	4	9.40	0.0091	ace=ctrl, ctrl=met, ace>met
	5	31.72	<0.0001	ace=ctrl>met
	Pooled	24.80	<0.0001	ace=ctrl>met
Total number of completed flights (Fig. 7)	1	0.27	0.8755	
	2	10.83	0.0044	ace=ctrl>met
	3	0.56	0.7547	
	4	3.62	0.1640	
	5	27.33	<0.0001	ace=ctrl>met
	Pooled	15.21	0.0005	ace=ctrl>met

met, methoprene; ace, acetone control; ctrl, untreated control.
All analyses were Kruskal–Wallis tests. Where this indicated a significant difference between groups, Dunn's *post test* was used to determine specific differences. Tied ranks were considered.

methoprene group had a shorter flight span than those in the acetone group in three (Fig. 5B,D,E) of the five replicates. The acetone group differed from the untreated group in replicate 5 only (Fig. 5E). When all the replicates were pooled, the methoprene group had a significantly shorter flight span than the acetone group whereas there was no significant difference between the acetone group and the untreated group (Fig. 5F).

Bees from the methoprene group had a reduced total time spent outside the hive in their lifetime compared with bees in the acetone group in two out of the five replicates (Table 1, Fig. 6D, E). No significant difference in total flight time was found between the acetone group and the untreated group in any replicates (Table 1, Fig. 6A–E). When all the replicates were pooled, the methoprene group spent significantly less time outside the hive than the acetone group, whereas there was no significant difference between the acetone group and the untreated group (Fig. 6F).

Methoprene-treated bees performed fewer trips than the acetone group in two out of the five replicates (Table 1, Fig. 7B,E). There was no difference between the acetone group and the untreated group in any the five replicates (Fig. 7A–E). When all the replicates were pooled, the methoprene group performed significantly fewer round trips than the acetone group, whereas there was no significant difference between the acetone group and the untreated group (Fig. 7F, Table 1).

Experiment 2: effect of methoprene treatment on short-range navigation

A subset of bees from experiment 1 were captured on return to the colony and displaced to one of three locations approximately 600 m away. In total, 659 different individual bees were captured and displaced. Of these 11 bees (1.29%) either lost their tags or failed to take off for more than 5 min after release and therefore were not used in the analysis. Some individuals were caught and tested more than once (Table 2), and in total we performed 856 displacement tests. The frequency of bees that were lost after displacement, returned on the same day or returned on a later day did not differ between the three treatment groups (Chi-square test, N=659, d.f.=4, χ^2=3.545, P=0.4711). Homing speed also did not differ across treatment groups (two-way ANOVA of $\log_{10}$-transformed speed, $F_{2,536}$=0.43, P=0.6518), replicate ($F_{1,536}$=0.11, P=0.7375) or their interaction ($F_{2,536}$=1.27, P=0.2827). Homing speed did not vary with displacement site (two-way ANOVA of $\log_{10}$-transformed speed, $F_{2,533}$=1.62, P=0.1987), treatment ($F_{2,533}$=0.43, P=0.6514) or their interaction ($F_{2,533}$=0.82, P=0.5148) when all the replicates were pooled.

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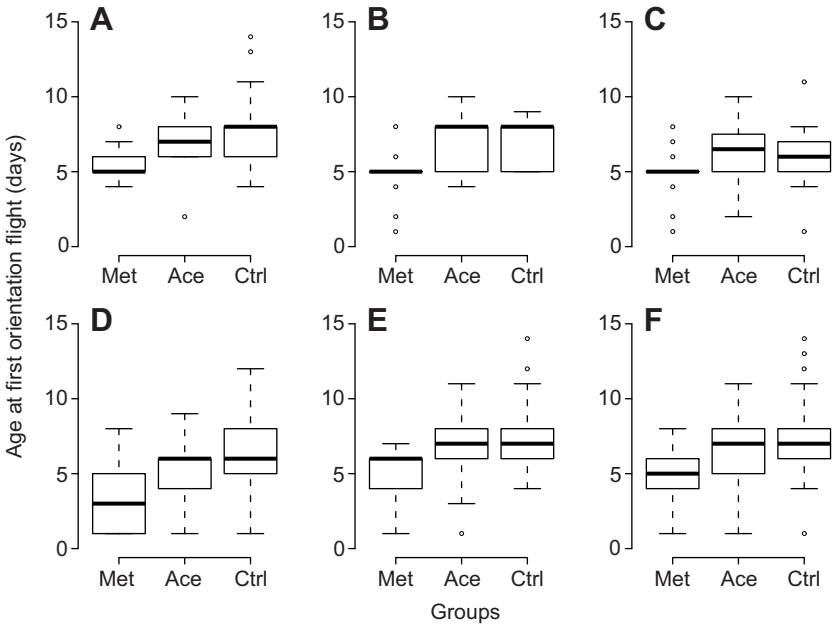

Fig. 2. Age at onset of orientation flight (first complete flight) for bees in the three treatment groups. Data are for methoprene (Met) acetone (Ace) and untreated (Ctrl) bees for five experimental replicates (A–E); (F) pooled data. The bar in the centre of the boxplot marks the median, boxes extend to quartiles and whiskers reach to 1.5× inter-quartile distance. Points beyond this are individually marked. A summary of statistical analyses is shown in Table 1.

Negative results being difficult to interpret, we examined the distribution of statuses across treatment groups more closely using a Bayesian perspective. We considered pooled results of the first return trip only. We considered only the 'same day' and 'lost' statuses, ignoring the low *N* values in the 'different' column: 82.3% of the methoprene group returned on the same day (135/164), whereas for the other two groups, about 85.8% returned (407/475). We compared the likelihood of obtaining the observed success rate of the methoprene group assuming that they had 85.8% 'true' success rate (the null hypothesis) against the likelihood of obtaining the observed success rate assuming other *a priori* 'true' success rates (alternative hypotheses). The ratio of the two likelihoods gives a measure of evidence in favour of the null. If the alternative hypothesis is 78% success (a value that would be statistically significant at our sample size), the null is slightly favoured: evidence ratio 1.25. If the alternative hypothesis is 75%, however, the null is strongly favoured: evidence ratio 6.12. And the alternative hypothesis of 70% success is vanishingly unlikely, with the evidence ratio in favour of the null being 343.16. We conclude that the data are not strongly in favour of the null against a possible weak effect, but it is highly unlikely that methoprene reduces the success rate by 10% or more.

DISCUSSION

Our data confirmed the effect of treatment with the JH analogue methoprene on the rate of honey bee behavioural development, but critically the RFID tagging method we used allowed a detailed examination of the consequences of methoprene-induced accelerated behavioural development for the performance of individual bees. While we observed differences in treatment effectiveness between replicates, overall, methoprene treatment accelerated the onset of orientation and foraging relative to the

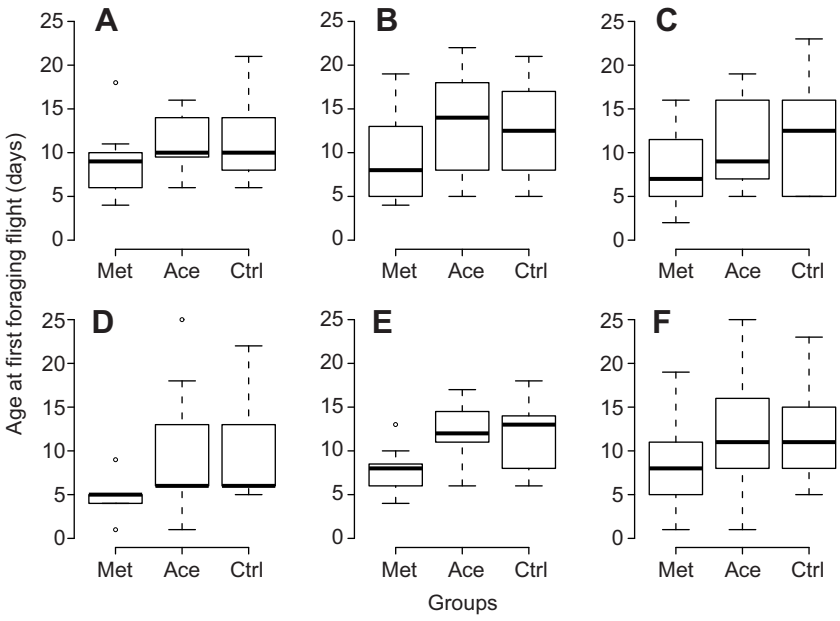

Fig. 3. Age at onset of foraging for bees in the three treatment groups. Data are for methoprene (Met) acetone (Ace) and untreated (Ctrl) bees for five experimental replicates (A–E); (F) pooled data. Boxplot details are as for Fig. 2. A summary of statistical analyses is shown in Table 1.

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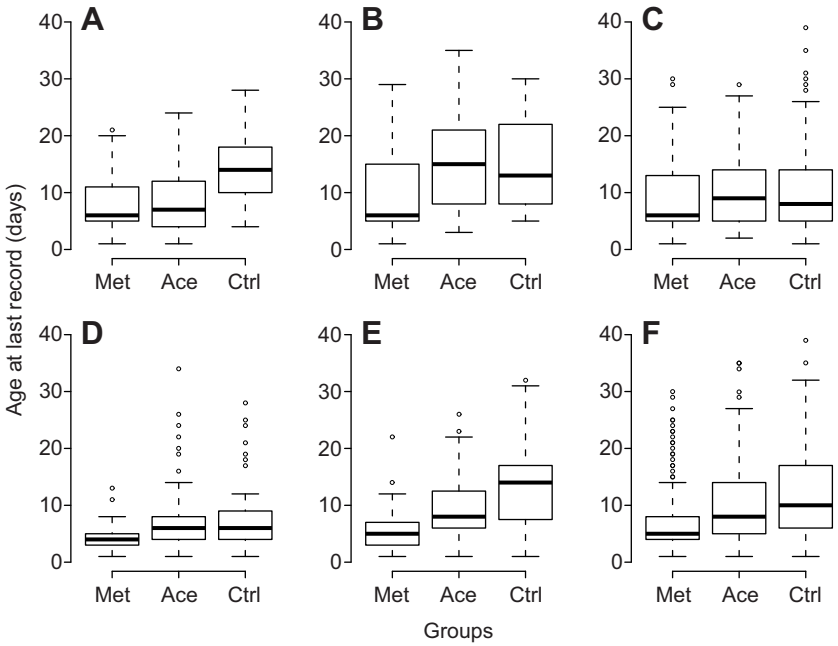

Fig. 4. Age at last recorded departure from the hive (effective age at death) for bees in the three treatment groups. Data are for methoprene (Met) acetone (Ace) and untreated (Ctrl) bees for five experimental replicates (A–E); (F) pooled data. Boxplot details are as for Fig. 2. A summary of statistical analyses is shown in Table 1.

untreated and acetone controls. The methoprene treatment, however, also reduced life span, flight span and the total number and duration of successful flights performed in the bees' lifetime. These data show that while methoprene treatment may accelerate behavioural development, it results in reduced lifetime foraging performance overall.

Our findings are in contrast to the foundational work by Robinson (1985), which reported the flight time of foragers was not adversely affected by methoprene. It should be noted, however, that the two studies used very different data collection methods. In Robinson's (1985) study, entry and exit of bees were manually scored for 1 h daily over 35 consecutive days. In the current study, nearly every single entry and exit was automatically recorded by the RFID system until the death of all the bees. The greater sample size, detail and measurement accuracy afforded by this method would have allowed statistical detection of smaller effects than manual focal observations. Furthermore, the effects we observed of methoprene on foraging performance were smaller than on the rate of behavioural development. The tendency of methoprene to reduce flight span, time outside the hive and number of foraging trips was seen in all five of our biological replicates, but differences between the methoprene and the two contol groups were not statistically significant in all individual replicates.

In this study we did observe an effect of the solvent acetone on behaviour in some replicates and some indices of performance (Table 1). Acetone was used to allow methoprene to penetrate the waxy cuticle. It is not surprising that this harsh solvent has some lasting effects on treated bees, but the inclusion of this critical solvent control has allowed us to separate the effects of methoprene from those of the solvent.

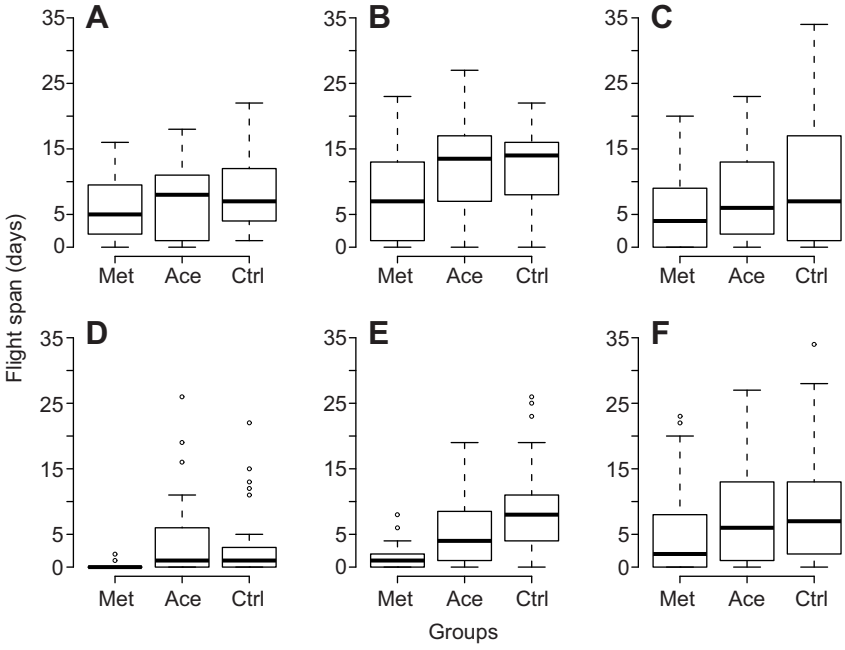

Fig. 5. Flight span (days between first completed orientation flight and last record of leaving the hive) for bees in the three treatment groups. Data are for methoprene (Met) acetone (Ace) and untreated (Ctrl) bees for five experimental replicates (A–E); (F) pooled data. Boxplot details are as for Fig. 2. A summary of statistical analyses is shown in Table 1.

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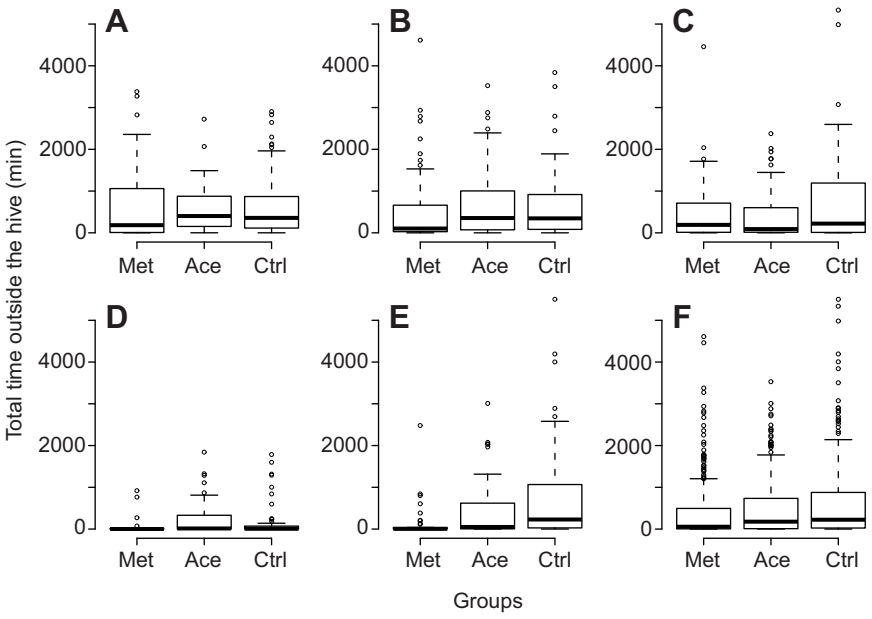

Fig. 6. Total time spent outside the hive for bees in the three treatment groups. Data are for methoprene (Met) acetone (Ace) and untreated (Ctrl) bees for five experimental replicates (A–E); (F) pooled data. Boxplot details are as for Fig. 2. A summary of statistical analyses is shown in Table 1.

We have shown that bees induced to forage precociously by methoprene treatment perform poorly as foragers, but there are two possible interpretations of this finding. Accelerated behavioural development could result in reduced foraging performance; alternatively methoprene-treated bees could be performing poorly as foragers because the methoprene treatment made them sick. Here, we also acknowledge Woyciechowski and Moron's (2009) hypothesis that precocious foraging could be a behavioural response to sickness in worker bees, and there are certainly a number of diseases and stressors that will induce precocious foraging (Higes et al., 2008; Schulz et al., 1998; Toth and Robinson, 2005). We note, however, that while the dose of methoprene we used has reliably accelerated behavioural development in several studies (Robinson, 1985, 1987; Robinson and Ratnieks, 1987; Schulz et al., 2002), there has been no evidence of methoprene making bees generally sick at this dose. Instead, we propose that accelerating behavioural development leads to reduced forager performance by precocious foragers.

Poor performance by precocious foragers was also seen when precocious foraging was induced in bees by establishing a single-cohort colony of 1 day old bees without an existing foraging force (Perry et al., 2015). In this situation, some bees rapidly become foragers, but the precocious foragers performed less well than bees that completed behavioural development at the normal rate (Perry et al., 2015). Here, precocious foraging was induced by a pharmacological treatment rather than a social manipulation, but the performance of precocious foragers was similarly reduced. This suggests there are costs to accelerating behavioural development of workers, reflecting a limitation to the developmental plasticity of worker bees.

Our findings appear to contradict those of Neukirch (1982), who found no significant influence of the chronological age of onset of foraging on total lifetime flight duration. Her data suggested that bee flight span was influenced by the amount of flight activity (Neukirch, 1982). Most bees had a similar total life span flight activity, which was independent of age at onset of foraging

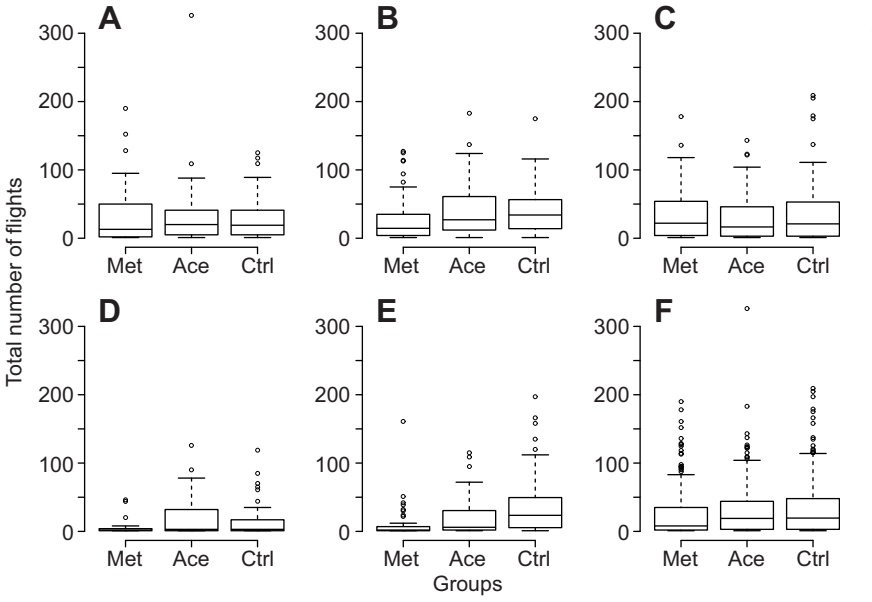

Fig. 7. Total number of flights for bees in the three treatment groups. Data are for methoprene (Met) acetone (Ace) and untreated (Ctrl) bees for five experimental replicates (A–E); (F) pooled data. Boxplot details are as for Fig. 2. A summary of statistical analyses is shown in Table 1.

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Table 2. Summary of bee fates in experiment 2 for the three treatment groups across the five experimental replicates

			Homing status			
Displacement	Replicate	Group	Lost	Same day	Different day	Total
First	1	Methoprene	1	9	1	11
		Acetone	2	6	1	9
		Untreated	5	25	3	33
	2	Methoprene	19	65	7	91
		Acetone	19	87	2	108
		Untreated	16	75	4	95
	3	Methoprene	6	53	0	59
		Acetone	6	44	1	51
		Untreated	6	65	0	71
	4	Methoprene	2	3	0	5
		Acetone	0	18	0	18
		Untreated	4	14	0	18
	5	Methoprene	1	5	0	6
		Acetone	3	22	0	25
		Untreated	7	51	1	59
	Pooled	Methoprene	29	135	8	172
		Acetone	30	177	4	211
		Untreated	38	230	8	276
	Subtotal		**97 (14.72%)**	**542 (82.25%)**	**20 (3.03%)**	**659**
Second	1	Acetone	1	0	0	1
		Untreated	1	3	0	4
	2	Methoprene	0	20	0	20
		Acetone	5	25	0	30
		Untreated	3	15	0	18
	3	Methoprene	0	26	0	26
		Acetone	3	6	0	9
		Untreated	2	22	0	24
	4	Acetone	1	4	0	5
		Untreated	1	1	0	2
	5	Methoprene	1	1	0	2
		Acetone	1	5	0	6
		Untreated	1	12	0	13
	Pooled	Methoprene	1	47	0	48
		Acetone	11	10	0	51
		Untreated	8	53	0	61
	Subtotal		**20 (12.5%)**	**140 (87.5%)**	**0**	**160**
Third	2	Methoprene	0	2	0	2
		Acetone	0	5	0	5
		Untreated	1	1	0	2
	3	Methoprene	2	2	0	4
		Untreated	0	6	0	6
	5	Methoprene	0	1	0	1
		Untreated	1	2	0	3
	Pooled	Methoprene	2	5	0	7
		Acetone	0	5	0	5
		Untreated	2	9	0	11
	Subtotal		**4 (17.39%)**	**19 (82.61%)**	**0**	**23**
Fourth	3	Untreated	0	3	0	3

Bees were captured arbitrarily for displacement, and in some cases the same bee was caught and displaced more than once. Bees displaced a second time are likely to have more navigational experience than bees displaced once; therefore, second, third and fourth displacements (displacement column) were considered separately.

Homing status of the bees was recorded as lost, returned on the day of the displacement or returned on a different day.

Subtotal values are given in bold.

(Neukirch, 1982). A bee's flight span might be related to a progressive depletion of glycogen in flight muscle (Neukirch, 1982). By contrast, Perry et al. (2015) reported a very strong relationship between age at onset of foraging and several aspects of foraging performance including flight span and total flight duration (Perry et al., 2015). This difference may be because here, and in Perry et al. (2015), manipulations were used to deliberately focus on precocious foragers that began foraging when less than 2 weeks old. It was for this age range that the age dependence of flight performance was most pronounced (Perry et al., 2015). In Neukirch's (1982) study, bees began foraging at the normal age of 3 weeks or greater. It would appear that foraging performance is only reduced if bees begin to forage at a very young age.

Why do bees that start foraging precociously perform badly? One possibility is that precocious foragers are not properly adapted for the demands of foraging (Schippers et al., 2010, 2006; Vance et al., 2009). Precocious foragers are heavier and less efficient fliers than normal-aged foragers (Vance et al., 2009), and the flight muscle biochemistry of precocious foragers differs from that of typical foragers (Schippers et al., 2010, 2006). In this study, however, we detected no difference in performance of precocious and normal foragers in a short-range homing task (Table 2). This would imply

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that if a difference in flight performance between precocious and normal foragers exists, it is rather subtle.

Our data suggest an important constraint to the phenotypic plasticity of worker honey bees: their behavioural development can be accelerated, but not without compromising the performance of the forager. This is significant because if a honey bee colony is under chronic population stress and losing a relatively high proportion (>25%) of its foragers daily, younger bees will become foragers precociously to compensate (Khoury, 2009; Khoury et al., 2011). But there is a risk this could result in a progressively younger and less effective foraging force, which could compound the population stresses on the colony (Perry et al., 2015).

This study emphasises that behavioural development in honey bees is a developmental programme. The programme shows a great deal of plasticity and can be accelerated to adapt to changes in colony condition. But that acceleration is not without cost. A precocious forager is not able to match the performance of bees that developed at a more normal, slower rate. This highlights an important constraint in honey bee biology that explains the vulnerability of a colony to sustained population or disease stresses.

MATERIALS AND METHODS

Study animals and location

A nucleus hive containing ∼2000 worker bees was created using three frames of nectar and pollen and their associated workers taken from a donor hive at the Macquarie University (MQ) apiary during mid-January 2012. The nucleus hive was placed inside a room of the MQ bee research facility where the temperature was maintained at ∼24°C. This hive was connected to the outdoors through a Plexiglas tunnel (inner height 20 mm, width 80 mm, length 800 mm) that contained within it at the end distal from the hive a smaller channel (inner height 10 mm, width 27 mm, length 200 mm) through which bees were funnelled and within which two RFID antennae were mounted. A young mated queen was introduced into the nucleus hive 2 weeks after the hive placement. We examined the queen for acceptance 2 days after her introduction. The hive was used for both experiments, with different foragers participating in each.

A RFID system (Invengo Information Technology Co. Ltd., Guangzhou, China) was used to record the flight activity of individual bees. RFID tags (∼920–925 MHz, XCTF-8018, diameter ∼3 mm, thickness 0.08–0.21 mm, mass ∼0.97 mg) had an effective detection distance of 10 mm. Tags were then individually programmed with a unique 24-digit code for individual identification. To visually identify bees of different treatment groups, we marked each tag with a dot of Tamiya enamel or Posca marker. We attached the tag to the dorsal thorax of a bee using commercial superglue. Two antennae were mounted on the top side of the RFID tunnel with antenna 1 closer to the outside and antenna 2 closer to the hive. These two antennae were connected to a tag reader (XCRF-860), which recorded the antenna number (either 1 or 2), the tag number and the time as a tagged bee passed underneath the antennae using customised software from Invengo. The sequence in which these two antennae read a tag indicated whether the bee was entering or exiting the tunnel.

Raw data were automatically saved into a text file each day by this software. The data were imported into a Microsoft Access database (Microsoft Office 2010). We visually checked the detection rate of the two RFID antennae for a total of 19.32 h. Less than 1% of the entries failed to be detected by the two antennae: 0.88% of 795 entries under antenna 1 and 0.25% of 789 entries under antenna 2.

Experiment 1: effect of methoprene treatment on onset of foraging and foraging performance

Frames containing emerging adult bees were collected from the source colonies and placed in a dark incubator (32°C, 50% relative humidity controlled by a dehumidifier). The next morning, newly emerged bees were assigned randomly across three treatments. Each bee received either 5 µl of acetone (solvent control) or 200 µg methoprene in 5 µl of acetone (all compounds from Sigma-Aldrich, Australia) applied to the dorsal abdomen. The untreated group received no treatment. Bees (110–120) from each of the methoprene, acetone and untreated groups were fitted with RFID tags and introduced into the nucleus hive on the day of the treatment. This experiment was replicated five times from 6 March 2012 to 26 May 2012. Flight activity of the bees was tracked with RFID from the introduction of the first replicate until the death or disappearance of the last bee of the last replicate.

Experiment 2: effect of methoprene treatment on short-range navigation

A subset of bees from experiment 1 were displaced from the hive on their return from a foraging trip, and their ability to successfully return to the colony determined. To easily collect returning bees for the displacement, the distal end of the broad section of the Plexiglas tunnel was modified to separate the inbound and outbound bees. During the collection period, the inbound entry was restricted to a small hole (1.2×1.2 cm) that allowed one bee to enter at a time. The outbound traffic was undisturbed at all times. During the sampling period, the small hole of the inbound entry was briefly blocked with a pencil (length 130 mm, diameter 11 mm) whenever we saw tagged bees returning. The tagged bees were collected individually in 15 ml tubes while crawling around the taped area. We scanned the identification of each bee through the tube and looked up their flight experience in the spreadsheet of accumulated flight times (see below).

It has been estimated that honeybee workers perform on average 5.6 orientation flights, averaging 5.5 min each, before they initiate pollen foraging (Capaldi et al., 2000). The cumulative number of round trips longer than 5.5 min was counted for each bee. This information was output to a table in the Access database to enable quick reference to a bee's flight experience. A bee was chosen for displacement when its flight experience met either of the following criteria: (1) it had been recorded leaving the hive for durations longer than 5.5 min at least 6 times, or (2) it returned with pollen or propolis regardless of the amount of flight experience. We considered both of these measures to be indicative of bees being experienced in flight around the local area.

Collected bees were transferred into red Plexiglas cages (height 70 mm, width 100 mm, length 100 mm). Honey was provided *ad libitum* through inverted gravity feeders modified from 1.5 ml Eppendorf tubes. Four honey feeders were attached vertically inside each cage by commercial Blu Tack. Collections were conducted on sunny mornings from ∼08:00–10:00 h until about 13:00 h. Collected bees were transported in cages to one of the three different sites that were all approximately 600 m from the hive. The site coordinates were measured by a hand-held GPS (eTrex H, Garmin International, Inc., KS, USA) to an accuracy of 6 m. The site distance to the hive was calculated using an Excel™ macro downloaded from Geoscience Australia.

Data analysis

Raw RFID data were processed and analysed with a self-written algorithm using SAS® 9.3 Base programming (SAS Institute Inc., Cary, NC, USA). We imported data ranging from 1 h before sunrise to 1 h post-sunset. During this period, data collected during which the bee traffic was not interrupted by the displacement activity were used for subsequent analysis.

The RFID system was set at the highest detecting power to minimise data loss; however, this also generated duplicated readings because a bee was detected more than once per entry. A genuine round-trip flight was assumed to last for at least 10 s. We therefore computed the duration of completed flights based on a 1-then-1 sequence with a between-time difference exceeding 10 s, which was indicative of a bee leaving the hive (last registered by the outermost antenna, antenna 1) and returning to the hive (next registered by the outermost antenna 1) with at least 10 s between detections to filter out bees that might have been walking back and forth in the tunnel.

Bees perform orientation flights before they commence foraging (Capaldi et al., 2000). The mean duration of these flights has been measured at 5.5 min. For experiment 1, we defined the onset of foraging as the time at which a bee performed its first flight of ≥14 min (AFF14), which was a

mean+1.28 s.d. of the orientation duration reported by Capaldi et al. (2000). We considered a ≥14 min flight a reasonably conservative indicator that foraging had begun. Flights before AFF14 were categorised as orientation flights whereas flights after AFF14 (regardless of duration) were classed as foraging flights.

Across the three treatment groups we compared six variables related to aspects of behavioural development and flight performance. These were: (1) the age at their first orientation flight; (2) the age at their first foraging flight; (3) the age at the last RFID record, which can be assumed to be an approximate indicator of the time of their death (Decourtye et al., 2011): more than 90% of these last records were recorded by antenna 1 (91.24%), indicating that the bee departed and never returned to the hive – bees whose last record was registered by antenna 2 were not counted, as it was possible bees may have lived for a period in the hive after their last detection; (4) flight span, calculated as the day difference between age at first orientation flight and age at the last record by antenna 1; (5) total time outside the hive, calculated by summing all trip durations for each individual bee; and (6) total number of flights. None of these variables were normally distributed (Shapiro–Wilk test: $P<0.05$ in each case). Comparisons between the acetone, untreated and methoprene group were made with Kruskal–Wallis tests, followed by Dunn's *post hoc* test (Elliott and Hynan, 2011). An SAS macro 'KW_MC' was used to make the comparison between any two groups of the three groups (Elliott and Hynan, 2011). All data management and statistical analyses were conducted using SAS 9.3 for Windows. Graphics were generated using R 2.15.3 (R Development Core Team, 2013).

For experiment 2, bees were considered to have returned to the colony after displacement if they were detected by an RFID antenna at the colony post-release. Across treatment groups we compared the proportions of displaced bees that did not return, that returned on the same day as release and that returned on a different day, with a Chi-square test. We also tested for differences in homing of bees from the three different release sites.

For bees that returned on the same day as release, we compared homing speed between treatments and release sites. Homing speed was found to be normally distributed in only two of the 15 replicate-by-treatment combinations and in none of the three groups when all the replicates were pooled. Transforming this variable with log base 10, however, was successful in normalising these data. The $\log_{10}$-transformed homing speed was therefore used for the subsequent analyses. Homogeneity of the $\log_{10}$-speed variance among the three treatment groups was affirmed in all the replicates.

All statistical analyses were conducted with SAS 9.3 Base programming. $P<0.05$ was considered to be statistically significant.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

L.-H.C. designed and executed the experiments, analysed the data and wrote the manuscript. A.B.B. designed the experiments and wrote the manuscript. K.C. designed the experiments and wrote the manuscript.

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