

## RESEARCH ARTICLE

# High experience levels delay recruitment but promote simultaneous time-memories in honey bee foragers

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## ABSTRACT

Honey bee (*Apis mellifera*) foragers can remember both the location and time of day food is collected and, even in the absence of a reward, reconnoiter the food source at the appropriate time on subsequent days. This spatiotemporal memory (time-memory) is linked to the circadian clock and enables foragers to synchronize their behavior with floral nectar secretion rhythms, thus eliminating the need to rediscover productive food sources each day. Here, we asked whether the establishment of one time-memory influences the formation of another time-memory at the same time of day. In other words, can two time-place memories with the same ‘time-stamp’ co-exist? We simultaneously trained two groups of foragers from a single hive to two separate feeders at the same restricted time of day. After 5 days of training, one feeder was shut off. The second feeder continued being productive 4 more days. Our results showed that (1) foragers with high experience levels at the first source were significantly more likely than low-experience foragers to maintain fidelity to their original source and resist recruitment to the alternative source, (2) nearly one-third of foragers demonstrated multiple, overlapping time-memories by visiting both feeders at the correct time and (3) significantly more high-experience than low-experience foragers exhibited this multitasking behavior. The ability to maintain and act upon two different, yet contemporaneous, time-memories gives the forager bee a previously unknown level of versatility in attending to multiple food sources. These findings have major implications for understanding the formation and management of circadian spatiotemporal memories.

**KEY WORDS:** *Apis mellifera*, Foraging behavior, Food-anticipatory activity, Time-memory, Circadian rhythm

## INTRODUCTION

Honey bees, *Apis mellifera*, have the remarkable ability to remember the time of day when food sources are most profitable. This time-memory, also described as circadian time–place learning (cTPL), has been shown repeatedly in *A. mellifera* (Butler, 1945; Corbet and Delfosse, 1984; Edge et al., 2012; Giurfa and Núñez, 1992; Kleber, 1935; Rabinowitch et al., 1993) and has now been demonstrated in the ants *Ectatoma ruidum* (Schatz et al., 1999) and *Paraponera clavata* (Harrison and Breed, 1987) and in the stingless bees *Melipona fasciculata* (de Jesus et al., 2014), *Trigona amalthaea*

(Breed et al., 2002) and *Trigona fulviventris* (Murphy and Breed, 2008). Evidence that honey bee time-memory is driven by a circadian clock comes from (1) experiments showing a free-running period of foraging behavior close to 24 h under constant conditions (Frisch and Aschoff, 1987; Renner, 1955, 1957), (2) phase shifts, with transients, in response to phase changes in light–dark (LD) cycles (Beier, 1968; Renner, 1959) and (3) a limited range (20–26 h) of entrainment under LD cycles (Beier, 1968). cTPL also occurs in vertebrates, including fish (Reebs, 1996), birds (Krebs and Biebach, 1989) and rodents (Boulos and Logothetis, 1990; Van der Zee et al., 2008). cTPL is adaptive because knowing both where and when to approach (or avoid) a location may optimize factors such as resource utilization and predator avoidance in the dynamic but predictably cyclic environment. In honey bees, time-memory enables foragers to match their flight activity with daily rhythms of nectar secretion in flowers. Experienced foragers resume exploiting known sources at the appropriate time of day without having to expend excess energy rediscovering them each day. Programmed into the bee time-memory system is the scheduling of anticipatory behavior, in which the individual forager inspects the food source at an earlier time than it was discovered on the previous day (Moore and Doherty, 2009). Such anticipatory flights may land the forager earlier within the time-window of nectar availability, thus prolonging the foraging opportunity (Van Nest and Moore, 2012).

The mechanism responsible for cTPL is not understood. Current thought suggests that cTPL is a cognitive function in which time-of-day information from a circadian clock is placed (‘time-stamped’) into memory as a contextual feature in association with other contextual features such as spatial and ‘nature of event’ attributes (Mistlberger et al., 1996; Mulder et al., 2013; Wilkie, 1995). Similarly, Bogdany (1978) previously described bee time-memory as a learned ‘Gestalt’ in which time of day is linked with several different signals for flower recognition (e.g. color, odor, shape and location). As with the time-compensated sun compass in bees (Lindauer, 1960; von Frisch, 1950), such mechanisms require a continuously consulted circadian clock (Pittendrigh, 1958) to accurately time-stamp the association of contextual features and to provide access to circadian phase so that established time-stamped associations can be matched with actual time of day and, thus, be available to influence behavior at the appropriate time of day.

We recently showed that a large proportion of foragers actively exploit alternative food sources during the window of availability of a previously productive artificial feeder even prior to the foragers’ discovery of the feeder’s ceased productivity (Wagner et al., 2013). Additionally, some individuals were observed interrupting flights to productive flowers in order to investigate the empty feeder. Thus, at least some members of the foraging group were maintaining time-memories of multiple, temporally overlapping food sources. In these experiments, it is unclear when the foragers learned of the alternative food sources. It is possible that they were already

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experienced at those sources prior to being recruited to our artificial feeder and that the alternative time memory was established at a different time point during the day. Another question remains as well: when an exploited food source ceases to be productive, how readily will a forager that was exploiting it be recruited to a new food source? Based on our prior experiments (Van Nest et al., 2016; Wagner et al., 2013), it is clear that the time-memory for the first source does not need to extinguish prior to a forager becoming employed at a new source as was previously thought (Moore et al., 1989; Moore, 2001; Saunders, 2002; Seeley and Towne, 1992). In the present study, we further explored the ability of honey bee foragers to schedule their foraging efforts to multiple food sources. Specifically, we examined how foraging experience at a time-specific feeder influences the likelihood that, once the feeder ceases being productive, a forager will be recruited to a new feeder available at another location but at the same time of day. In other words, does the existence of one spatiotemporal memory interfere with the formation of another spatiotemporal memory at the same circadian phase?

We proposed three hypotheses. Hypothesis 1: prior experience at a food source has no influence on the likelihood of recruitment to another source at the same time of day. A prediction from hypothesis 1 is that foragers with different levels of experience with the training feeder will not differ significantly in the recruitment rate to the new feeder. The observation that both persistent foragers (which tend to have high levels of foraging experience at their source) and reticent foragers (which tend to have less such experience) appear to exploit multiple food sources (Wagner et al., 2013) suggests that hypothesis 1 is plausible. Hypothesis 2: formation of a time-memory at a particular time of day precludes recruitment to another food source at that same time of day. In other words, two spatiotemporal memories cannot occupy the same temporal phase. Hypothesis 2 is suggested by von Frisch's assertion that foragers can be trained to multiple food sources per day, but the sources must have different times of availability (von Frisch, 1967). Gould and Towne (1988) reiterate this assertion. A prediction from hypothesis 2 is that there will be no recruitment to the second feeder for any forager exhibiting a time-memory for the first feeder. Hypothesis 3: formation of a time-memory at one food source influences, but does not preclude, the ability of the forager to be recruited to another food source at the same time of day. In this case, the potential for recruitment at the same time of day depends upon the perception of salience for the original food source. Two predictions follow from H3. First, there should be a difference between high-experience and low-experience foragers in their ability to be recruited to the new feeder. Second, there should be a decline in salience (and therefore an increase in recruitment to the new source) with the passage of time. In support of the plausibility of hypothesis 3 is the observation that the rate at which a forager ceases making reconnaissance flights to a previously productive food source is influenced by the amount of experience the forager gained at that source (Moore et al., 2011; Van Nest et al., 2016). The

results of these experiments have major implications for understanding how honey bees manage their foraging behaviors with respect to alternative food sources in the environment as well as the mechanisms by which time memories are maintained and executed by the underlying circadian clock system.

## MATERIALS AND METHODS

### Animals and study site

Western honey bees (*Apis mellifera* L.) were housed at the former Marine Corps Armory in Johnson City, TN, USA (36°18'17.8"N, 82°23'24.4"W). This 12 ha site consists of a mix of wooded areas and grassy open spaces supporting a large variety of flowering plant species, including blackberry, Joe-Pye weed, iron weed, butterfly weed, dogbane, *Clematis* spp., everlasting pea, sumac and a variety of asters and goldenrods. Up to 12 colonies were housed on site, either in standard Langstroth box hives or in three- or four-frame glass-walled observation hives within darkened sheds.

### Experimental design

Four trials were performed using colonies headed by genetically unrelated queens (Table 1). In each trial, two different groups of foragers were trained from the same hive to two different feeders (the training station and the recruitment station) using standard training methods (Moore and Rankin, 1983; Moore and Doherty, 2009; Moore et al., 2011; Van Nest et al., 2016; von Frisch, 1967; Wagner et al., 2013). Briefly, this entailed luring bees at the hive entrance to a feeder filled with 2 mol l<sup>-1</sup> sucrose placed immediately in front of the hive. As bees began returning to the feeder, the feeder was incrementally moved to the desired location. The bees involved in this step were not included in the analyses but were used to recruit the experimental subjects from their hive via the waggle dance (von Frisch, 1967). The stations were both 100 m from the hive and separated by a minimum of 60 deg (e.g. one to the north and one to the east). The feeders consisted of 15 mm Petri dishes trimmed to a height of 5 mm, filled with 2 mol l<sup>-1</sup> sucrose, and placed on a circular sheet of filter paper scented with a few drops of essential oils (Table 1). Two different scents were used to help differentiate the two stations. All bees trained to the training station were individually marked with combinations of enamel paint dots (Testor Corporation, Rockford, IL, USA) applied to the dorsal thorax and abdomen during the bee's first feeding bout at a station. All bees trained to the recruitment station received the same population color code (a single dot of paint on the dorsal thorax). To maintain independent groups during the training phase, any bees visiting both stations on training days were killed and removed from the study. This occurred only 12, 2, 7 and 4 times in trials 1–4, respectively. Training was provided for 5 consecutive days during a restricted time frame of 60–120 min (Table 1). This allowed for cohorts of foragers with 1–5 days of experience at the feeders. The duration of the training time was chosen on the first training day of the trial such that at least 20 new recruits arrived each day.

**Table 1. Date, scent, training time and hive type for each trial**

Trial	Date	Scent	Training time	Hive type
1	4–7 August 2003	Anise/peppermint	11:00 h–12:00 h	4-frame observation
2	29 July–1 August 2005	Lavender/lilac	11:00 h–13:00 h	3-frame observation
3	29 July–1 August 2006	Anise/almond	10:00 h–11:15 h	Langstroth box
4	27–30 September 2006	Anise/almond	14:30 h–15:30 h	3-frame observation

Scent refers to the training station/recruitment station scent.

For 4 days immediately following the 5 training days, sucrose availability was continued at the same restricted time of day at the recruitment station, but the training station had only an empty, unscented feeder. All visits made by individually marked bees were recorded from 4 h prior to the onset of the training time to 4 h past the end of the training time at both stations on all 4 test days. Because experienced foragers gather on the hive's dance floor in anticipation of their appropriate training time (Moore et al., 1989; Van Nest et al., 2016), foragers from the training station were positioned favorably for encounters with recruitment waggles performed by foragers from the recruitment station. Trials 1, 2 and 4 were performed on observation hives (Table 1) with colonies of approximately 6000–8000 bees. Trial 3 was performed on a standard Langstroth box hive with a colony of approximately 20,000 bees in order to test that trends observed in trials 1, 2 and 4 persisted in larger colonies. Previous work showed foraging behavior differed little between small and large colonies (Beekman et al., 2004). In trials 1, 2 and 4, a visual census of the observation hive was taken three times each test day to determine which marked bees were alive: one before, one during and one after the training time. In trial 3, after the last test day, a re-recruitment day was added on which both feeders were once again filled with sucrose solution, and the original scents were applied. The foragers making reconnaissance flights to the feeders then re-recruited the foragers that had visited the feeders on previous days (Moore et al., 2011). The record of foragers seen in re-recruitment was used as the hive census.

### Statistical analyses

Foragers that skipped any training days were removed from the study. We used two different methods to quantify the amount of experience gained at the training station. In method 1, we used the number of consecutive days foraging at the training station. In method 2, we used the mean number of rewards received per day at the training station.

### Method 1

Individually marked foragers with 1 or 2 days of experience were pooled (the low-experience cohort), and bees with 4 or 5 days of experience were pooled (the high-experience cohort). All high- and low-experience cohorts then had 19–60 individuals on all test days of all 4 trials (Table 2). Foragers with 3 days of experience were not included in this method. To test how experience affected the likelihood of switching stations, the proportion of high- and low-experience foragers known to still be alive that visited the feeders were compared using Fisher's exact test ( $\alpha=0.05$ ). Then, to see how the effect of experience changed from day to day, proportions of the still-living foragers visiting the feeders were compared among test days using Tukey-type multiple comparisons of proportions with arcsine transform ( $\alpha=0.05$ ) (Zar, 2010).

**Table 2. Number of high-experience and low-experience foragers (as calculated from method 1) from each census for each trial**

Trial	Test day 1		Test day 2		Test day 3		Test day 4	
	High	Low	High	Low	High	Low	High	Low
1	43	60	43	59	43	58	42	57
2	21	49	21	47	20	42	19	37
3	34	43	31	40	26	36	24	32
4	35	46	32	45	28	43	24	42

### Method 2

The total number of visits made on the 5 training days to the training station by each individually marked forager was divided by the number of days that forager visited the training station (a metric of foraging intensity). This mean was then compared between groups of foragers that visited the stations on test days using the Mann–Whitney *U*-test (95% confidence interval, CI). We also examined how the mean number of rewards per day influenced a forager's behavior separately for the high- and low-experience cohorts from method 1.

### RESULTS

In each of the 4 trials, after 5 training days, one feeder (the training station) was shut off, and the second feeder (the recruitment station) continued to offer sucrose solution for 4 more days (test days). Both stations were monitored, and the arrivals of individually marked bees were recorded. A total of 366 individually marked bees were recorded across all four trials. Nearly half of these foragers (175, or 47.8%) visited both feeders on at least one test day.

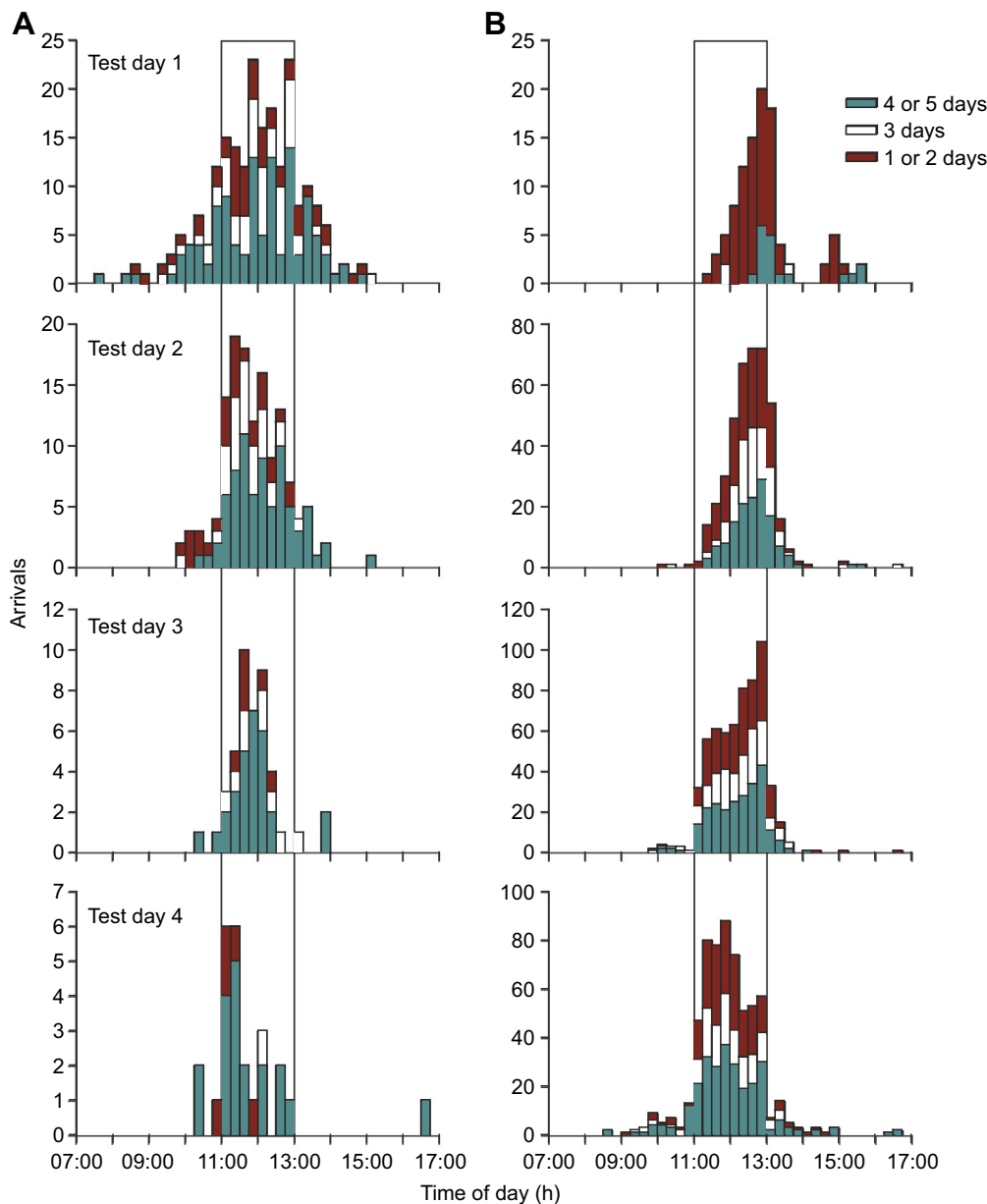
### Temporal accuracy of flights to feeders

It was important to confirm, in all four trials, that the individually marked, time-trained foragers had indeed established accurate time-memories for the training station. In accord with acquisition of an accurate time-memory, most reconnaissance flights to the training station took place during or near the original training time on all 4 test days for the high-experience (4 or 5 days), medium-experience (3 days) and low-experience (1 or 2 days) cohorts of foragers. As a representative example, trial 2 reconnaissance flights to the training station are shown in Fig. 1A. This trend is consistent with previous studies (Moore, 2001; Moore and Doherty, 2009; Moore et al., 2011; Van Nest et al., 2016). Consistent with the formation of a new time-memory, the individually marked foragers that made visits to the recruitment station did so with similar temporal accuracy on all 4 test days (Fig. 1B). It is important to note that prior to test day 1, none of these bees had ever visited the recruitment station.

### Influence of experience on recruitment: method 1

Using Fisher's exact test, we compared the proportions, pooled across all trials, of the high- and low-experience forager cohorts that visited the feeders versus the number that did not visit the feeders on each test day (Fig. 2). High-experience foragers were significantly more likely to remain faithful to the training station (i.e. visit the training station but not the recruitment station) than low-experience foragers on any test day ( $P<0.0001$  for test days 1–3,  $P<0.001$  for test day 4; Fig. 2A). In contrast, low-experience foragers were significantly more likely than high-experience foragers to visit only the recruitment station on any test day ( $P<0.0001$  for test days 1–3,  $P<0.01$  for test day 4; Fig. 2B). High-experience foragers were significantly more likely to be observed at both feeders on test days 2–4 but not test day 1 ( $P>0.05$  for test day 1,  $P<0.0001$  for test days 2–4; Fig. 2C). Finally, low-experience foragers were significantly more likely to be observed at neither station on test days 1–3 but not test day 4 ( $P<0.0001$ ,  $P<0.01$ ,  $P<0.001$  and  $P>0.05$ , respectively for test days 1–4; Fig. 2D). These analyses were also performed on each trial individually with similar results (Fig. S1).

To examine how the proportions of foragers visiting the feeding stations changed from test day to test day, we performed Tukey-type multiple comparisons of proportions by arcsine transforming the proportions (Zar, 2010) for all four trials pooled (Fig. 2). Fidelity to



**Fig. 1. Temporal accuracy of visits to the feeding stations from trial 2.** The number of arrivals of individually marked foragers from three cohorts with different experience (4 or 5 days, 3 days and 1 or 2 days at the training station) is plotted against time of day. Training time is indicated by the vertical boxes. (A) As in previous reports (Moore, 2001; Moore and Doherty, 2009; Moore et al., 2011; Van Nest et al., 2016), time-memory response at the training station diminishes with successive test days but retains accuracy. Most inspections occur within the time-training window. (B) Most visits by individually marked bees to the recruitment station also occur during the time-training window, where a reward was still available. Arrivals are plotted in 15 min intervals from 07:00 h to 17:00 h. Time training was from 11:00 h to 13:00 h.

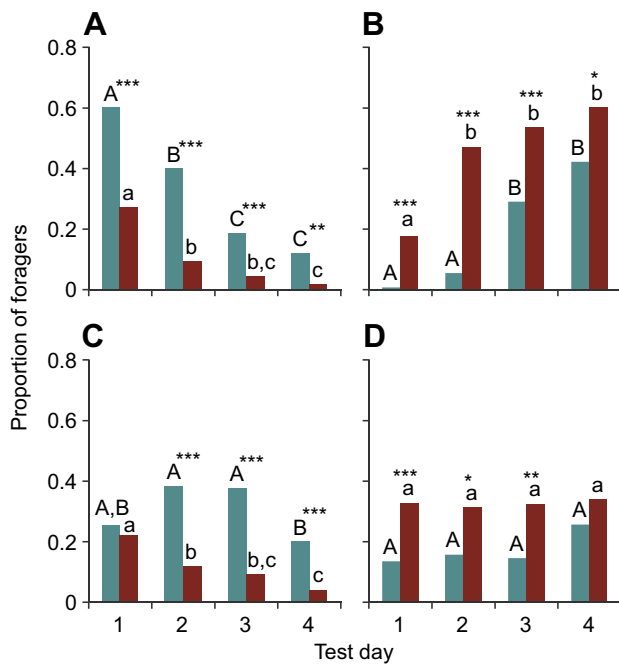
the training station dropped across the test days for both the high- and low-experience foragers (Fig. 2A). In concert with those declines, the proportion of both high- and low-experience foragers visiting the recruitment station exclusively showed significant increases, after test day 2 in the high-experience foragers and after test day 1 in the low-experience foragers (Fig. 2B). Among high-experience foragers, the proportion of foragers visiting both stations did not appear to change much over the 4 test days, while the proportion dropped for the low-experience foragers (Fig. 2C). Finally, there was no evidence that the proportion of foragers visiting neither station changed across test days for either experience cohort (Fig. 2D). The same basic trends can be seen for each of the four trials individually (Fig. S1).

#### Influence of experience on recruitment: method 2

In method 2, we compared the mean number of rewards per day received at the training station between foragers that exhibited different foraging behaviors. Values for all four trials were pooled.

First, we compared these means between foragers that continued to make reconnaissance flights to the training station versus those that did not (regardless of whether they visited the recruitment station or not). This was a test of abandonment of the training station. On all 4 test days, foragers not abandoning the training station were found to have experienced a significantly higher number of rewards per day than foragers that discontinued all visits to the training station ( $P < 0.0001$ ; Fig. 3).

Next, we compared the mean number of rewards per day between foragers that were recruited to the recruitment station (whether or not they continued reconnoitering the training station) versus those that continued reconnoitering the training station only (Fig. 4A). This was a test of recruitability. Foragers that were not seen at either station were omitted from this test. On all 4 test days, foragers that were recruited to the recruitment station were found to have experienced significantly fewer rewards per day than foragers remaining faithful to the training station ( $P < 0.0001$ ,  $P < 0.0001$ ,  $P < 0.001$  and  $P < 0.01$ , respectively, for test days 1–4). We repeated this analysis on the high-



**Fig. 2. Proportion of individually marked high- and low-experience foragers making visits to the feeding stations.** High-experience foragers (4 or 5 days) are shown in blue; low-experience foragers (1 or 2 days) are shown in red. (A) Proportion of bees visiting the training station only (versus bees visiting the recruitment station only, both stations or neither station). (B) Proportion of bees visiting the recruitment station only (versus bees visiting the training station only, both stations or neither station). (C) Proportion of bees visiting both stations (versus bees visiting only one or neither station). (D) Proportion of bees visiting neither station (versus bees visiting one or both stations). Comparisons between high- and low-experience cohorts using Fisher's exact test: \* $P < 0.01$ ; \*\* $P < 0.001$ ; \*\*\* $P < 0.0001$ . Comparisons of proportions across test days using Tukey-type multiple comparisons with arcsine transformations: separately within each panel, groups that do not share an uppercase letter (for high-experience bees) and groups that do not share a lowercase letter (for low-experience bees) are significantly different ( $P < 0.05$ ).

and low-experience cohorts as determined in method 1. Among high-experience foragers, there was no significant difference in the mean number of rewards between foragers that were recruited to the recruitment station and those that were not on any test day ( $P > 0.05$ ; Fig. 4B). However, among low-experience foragers (Fig. 4C), recruited bees received significantly fewer rewards per day than those

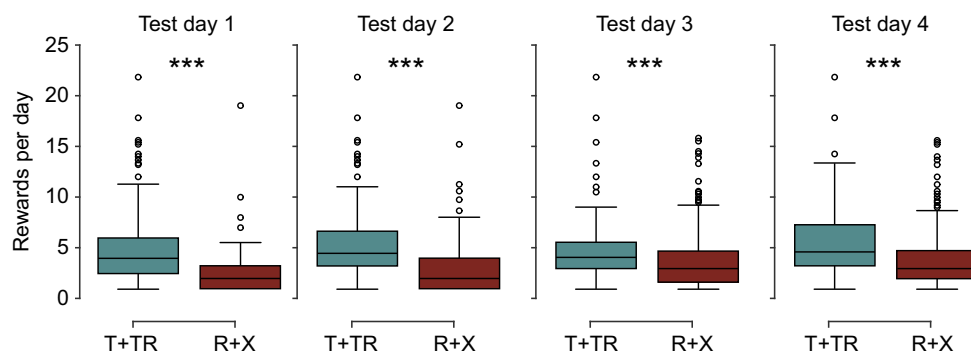
not recruited, for the first 3 test days but not for the last test day ( $P < 0.001$ ,  $P < 0.001$ ,  $P < 0.01$ ,  $P > 0.05$ , respectively, for test days 1–4).

### Examination of the foragers that visited both feeding stations

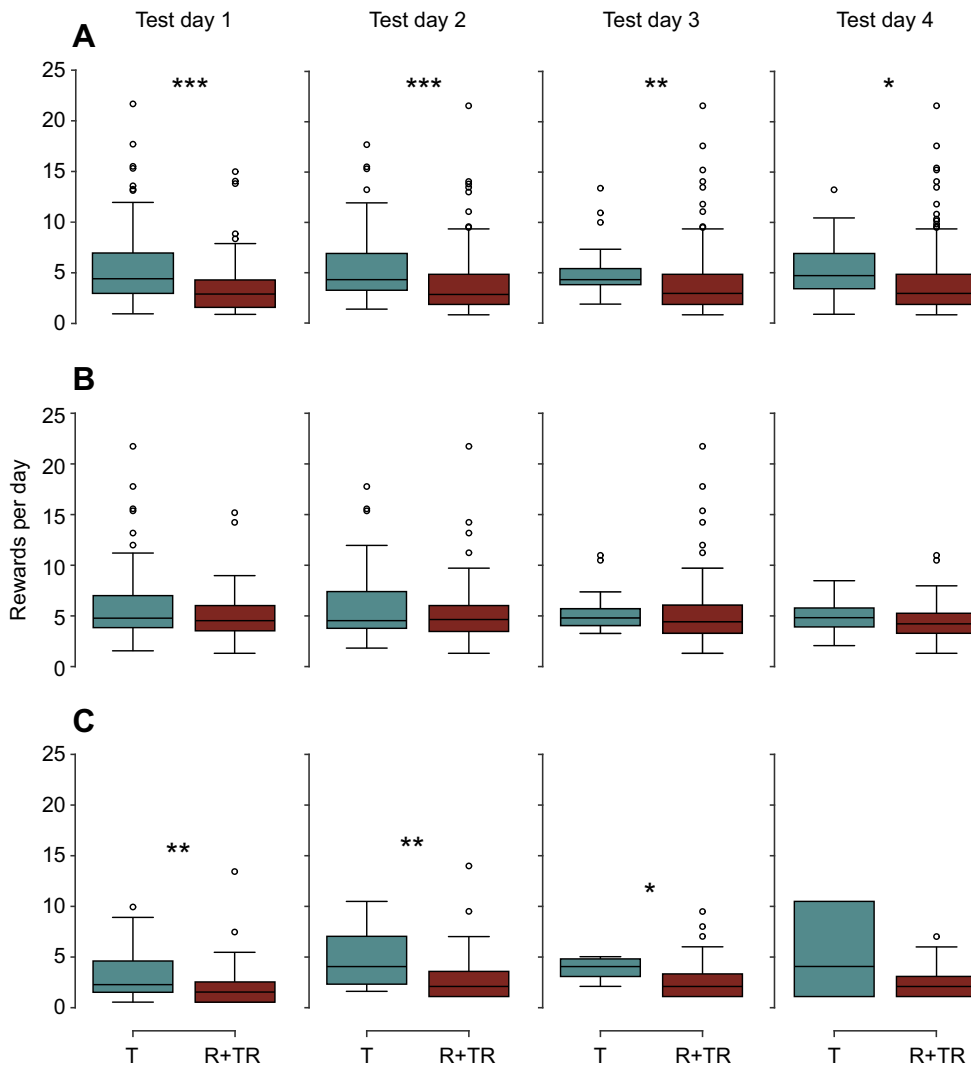
Nearly half of all the individually marked bees recorded in this study (175 of 366, or 47.8%) visited both feeders on at least one test day. Only about one-third of these 175 bees (53, or 30.3%) switched permanently to the recruitment station after receiving a reward there and never returned to the training station (e.g. Fig. 5A). Of these 53 permanent switchers, 23 (43.4%) were high-experience bees (4 or 5 days of training) and 30 (56.6%) were low-experience bees (1 or 2 days of training). The permanent switchers apparently abandoned their original feeder immediately after being successfully recruited. However, these proportions dropped significantly throughout the test days (32.6%, 19.3%, 5.6% and 5.4%, respectively, on test days 1–4; Fig. 6). The largest proportion of the 175 bees that visited both stations on at least one test day (110, or 62.9%) made visits to both feeders even after receiving a reward at the recruitment station. Most of these 110 bees (75, or 68.2%) were high-experience bees, and the remaining 35 (31.8%) were low-experience bees. Many of these multi-taskers resumed inspecting the training station only after successfully collecting from the recruitment station and then failing to find a reward there outside its hours of availability (e.g. Fig. 5B,C). Some, however, continued to visit both feeders repeatedly throughout the training time (e.g. Fig. 5D). In either case, this larger group of bees (30.1% of all 366 trained bees) clearly maintained simultaneous time-memories for the two feeders, and their proportions increased significantly across the training days (64.1%, 75.0%, 87.3% and 94.6%, respectively, on test days 1–4; Fig. 6). The remaining 12 (6.9%) of the 175 bees that visited both stations on at least one test day appeared to be recruited to the recruitment station but made visits there either too early or too late to receive a reward. Of the bees that missed the time-window, 10 (83.3%) were high-experience bees and 2 (16.7%) were low-experience bees. These proportions remained low and did not vary significantly across the test days (Fig. 6).

### DISCUSSION

Our primary objective was to determine whether the existence of one time-memory influences the establishment of a second time-memory at the same time of day in honey bee foragers. We addressed this by testing three hypotheses. Because foragers with lower levels of experience at the training station were recruited to the



**Fig. 3. Mean number of rewards received by foragers that visited the training station versus those that did not visit the training station.** The medians were significantly different on all 4 test days. Boxes represent first quartile, median and third quartile; whiskers represent minimum and maximum values, excluding outliers (open circles; values greater than 1.5× the interquartile range). T, bees that visited the training station only; TR, bees that visited both stations; R, bees that visited the recruitment station only; X, bees that visited neither station. Mann–Whitney  $U$ : \*\*\* $P < 0.0001$ . Data were pooled across all four trials.



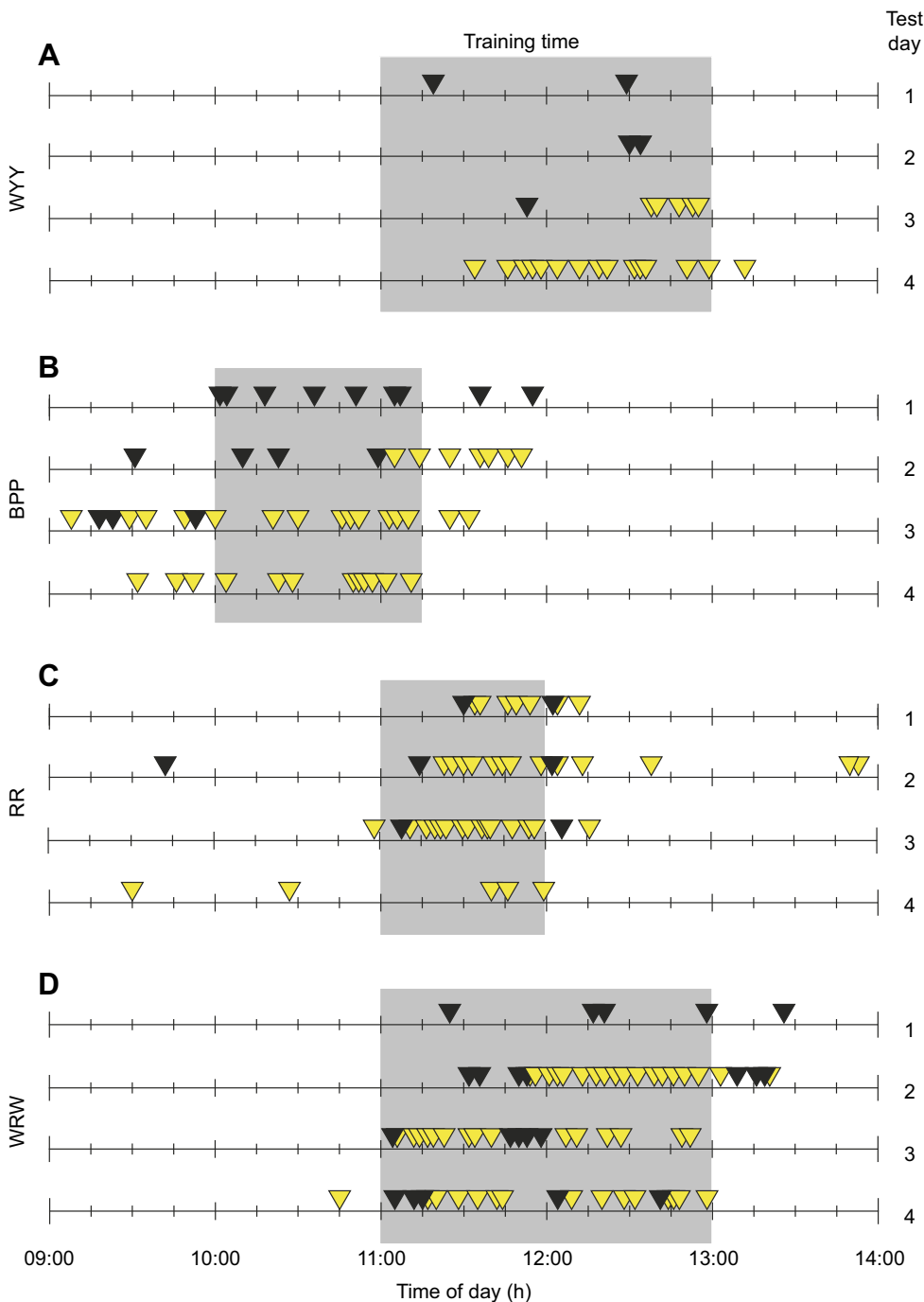
**Fig. 4. Mean number of rewards received by foragers that visited the training station only versus those that visited the recruitment station.** (A) All bees: the medians were significantly different on all 4 test days. (B) High-experience bees (those with 4 or 5 days of experience): the medians were not significantly different on any test day. (C) Low-experience bees (1 or 2 days of experience): the medians were significantly different on test days 1–3. Boxes represent first quartile, median and third quartile; whiskers represent minimum and maximum values, excluding outliers (open circles; values greater than 1.5× the interquartile range). T, bees that visited the training station only; TR, bees that visited both stations; R, bees that visited the recruitment station only. Mann–Whitney *U*: \**P*<0.01; \*\**P*<0.001; \*\*\**P*<0.0001. Data were pooled across all four trials.

recruitment station at significantly higher proportions than high-experience foragers, hypothesis 1 (no influence of experience on recruitment) was not supported. Hypothesis 2 (two spatiotemporal memories cannot occupy the same temporal phase) was also not supported because 30.1% of our time-trained foragers clearly exhibited time-memories to both the training and recruitment stations. Hypothesis 3 stated that experience at one food source influences but does not prevent recruitment to another food source at the same time of day, with the salience of (i.e. amount of experience with) the original source being the major factor controlling the likelihood of being recruited. Our findings supported two predictions from this hypothesis, as detailed below.

Consistent with the first prediction, several metrics revealed that the amount of experience at the training station influenced recruitability to the recruitment station. For example, high-experience foragers (4–5 days of training) were significantly less likely than low-experience foragers (1–2 days of training) to visit the recruitment station exclusively on all 4 test days but significantly more likely to visit both stations on test days 2–4. Furthermore, when the results for both low- and high-experience foragers were pooled, it was found that, for all 4 test days, foragers remaining faithful to the training station received significantly more rewards per day than foragers that were recruited to the recruitment station (whether or not they continued to reconnoiter the training station).

This relationship between food source faithfulness and experience level is consistent with previous studies (Moore and Doherty, 2009; Moore et al., 2011; Wagner et al., 2013). Furthermore, the differences in recruitability based on rewards per day were significant within the low-experience but not the high-experience cohort, suggesting the existence of a certain experiential threshold for the food source that, once reached (especially in high-experience cohorts), compels the forager to continue daily reconnaissance of that source despite the absence of reward.

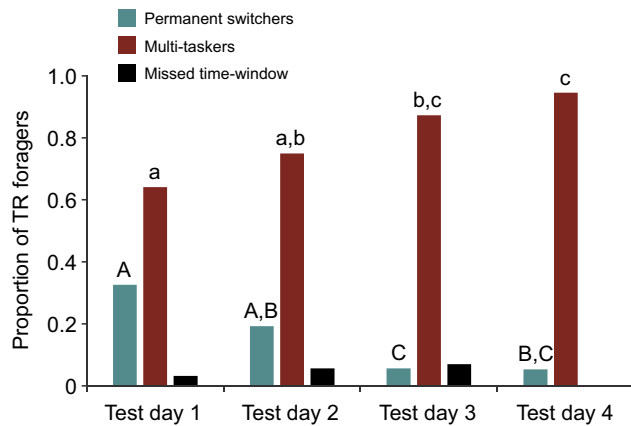
The second prediction assumed that the perceived salience of the training station should decline with the passage of time, thus permitting a progressive increase in recruitment to the recruitment station. As predicted, there was a significant increase through test days 1–4 in the proportion of both high- and low-experience foragers visiting only the recruitment station. These bees had permanently switched to the recruitment station and never returned to the training station. There also was a significant decrease through test days 1–4 in the proportion of low-experience foragers visiting both stations. This progressive decline represents foragers that visited both stations on one or more test days but, on a subsequent test day, visited only the recruitment station. The proportion of high-experience foragers visiting both stations did not show this same decline through test days 1–4, further illustrating the differential effects of prior experience on foraging behavior.



**Fig. 5. Individual records from foragers that visited both stations.** Visits to the training station are shown in black; visits to the recruitment station are shown in yellow; training times are indicated by the gray shading. Of the 175 bees that visited both stations, 53 (30.3%) inspected the training station for one or more days, then, upon discovery of the recruitment station, were never again observed at the training station. (A) An example of this behavior can be seen in bee WYY from trial 2. After 5 days of training, WYY switched to the recruitment station on test day 3 and never returned to the training station. Of the 175 bees, 110 (62.9%) inspected the training station at least once after receiving a reward at the recruitment station. These bees clearly show time-memories for two temporally overlapping food sources. They account for nearly two-thirds of all the bees that visited both stations and for 30.1% of all 366 trained bees. Examples of this behavior follow. (B) Bee BPP from trial 3 received 4 days of training. After discovering the recruitment station late on test day 2, BPP inspected both stations early on test day 3 but did not return to the training station thereafter. (C) Bee RR from trial 1 received 5 days of training. After discovering the recruitment station on test day 1, RR received several rewards at the recruitment station. At the end of test day 1, RR inspected both stations. On test day 2, RR inspected the training station early, then received rewards throughout the remainder of the training time at the recruitment station. After rewards were no longer available there, RR inspected both stations again. On test day 4, RR appeared to abandon the training station and only visited the recruitment station. (D) Bee WRW from trial 2 received 5 days of experience. WRW inspected the training station only on test day 1 and discovered the recruitment station on test day 2. WRW received rewards there for the remainder of the training time and then inspected both stations thereafter. On test days 3 and 4, WRW interrupted its food collection activity at the recruitment station several times to inspect the training station. At the end of the training time, WRW visited neither station on those days.

Our results clearly are consistent with hypothesis 3 and demonstrate that a second time-memory can be initiated during a time of day that is already occupied (and, in many cases, currently acted upon) by a previously established time-memory. Furthermore, the level of experience (salience) associated with the first time-memory influences both the recruitability of the forager to another food source at the same time of day and the forager's likelihood of maintaining two time-memories simultaneously. Thus, it is possible that the foragers visiting both stations in the present study are doing so not because high levels of experience may induce a forager to 'multi-task' but because the extensive experience at, and perceived high importance of, the training station warrants making occasional reconnaissance flights to it regardless of whether or not the forager was recruited elsewhere.

Perhaps our most compelling finding is the capability of time-trained foragers to attend to two different time-memories at the same time of day. This ability apparently is related to the amount of experience the forager acquires at the first source, presumably determining the perception of salience associated with that food source. Foragers with greater experience with a food source (1) take longer to abandon that source when it is no longer productive than foragers with less experience at the source and (2) take longer to be recruited to a second source at the same time of day. Nevertheless, they have a significantly greater probability of continuing to investigate the non-productive first source even after they get recruited to the second source. We have direct evidence that a relatively large proportion of time-trained foragers (30.1%) accomplish such simultaneous time-memories. In fact, this may



**Fig. 6. Proportion of foragers that visited both stations.** Permanent switchers are those that were recruited to the recruitment station and never returned to the training station. Multi-taskers are those that made at least one repeat visit to both feeders. Missed time-window refers to foragers that missed the time-window of the recruitment station and continued inspecting the training station. Comparison of proportions across test days was carried out using Tukey-type multiple comparisons with arcsine transformations: groups that do not share an uppercase letter (for permanent switchers) and groups that do not share a lowercase letter (for multi-taskers) are significantly different ( $P < 0.05$ ). Proportions of those that missed the time-window did not change significantly across test days ( $P > 0.05$ ).

be an underestimation of the true proportion. Because our experiments were performed in the field, it is possible that some foragers observed visiting only the training station on test days were recruited to a natural (unmonitored) food source rather than the recruitment station. It is also possible that some foragers observed at neither station on test days were working multiple natural sources. Also, we have assumed that individually marked foragers visiting the recruitment station were recruited there by other foragers actively collecting from it. While it is probable that most of these bees were recruited there, it is possible that some found it through their own scouting. In either case, whether scouts or recruits, these foragers were exhibiting two overlapping time-memories if they continued reconnoitering the training station.

There is some variation in the manner in which the ‘multi-tasking’ foragers attend to the feeders. Some inspect the training station only before or after the food is available at the recruitment station. Remarkably, others intersperse their collecting visits to the currently productive recruitment station with investigative flights to the previously productive training station. In all cases, the multi-tasking forager directs its behavior to a currently productive food source in addition to another source that has provided no food for as many as 4 days. How long will the time-memory for the training station remain? Previous experiments have shown that scent from a previously productive food source injected into the hive is capable of reactivating forager honey bees to visit that source (Reinhard et al., 2004; von Frisch, 1967) and that, in the absence of alternative food sources, some foragers can be reactivated in this manner 1 week or more after the source has ceased being productive (Beekman, 2005). What is uncertain, however, is whether scent-triggered recollection of place still retains a linkage with time of day. In experiments not relying on scent reactivation, a large proportion (about 40%) of foragers with a high level of previous experience at a food source will continue to reconnoiter that source, at the appropriate time of day, 4 days or more after the source last offered food (Moore et al., 2011). Thus, in this case, the linkage of time with place apparently is still intact.

The results we report here and elsewhere (Wagner et al., 2013) are not the first findings of honey bee foragers working multiple sources at the same time of day, although this phenomenon has been largely overlooked. Seventy years ago, Ribbands (1949) followed honey bee foragers through a garden and reported that a small number of bees visited multiple flower species during a single flight. In contrast, the ability of individual foragers to remember the locations of two or more distinct food sources, each providing food at a different time of day, has been noted previously (Gould and Towne, 1988; von Frisch, 1967). These findings conflict with the long-held notion that individual foragers are specialists, each exhibiting ‘flower constancy’ by focusing on a single species of flower (Butler et al., 1943; Butler, 1945; Chittka et al., 1999; Free, 1963; Hill et al., 1997; Waser, 1986). The ability to shift attention from a currently productive source to a previously productive source provides the forager with a previously unknown level of versatility. However, one might question whether interrupting food collection from a currently productive source to inspect another source that may or may not be productive constitutes an adaptive behavior. Such inspection flights may be worth the effort, considering the nearly negligible energy expenditure required to power a diverted flight relative to the energy reward associated with a renewed food source (Van Nest and Moore, 2012).

Our results provide some insights into the mechanisms underlying the formation and management of time-memories in honey bee foragers. First, the finding that highly experienced bees were more likely to visit both sites suggests that the amount of experience at a productive food source increases the salience of a particular spatiotemporal memory (and its associated contextual features). Second, multiple time-memories with virtually the same time-stamp can co-exist. Third, the remarkable finding that some foragers (Fig. 5D) interrupted their collecting flights at the currently productive feeder to reconnoiter the previously productive feeder suggests that the two sources, in their current states, held equivalent importance to the forager, despite the fact that one of them was last productive 3–4 days in the past. These foragers switched back and forth between the feeders rather than choosing one over the other. This phenomenon may be analogous to multistable perceptions, such as the cognitive illusions known as ambiguous figures (e.g. the Necker cube, duck–rabbit and face–vase illusions) in which perception continuously shifts from one plausible interpretation to the other (Palmer, 1999). For those other foragers that visited both stations on the same day but did not interrupt their food collection at the currently productive station (Fig. 5B,C), the reconnaissance flights to the previously productive station were still time linked, occurring just before or just after the successful collection flights rather than spread randomly through the day.

Other aspects of multiple time-memories remain to be examined. For instance, because foragers make anticipatory flights to a known source each day to ‘find’ the onset time of nectar availability (Moore and Doherty, 2009), their time-stamps may be imprecise. What is the minimal time difference necessary to discriminate between two time-stamps? Koltermann’s (1971) experiments with flower odors at a single feeder suggest that foragers can discriminate changes that occur within about 20 min. It might be interesting, then, to repeat the present experiment with feeders with different relative start times (e.g.  $\pm 10, 20, 30, 60$  and 120 min). It might also be valuable to examine the effects of patriliness, age and life-long foraging experience on the expression of multiple time-memories. These factors are known to affect other aspects of foraging (Dukas and Visscher, 1994; Kraus et al., 2011; Mattila and Seeley, 2014; Robinson and Page, 1989; Seeley, 1982, 1995).



More importantly, perhaps, the next step in understanding the neural mechanisms underlying the honey bee time-memory (and cTPL in general) will be to consolidate behavioral findings with brain gene expression profiles. For example, microarray analyses of the brains of foragers trained to a morning or an afternoon feeder yielded a distinct set of gene expression differences (neurogenomic signatures) associated with distinct spatiotemporal memories (Naeger et al., 2011). Both groups also showed differentially expressed genes associated with the state of food anticipation, the time of training and the time of collection (independent of time of training), thus suggesting a modular composition for spatiotemporal memories. More recently, Shah et al. (2018) showed that the immediate early gene *Egr-1* is upregulated in time-trained honey bee foragers, in anticipation of the training time, and therefore may be a molecular contributor to anticipatory behavior. For a complete understanding of the relationship between molecular processes and time-memory behavior, a number of questions will need to be addressed. For instance, how long do these neurogenomic signatures continue at the appropriate time of day in the absence of a food reward? Time-training experiments suggest that the time-memories of high-experience bees may last at least 4 days and probably more (Moore et al., 2011; present study). What is the relationship between anticipatory gene expression changes and those associated with specific spatiotemporal memories? Do the gene expression patterns associated with specific time-memories strengthen with experience, as suggested in the present study? How closely do these molecular processes match the behavior, especially with respect to changes in the degree of anticipation due to time of day (Moore et al., 1989) or amount of training (Moore and Doherty, 2009)? And, finally, can they discriminate between two different time-memories that have the same time-stamp? Whatever the results, the honey bee mini-brain (Menzel, 2001) continues to impress upon us that a tremendous amount of cognitive sophistication can be accomplished with a rather limited number of neurons.

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#### Competing interests

The authors declare no competing or financial interests.

#### Author contributions

Conceptualization: M.W.O., D.M.; Methodology: B.N.V.N., M.W.O., D.M.; Formal analysis: B.N.V.N., M.W.O., D.M.; Investigation: B.N.V.N., M.W.O., D.M.; Resources: D.M.; Data curation: B.N.V.N., M.W.O., D.M.; Writing - original draft: B.N.V.N.; Writing - review & editing: B.N.V.N., M.W.O., D.M.; Visualization: B.N.V.N., D.M.; Supervision: D.M.; Project administration: M.W.O.; Funding acquisition: M.W.O., D.M.

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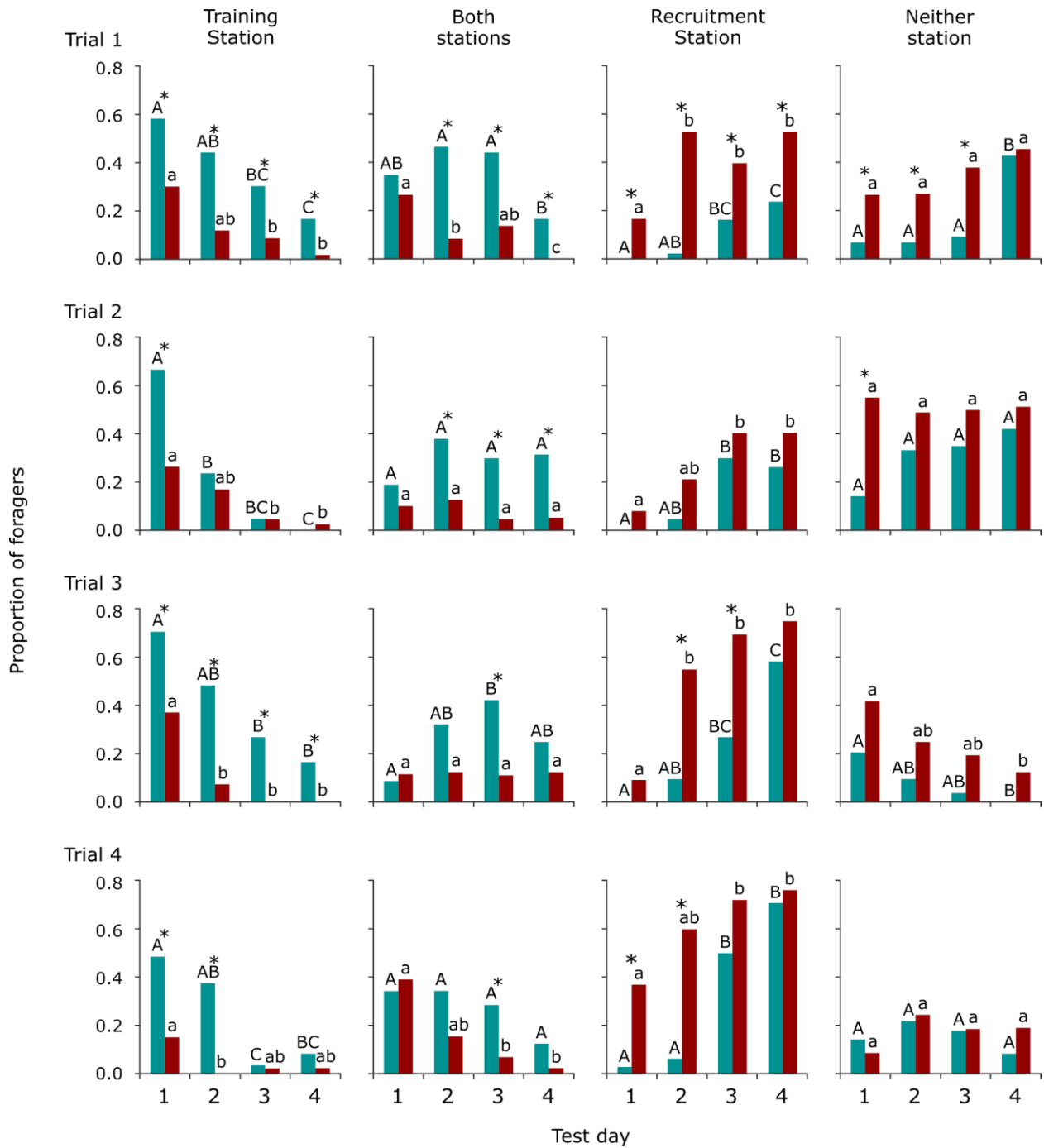
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**Fig. S1. Proportions of individually marked high- and low-experience foragers making visits to the feeding stations.** High-experience foragers (4 or 5 days) are shown in blue; low-experience foragers (1 or 2 days) are shown in red. Comparisons between high-experience and

low-experience cohorts using Fishers exact test: \*  $P < 0.05$ . Comparisons of proportions across test days using Tukey-type multiple comparisons with arcsine transformations: separately within each panel, groups that do not share an upper-case letter (for high-experience bees) and groups that do not share a lower-case letter (for low-performance bees) are significantly different ( $P < 0.05$ ). Values pooled across the 4 trials are shown in Fig. 2.