Insect. Soc. 52 (2005) 139 – 146
0020-1812/05/020139-08
DOI 10.1007/s00040-004-0784-7

Insectes Sociaux

Research article

The influence of marking upon bee behaviour in circle tube experiments with a methodological comparison among studies

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Received 15 December 2003; revised 12 September 2004; accepted 15 October 2004.

Summary. Circle tubes were used to investigate the influence of marking upon the behavioural profiles of two halictid species; one, *Penapis toroi* Rozen, putatively solitary, the other, *Corynura chloris* (Spinola) thought to be semisocial. Previous studies have shown that solitary bees tend to exhibit avoidance behaviour and those with a reproductive division of labour tend to behave aggressively within circle tube arenas. Marked pairs of *C. chloris* were significantly more cooperative than unmarked pairs and marked *P. toroi* showed significantly increased aggression and decreased cooperation. These findings suggest that marking bees may reduce behavioural differences among taxa with different types of social organization. Recommendations are made on how to use the circle tube apparatus to detect bee populations that might be worth more detailed studies of social organization and also for standardization in the way results of these studies are presented. Further experiments are suggested to clarify the effects of certain variables upon bee behaviour using this apparatus. The data presented here are the first for any member of the subfamily Rophitinae and the first for any bee thought to be primarily semisocial. A narrower range of behaviours were observed in the Rophitine, *Penapis toroi* Rozen, in comparison to species from other subfamilies of Halictidae and *Corynura chloris* Spinola had a behavioural profile similar to that of eusocial species.

Key words: Halictidae, social behaviour, experimental methods, *Penapis, Corynura*.

Introduction

The circle tube is a simple apparatus used to study the behaviours of interacting pairs of bees (Breed et al., 1978). Two individuals are introduced into opposite ends of a length of clear plastic tubing the ends of which are then apposed. The result is an annulus-shaped arena in which the two individuals are forced to either remain stationary or interact repeatedly. It was initially used to compare the behaviours within and among castes of a eusocial halictine (Breed et al., 1978; use of behavioural terms follows Michener, 1974). This subfamily of bees contains a greater social diversity than does any group of similar taxonomic rank (Packer, 1997) and so it is not surprising that the use of this apparatus has been extended to study the behavioural repertoires of species with different types of social organization: data are now available for solitary, communal and eusocial species (Kukuk, 1992; McConnell-Garner and Kukuk, 1997; Smith and Weller, 1989; Wcislo, 1997). The circle tube has recently been used in interspecific comparisons of behaviour (McConnell-Garner and Kukuk, 1997) and even to help predict the social organisation of species for which detailed sociobiological data are unavailable (Packer, 2000; Packer et al., 2003). Data presently at hand suggest that communal bees interact predominantly cooperatively, those that are eusocial exhibit high levels of aggressive behaviour, whereas solitary species tend to avoid conspecifics in circle tube arenas (McConnell-Garner and Kukuk, 1997; Packer et al., 2003). Unfortunately, details of the methods used in circle tube experiments have not been very well standardized; indeed, some important details are not presented in some of the papers (see Discussion). Furthermore, the effect of marking upon bee behaviour has not been investigated. In this paper I present the results of circle tube experiments upon two species of halictid bee with markedly different behavioural profiles and demonstrate that the relative frequency of behaviours commonly used to categorise behavioural interactions are sometimes significantly altered by the act of marking. A plea is made for more carefully described methods and greater standardization of approaches when using the circle tube apparatus.

Methods

Bees were collected from flowers: *Penapis toroi* Rozen from *Nolana alba* (Nolanaceae) at the 1005 kilometre marker of the Pan American Highway, East of Chañaral, Region III, Chile on October 14th 1997 and October 23rd 2000 and *Corynura chloris* Spinola from various Asteraceae, 23km NE of Valdivia, Region VIII, Chile on the 11th and 17th of October, 2000. These dates represent late spring in this part of the world. In the Chañaral region, rain (if any) falls primarily in the winter and so there is generally only one period of bloom. Valdivia is a much wetter environment with rains throughout the year but with cold winters such that bloom is available from spring to fall. *Penapis* is a member of the halictid subfamily Rophitinae, all species of this subfamily for which nests have been discovered are solitary. *Corynura chloris* is a member of the subfamily Halictinae and it is believed to have small semisocial colonies (Claude-Joseph, 1926). Voucher specimens are in the Packer collection at York University.

Upon capture, bees were stored individually in 1.5 ml eppendorf tubes until used. Experiments began at most 30 minutes following capture of the first bee, although usually substantially less time elapsed between capture and experimentation. This maximum time period was based upon observations (of *Halictus ligatus* Say) of changes in ovarian condition of bees kept in captivity for half an hour and the possibility that these physiological changes could also be reflected in behavioural differences (Pabalan et al., unpubl. data). Marked bees received a small dot of either red or grey Testors PLA enamel paint applied to the top of the head. Unmarked bees were manipulated for a similar length of time and in a similar manner to ensure that differences between marked and unmarked bees do not result simply from handling during marking. None of the analyses presented here rely upon being able to identify which individual was which. One individual was placed inside each end of a piece of clear plastic tubing 20 cm in length and 5 mm in internal diameter, wide enough to permit individuals to somersault within the tube and for bees to pass one another. The bees were introduced simultaneously to avoid 'ownership' effects (Wcislo, 1997) and the two ends were then apposed thereby closing the circle. Each piece of tubing was used only once. Experiments took place in the shade in the field.

Behaviours were observed for between 15 and 30 min but for purposes of comparison with other studies, only data from the first 10 min are referred to here. Behavioural outcomes were scored for each frontal encounter (FE), defined as the bees coming within one body length of each other. Based upon earlier studies (Breed et al., 1978; Smith and Weller, 1989; Kukuk, 1992; McConnell-Garner and Kukuk, 1997; Wcislo, 1997; Pabalan et al., 2000; Packer et al., 2003), behaviours were broadly classified as avoidance (one or both bees moving away from the other/each other), cooperation (passing or backing with following) or aggression (biting, nudging, lunging, fighting, pushing or exhibition of the C-posture). For a more detailed description of the various behaviours see any of the references listed above. Because any interaction has to result in the bees parting before a subsequent FE can occur, each instance of aggressive or cooperative behaviour was necessarily accompanied by a subsequent example of avoidance. Consequently, avoidance was only scored if it was the only behaviour resulting from an FE. The only situation in which more than one outcome was scored for a single FE was when an aggressive behaviour was followed by a cooperative one (the reverse sequence was never observed within an FE) before the bees parted. This occurred only twice, once for an unmarked pair of *P. toroi* and once for a marked pair of *C. chloris*. Consequently, the summed frequencies of behaviours per FE are either one or very close to one.

Pairs differed in the number of interactions scored and simple summation of the number of behaviours across circle tube bouts within a species would bias the results in favour of the repertoires of the most active pairs. Consequently, data are presented both as total number of occurrences of the three different behavioural categories per pair as well as the average of the frequencies across all pairs. Comparisons between marked and unmarked bees were made using two-tailed Mann-Whitney U tests because sample sizes are small (Zar, 1996).

Results

The number of aggressive, avoidance and cooperative behaviours exhibited by each pair of bees is shown in Table 1 for *P. toroi* and Table 2 for *C. chloris*, separately for marked and unmarked individuals. Also shown is the total number of FE's exhibited for marked and unmarked bees as well as the average frequency of occurrence of the three types of behaviour

Table 1. Number of aggressive, avoidance and cooperative behaviours in pairs of unmarked and marked *Penapis toroi*. Frequency 1 counts each interaction as equivalent, Frequency 2 counts each pair as equivalent

	Pair	Aggressive	Avoidance	Cooperative	Total FE's
Unmarked bees	1	1	12	3	16
	2	1	12	2	15
	3	5	10	0	15
	4	2	24	1	27
	5	0	26	0	26
	6	4	7	2	13
	7	1	11	6	17
	8	0	4	0	4
	Total	14	106	14	134
	Frequency 1	0.1	0.79	0.1	
	Frequency 2	0.11	0.78	0.11	
Marked bees	1	8	5	0	13
	2	0	4	0	4
	3	10	2	0	2
	4	3	4	0	7
	5	19	5	0	24
	6	9	20	0	29
	7	7	10	0	17
	8	7	20	0	27
	9	7	23	0	30
	Total	70	93	0	163
	Frequency 1	0.43	0.57	0.00	
	Frequency 2	0.43	0.57	0.00	

Table 2. Number of aggressive, avoidance and cooperative behaviours in pairs of unmarked and marked *Corynura chloris*. Frequency 1 counts each interaction as equivalent, Frequency 2 counts each pair as equivalent

	Pair	Aggressive	Avoidance	Cooperative	Total FE's
Unmarked bees	1	4	8	2	14
	2	4	7	0	11
	3	9	2	0	11
	4	4	2	0	6
	5	11	10	1	22
	6	5	6	0	11
	7	9	9	2	20
	8	6	6	0	12
	9	6	4	0	10
	10	10	12	0	22
	Total	68	66	5	139
	Frequency 1	0.49	0.47	0.04	
	Frequency 2	0.51	0.46	0.03	
Marked bees	1	5	4	5	13
	2	3	5	3	11
	3	3	4	2	9
	4	3	2	0	5
	5	5	3	1	9
	Total	19	18	11	48
	Frequency 1	0.40	0.38	0.23	
	Frequency 2	0.43	0.39	0.20	

across all pairs within a treatment. Some behaviours previously found in studies of halictine bees in circle tubes were not detected in *P. penai*: no instances of biting, fighting (prolonged agonistic encounters) or the C-posture were observed. In contrast, C-postures were the commonest single behavioural outcome of an FE for *C. chloris*, forming one third of all interactions and 59% of the aggressive ones.

There was no significant difference in activity levels (number of FE's) between marked and unmarked bees for either species (for *P. toroi* $U = 34.5$ $p \gg 0.2$; for *C. chloris* $U = 38.5$, $p > 0.05$). For *P. toroi* there was a significant increase in aggressive behaviours and a significant decrease in cooperative interactions between marked bees in comparison to unmarked ones ($U = 60$, $p < 0.05$ and $U = 58.5$, $p < 0.05$ respectively). Indeed, none of the marked bees exhibited a single example of cooperative behaviour whereas 5 of 8 unmarked bees exhibited at least one cooperative act (this difference is significant, Fisher Exact Test $p < 0.01$). There was a significant increase in frequency of cooperative behaviour in *C. chloris* ($U = 42.5$, $p < 0.05$), but no difference

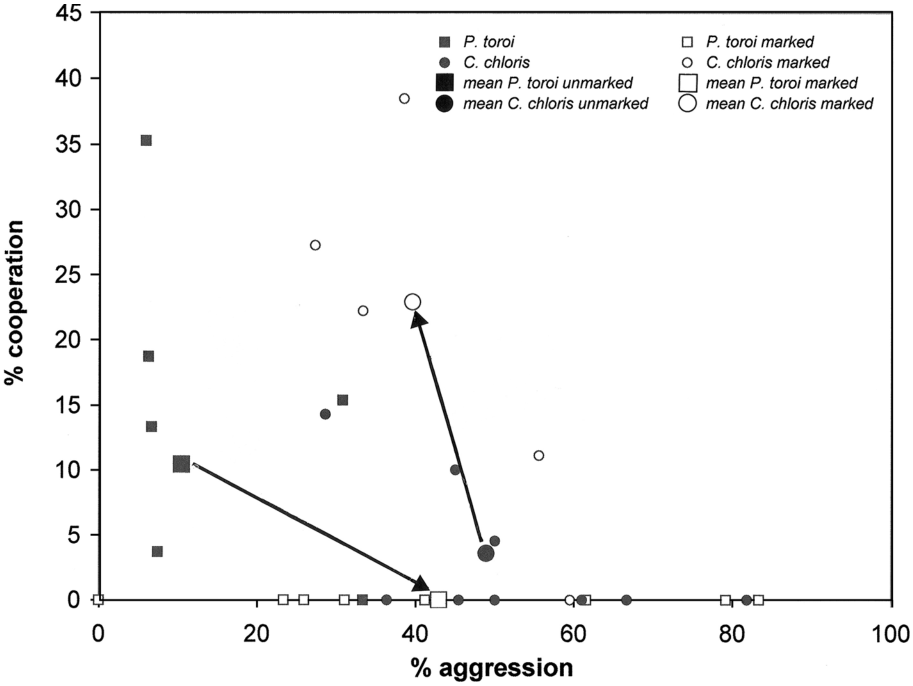

Figure 1. Plot of percentage of frontal encounters that resulted in cooperation versus those that resulted in aggression for marked and unmarked pairs of bees. The arrows connect the means and point from the mean for unmarked to the mean for marked individuals

in the frequency of aggressive behaviour (U = 33.5, $p \gg 0.2$). The frequency of avoidance behaviours did not differ between treatments in either species (for *P. toroi* U = 45, p = 0.2, for *C. chloris* U = 37, p = 0.2). Figure 1 plots the frequency of occurrence of cooperative behaviour against aggressive behaviour for all pairs with average values for each of the four species/marking combinations also shown. Differences in the mean frequency of behaviours between marked and unmarked bees are shown with arrows for each species. Pairs further to the right are more aggressive, those higher on the y-axis are more cooperative and those closer to the origin exhibit primarily avoidance behaviour. Figure 2 shows the means for marked and unmarked pairs along with means for other species for which circle tube data have been published. Unmarked pairs of *P. toroi* are mostly to the lower left, consistent with a behavioural profile of a solitary species in which FE's usually result in avoidance (Fig. 2), while unmarked *C. chloris* are lower down in the plot and further to the right a position that is more aggressive and less cooperative than either the solitary species *L. figueresi* studied by Wcislo (1997) or the eusocial *L. pauxillum* studied by Smith and Weller (1989). Both of these studies used marked bees and if the direction of change due to marking is consistent with those I have found, we might expect unmarked pairs of the former to behave less aggressively (i.e. move to the left) and those of the latter to behave less cooperatively. These relocations on the plot would leave *C. chloris* between the two eusocial taxa, perhaps consistent with a reproductive division of labour. A move away from the behavioural frequencies associated with solitary behaviour towards a more aggressive profile can be seen in marked *P. toroi* pairs: five pairs fall outside of the range of unmarked conspecifics but within the range of unmarked *C. chloris* in Figure 1. It is clear from both figures, that marking has the effect of reducing the differences in behavioural profiles found in the two species.

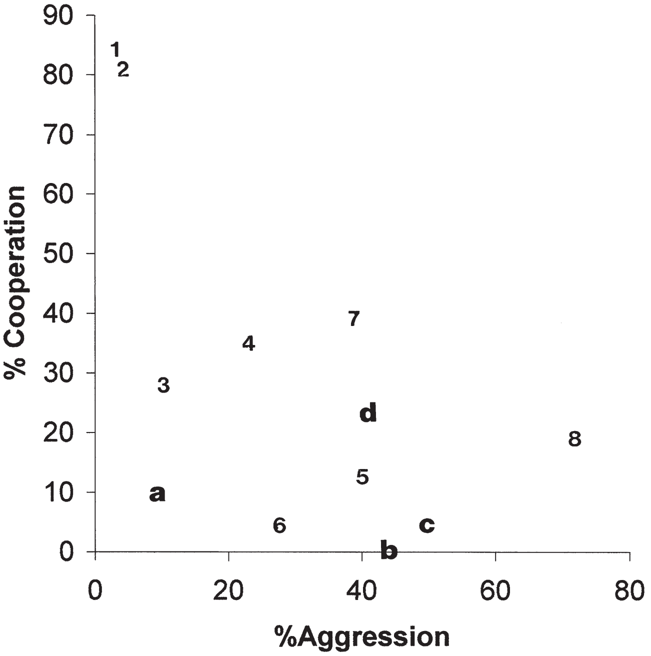

Figure 2. Plot of proportion of frontal encounters that resulted in cooperation versus those that resulted in aggression. Points are averages for published studies of Halictidae. Numbers refer to previous studies as follows: 1 and 2 are communal: 1 – *Lasioglossum hemichalceum* (Cockerell) (McConnell-Garner and Kukuk, 1997), 2 – *Ruizantheda mutabilis* (Spinoza) (Packer unpublished data); 3 to 6 are primarily solitary: 3 – *Lasioglossum platycephalum* (Rayment) (McConnell-Garner and Kukuk, 1997), 4 – *Lasioglossum* sp. (McConnell-Garner and Kukuk, 1997); 5 – *Lasioglossum figueresi* Wcislo (Wcislo, 1997), 6 – *Thrincohalictus prognathus* (Pérez) (Packer, 2000); 7 and 8 are from eusocial populations, 7 – *Lasioglossum pauxillum* (Schenck) (Smith and Weller, 1989), 8 – *Halictus lanei* (Moure) (Packer et al., 2003). Data shown by letter refer to the means for unmarked and marked bee pairs described herein – **a** and **b** are unmarked and marked *Penapis toroi* and **c** and **d** are unmarked and marked *Corynura chloris* respectively

Discussion

The bee family Halictidae contains the greatest social diversity for any group of organisms of similar taxonomic rank, with many solitary species, many communal ones and eusocial colonies with the number of workers ranging from an average maximum of 0.5 (Packer, 1990) to colonies with hundreds of bees (Plateaux-Quénu, 1959; Sakagami and Okazawa, 1985). Of the four subfamilies within the Halictidae (Rophitinae, Nomiinae, Nomioidinae, Halictinae with the latter divided into the tribes Halictini and Augochlorini), only the Halictinae are known to exhibit forms of sociality that involve a reproductive division of labour, either between generations in eusociality or within a generation in semisocial societies. All subfamilies with the exception of the Rophitinae (for which only solitary behaviour is known) have some communal species (Michener, 2000).

This paper is the first to describe circle tube interactions either for a member of the subfamily Rophitinae or for a primarily semisocial halictine species. Evidence that these behavioural categorizations are broadly correct for the populations studied comes from dissections of the bees used in the experiments. Nine out of the 30 *C. chloris* completely lacked ovarian development despite all having at least some mandibular and/or wing wear and being caught collecting pollen. This is suggestive of a division of labour as solitary or communal bees that forage for pollen and exhibit signs of not being recently emerged would all be expected to have some ovarian development. In contrast, all individuals of *P. toroi* were ovarially developed, indeed, all had the summed equivalent oocyte mass of at least one fully developed egg.

As expected for a species that almost certainly has had no communal behaviour or reproductive division of labour at any time in its evolutionary history, unmarked *P. toroi* females avoided one another most of the time and exhibited few instances of passing (only 4 of 8 pairs passed at all and only one pair passed more than once). This species clearly falls among the solitary species in Figure 2. Additionally, *P. toroi* females lacked the C-posture, did not bite one another and did not engage is sustained aggressive behaviour (fighting). Their agonistic encounters consisted solely of nudges (observed in 3 pairs), lunges (8 pairs) or pushes

(2 pairs). In contrast, unmarked *C. chloris* females were primarily aggressive (41% of all interactions were aggressive) and they exhibited the complete repertoire of aggressive interactions found among social halictines. This species falls among the eusocial species in Figure 2, indicating that aggressive interactions may typify circle tube interactions among putatively unrelated (i.e. collected from flowers rather than nestmates collected from nests) females of species with a reproductive division of labour whether in semisocial or eusocial contexts. Both of these behavioural profiles are in contrast to the sometimes extremely cooperative behaviour that has been observed in non-nestmate communal species (Kukuk and Crozier, 1990; Packer, unpubl. data).

Given the vast number of species of halictid bees and the fact that different populations may exhibit markedly different behaviours (reviewed by Packer, 1997; Wcislo and Danforth, 1997), the vast majority of them has not been, and will not be, studied sociobiologically in the field. This suggests the importance of finding a simple way in which the social organization of a population may be estimated without the difficulties associated with finding and excavating large numbers of nests. It seems possible that the circle tube may fulfill this task as the behaviours of solitary, communal and eusocial species seem to differ substantially when this apparatus is used (McConnell-Garner and Kukuk, 1997; Packer et al., 2003; Packer, unpubl. data). At the very least, this methodology could be useful to hypothesize the social organization of populations or for detecting those that seem atypical in comparison to the larger taxonomic group to which they belong. For example, the discovery of primarily aggressively interacting individuals in a population of a species that is thought to be communal (and thus expected to have predominantly cooperative behaviour) would indicate that this population would be worthy of more detailed investigation. However, for accurate comparisons among populations or species using the circle tube apparatus, standardization of methods is of crucial importance.

I have demonstrated that the behaviours of bees in circle tubes may differ substantially as a result of marking and that this may be the case both with bees that are solitary and those which have a reproductive division of labour. Of importance for those wishing to use the circle tube apparatus to test hypotheses pertaining to the social organization of a population was the direction of change in the relative frequency of behaviours. As can be seen from Figure 1, marked individuals of the solitary species showed a more aggressive behavioural repertoire, more typically associated with species with a reproductive division of labour, whereas the increase in frequency of cooperative behaviour in the semisocial species resulted in some pairs exhibiting a behavioural profile perhaps more typical of a solitary species. All previous studies by other researchers have involved marking one or both bees in a pair. Despite this, substantial differences remain among taxa with different social organisations (McConnell-Garner and Kukuk, 1997; Pabalan et al., 2000). It would be interesting to repeat some of these studies with unmarked bees to see if the differences among taxa with different social organizations increases in magnitude as expected based upon the results presented herein.

Other than marking or not marking the bees, there are a large number of variables that differ among circle tube studies of halictine bees which might be usefully standardized in large scale comparative studies. These are listed in Table 3 and are discussed in turn below.

My experiments have been performed with the experimental condition being marking of the bees on the vertex of the head. This is a marking location that permits identification of bees at nest entrances. However, it should be noted that many sweat bee researchers mark the bees on the thorax and/or gaster (although I have found that the paint comes off more easily from the thorax, perhaps as a result of the vibrations caused by the act of flight) and it is possible that different locations of paint marks will influence the behaviour of marked bees somewhat differently.

In some studies, bees were chilled prior to experimentation. The effect this has upon subsequent behaviour has not been investigated, although most authors that chilled their bees state that interactions were recorded only once the bees had 'warmed up'. Some studies used bees from excavated nests whereas others used bees caught from flowers. Presumably the experience of the fairly long disturbance associated with having their nest excavated might influence the physiological state and behavioural responses of bees, especially as it has been shown that captivity has an influence on ovarian condition within half an hour of capture (see below, Pabalan et al., unpubl. data) and nest excavation commonly can take much longer than this. The influence of nest excavation could be studied by comparing interactions among bees from different nests captured through excavation with those collected returning to the nest, although the confounding effect of possible behavioural differences between primarily 'nest bees' and foragers would have to be considered. Of course, there is a problem associated with using bees collected from flowers as one knows nothing of their provenance and it is possible that some individuals might actually be nestmates. Several studies have compared interactions among nestmates and non-nestmates. Such comparisons could be used as 'calibrations' to predict the difference in direction and magnitude of changes between nestmates and non-nestmates for populations in which nests have not been discovered. In *Halictus lanei*, I found one cooperative pair out of ten largely agonistically interacting pairs and suggested that these two individuals might have been nestmates (the species involved is thought to have very large colony sizes) (Packer et al., 2003). Circle tube studies of bees that nest in stems would also be a way of avoiding the long term trauma associated with extended nest excavation as bees in twigs can be extracted fairly quickly (e.g. Arneson and Wcislo, 2003). The same is true of species that make brood cell clusters that are very shallow and can be excavated within a few minutes (e.g. *Lasioglossum comagenense*; Packer et al., 1989).

The time elapsed between capture and experimentation varies among the studies from less than ten minutes to up to 24 h. In the eusocial halictine *Halictus ligatus*, detectable changes in ovarian development occur within 30 min of

Table 3. Methodological variation in circle tube experiments, bees are in the family Halictidae unless otherwise stated

Species/family	Marked Bees/location	Cooled bees	Excavated bees	Time post-capture	Duration of observations	Mean # FE's/trial	Simultaneous placement	Tube diameter	Tube length	FE distance	Reference
Penapis toroi Rozen	Some/head	No	No	< 30 min	10 min	17.4	Yes	5 mm	20 cm	1 body length	This paper
Corynura chloris Spinola	Some/head	No	No	< 30 min	10 min	12.4	Yes	5 mm	20 cm	1 body length	This paper
Megalopta genalis Meade-Waldo	Yes/not stated	No	From stems	< ~18 h	30 min	Not given	~64 s difference	8 mm	30–32 cm	7 cm	Arneson and Wcislo, 2003
Lasioglossum zephyrum (Smith)	Both/thorax	No	No	Not stated	10 min	Between 6 and 9	Not stated	5 mm	30 cm	'face to face'	Breed et al., 1978
Lasioglossum pauxillum (Schenck)	Both/thorax	Yes	No	'later same day'	15 min	Between 16 and 26	Not stated	'= to nest diameter'		20 cm	Not stated Smith and Weller, 1989
L. hemichalceum (Cockerell)	Both/thorax	Yes	Yes	Not stated	10 min	Between 11 and 26	No	Not stated	Not stated	≤ 2 mm	Kukuk, 1992
L. figueresi Wcislo	Both/thorax	No	Yes	Not stated	10 min	Between 7 and 10	Yes	8 mm	20 cm	1 body length	Wcislo, 1997
L. platycephalum (Rayment) and *L.* (*Ctenonomia*) sp.	One/thorax	Yes	Yes, plus lab nests	< 24 h	10 min	Between 5 and 7	Not stated	3 mm	20 cm	≤ 2 mm	McConnel-Garner and Kukuk, 1997
Thrincohalictis prognathus (Perez)	No	No	No	< 10 min	20–30 min	16	Not stated but Yes	Not stated but 8 mm	Not stated but 20 cm	Not stated: 1 body length	Packer, 2000
Halictus ligatus Say	No	Sightly	Some pairs	> 30 min	75–120 min	Highly variable	Not stated	5 mm	20 cm	Not stated	Pabalan et al., 2000
Halictus lanei (Moure)	No	No	No	< 30 min	30 min	55.5 (18–181)	Yes	Not stated but 5 mm	Not stated but 20 cm	1 body length	Packer, et al., 2003
Acamptopoeum submetallicum (Spinola) Andrenidae	No	No	No	≤ 5 min	15 min	9.2	Not stated but?	5 mm	20 cm	Not stated but	Grixti et al., 2004
Nolanomelisa toroi Rozen Andrenidae	No	No	No	≤ 5 min	15 min	19.3	Not stated but	5 mm	20 cm	Not stated but	Grixti et al., 2004
Andrena scotica Perkins Andrenidae	Not stated	Yes	No	~ 8 h	10 min	70.7	Yes	8 mm.	45 cm	5 mm	Paxton et al., 1999
Panurgus calcaratus Scopoli Andrenidae	Not stated	Yes	No	~ 8 h	10 min	14.1	Yes	5 mm	25 cm	2 cm	Paxton et al., 1999

capture and these changes can be massive after 12 h (Pabalan et al., unpubl. data). It is known that ovarian development variables and levels of aggression are linked (Roseler et al., 1980; Pabalan et al., 2000). It is likely that the physiological changes associated with extended periods of captivity may also influence the relative expression of aggressive and cooperative behaviours, something that should be investigated empirically.

The duration of circle tube experiments has generally been only 10 min. Pabalan et al. (2000) showed that some interesting behaviours were missed in such short periods of observation; sustained aggressive behaviours in *Halictus ligatus* becoming manifest only after a period of 45 min of interaction. One problem with short duration observations is that the sample size for number of behaviours actually observed is small. Especially considering that behavioural interactions in circle tubes often occur in chains of similar response (pers. obs.), an average of less than 10 FE's per pair in 10 min trials (as occurred in three of the published studies) seems rather low. Despite this, researchers commonly report significant differences among treatments (caste, bees from the same or different nests etc.) with even lower mean FE's per trial (for example, 4.75 for *Lasioglossum (Ctenonomia)* sp. in McConnell-Garner and Kukuk, 1997). This suggests that short periods of observation may suffice for most purposes.

Wcislo (1997) showed that individuals that were placed into the circle tube first were more aggressive than the second bee to be introduced. This 'ownership' effect may arise in all studies in which bees are not introduced approximately simultaneously (Wcislo showed that one minute was sufficient to lead to differences in levels of aggression between 'owners' and intruding bees). It is physically difficult to introduce bees into opposite ends of a plastic tube at the same time; nonetheless, with practice it can be done. However, it is worth noting that under natural conditions of a nest tunnel, it would be impossible for two individuals to enter at the same time (unless perhaps they had emerged from their brood cells simultaneously, itself somewhat improbable). Thus, introducing two bees to the circle tube at the same time is even less 'natural' than letting one establish ownership. Comparisons between the results obtained for unmarked bees when introduced simultaneously and at different times for taxa with different social organizations would be useful.

The internal diameter of the circle tube in relation to the size of the bees introduced therein might be expected to influence subsequent behaviours. The largest discrepancy between tube and bee sizes occurs when comparing the study of Wcislo (1997) of *Lasioglossum (Dialictus) figueresi*, a comparatively small halictine studied in tubes with the comparatively large internal diameter of 8 mm, and the larger *Halictus ligatus* in which 5 mm tubes were used (Pabalan et al., 2000). Both species have similar nest burrow diameters, averaging 5.5 mm for *L. figueresi* (Wcislo et al., 1993) and 5.7 mm for *H. ligatus* (Packer and Knerer, 1986). No data on the effect of bee size:tube size have been published, although it is possible that behavioural profiles are similar as long as there is sufficient space for the bees to both turn around to avoid one another and to pass each other. Nonetheless, it would be useful to present the average width of bees as well as circle tube internal diameters in experiments involving this apparatus. The mean head width of *P. toroi* in the experiments reported here was 2.15 mm (SD = 0.07, n = 32) and for *C. chloris* 2.49 mm (SD = 0.12, n = 30).

The length of tubing used in the experiments varies somewhat. Most studies have used 20 cm lengths irrespective of the size of the bees being studied. However, this is a considerably longer distance for a small bee to travel than it is for a larger one. Indeed, it is 60 times the body length of a small *Lasioglossum (Chilalictus)* but less than 20 times the length of *Halictus ligatus* queens. It seems sensible for the length of tubing used to be approximately proportional to the mean body lengths of the species being studied, although there may be physical limitations associated with bending a very short tube into a circle. Although the possible influence of this upon the relative proportions of different behaviours remains to be investigated, for a given pace of bee movement, the shorter the length of tubing used, the more often the bees will interact.

What exactly represents a frontal encounter varies among studies. Some authors use a fixed distance. This has ranged from 0.2 cm which is approximately half the body length of the species studied (Kukuk, 1992) to 7 cm, approximately 3.5 times the body length of the species studied (Arneson and Wcislo, 2003). Others have used a fixed proportion of the body length to delimit an FE between interactants. Both aggressive interactions and cooperative ones involve direct contact (although C-postures can occur at quite some distance from the opponent as for example in *H. lanei*; Packer et al., 2003). Consequently, the use of different distances to represent an FE is likely to only influence the number of avoidance behaviours, although thereby the relative frequency of the others. Smaller bees would receive higher scores for avoidance and lower scores for the other types of behaviour in comparison to larger bees when fixed distances are used to suggest an FE because smaller bees will be registered for an FE at a larger proportion of their body length than will larger bees. It would seem that delimiting an FE by a given proportion of the body length of the interactants within a similarly controlled length of tubing might be the best protocol.

Lastly, circle tube experiments usually take place under lit conditions whereas halictine bees normally interact in the dark of their subterranean nests (although some nest in twigs and wood). Some experiments have taken place with infra red illumination (e.g. Arneson and Wcislo, 2003). The effect of visual communication on interactions was apparent in an earlier study of *Halictus lanei* (Packer et al., 2003), in which individuals adopted the C-posture as soon as the 'opponent' came into view at a distance greater than normally considered to reflect a frontal encounter. Another aspect of interaction that remains to be investigated is the potential of auditory or vibrational communication. I have found this to be the major form of communication among individuals in two pairs of *Systropha* sp. (Halictidae: Rophitinae) in which after the initial FE, bees spent the rest of the observation period stationary, at some distance from one another, with repeated buzzing (Packer, unpubl. data).

The results and comparisons performed herein suggest some changes to the protocols used in circle tube experiments particularly if they are to be used to guess at the social organization of a species of bee for which this information is unknown. First, experiments should be performed on unmarked bees because marking influences behaviour and seems to do so in a way that blurs differences in behavioural profiles of bees with different types of sociality. Second, it might be useful to use bees that have not undergone the stresses of nest excavation, chilling or prolonged periods of captivity in order to establish behavioural profiles. Thirdly, it would be useful if the diameter and length of the tubing used and the distance between interactants taken to delimit a frontal encounter were standardized as far as possible with respect to the size of the bees rather than as absolute measurements. Internal tube diameters of approximately twice the head width, tubes approximately 40 times the average length of the bees and one body length separating individuals to delimit an FE would seem to be good choices for such standardization. Lastly, all of these methodological details should be presented, for this omission, I am the worst culprit (Packer, 2000).

Given that marking influences the subsequent behaviours of bees in circle tubes, it is worth pondering the influence of marking upon interactions among individuals within the nest in the field. Marked females of the semisocial *C. chloris* were more cooperative than unmarked individuals. Bees that have received acts of aggression from nestmates tend to regress their ovaries and act more cooperatively than do control individuals (Pabalan et al., unpublished) and aggression has long been known to modulate differences in ovarian development in social insects (Roseler et al., 1980). Marking may have a physiological effect similar to that of an act of aggression among nestmates and marked bees may be more cooperative as a result. Marking individuals is a common component of field research on halictines and whether there is an effect of marking upon intranidal social interactions and, if so, how long this lasts is something we need to know about. It remains possible that marking of workers may have the long term effect of increasing the inhibition of their ovarian development and thereby the degree of physiological caste determination deduced by dissection or the degree of reproductive skew detected using genetic methods.

Acknowledgements

This work was supported by an NSERC research grant. I am grateful to Dr. Luisa Ruz, Sr. P. Araya and the late Haroldo Toro for greatly facilitating my studies in Chile. I am grateful to Jen Grixti, Bill Wcislo and an anonymous reviewer for commenting upon the manuscript.

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