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Autumnal helpers of *Polistes dominulus* represent a distinct behavioural phenotype

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Division of labour is a major feature of insect societies. Behavioural differences can be present also during non-colonial stages of the life cycle, when it is difficult to discriminate between distinct behavioural phenotypes and by-products of differences in overall activity levels. We used the social wasp *Polistes dominulus* to address this issue. In pre-hibernating aggregations some individuals (helpers) perform external tasks by collecting food and providing it to cluster mates. Such helpers have been so far identified using only a descriptive approach, and their behaviour was not disentangled from a possible higher level of overall activity. Here we provide an operational definition of the helper's trait and we then compare behavioural patterns of helpers and non helpers, verifying that helpers actually represent a peculiar behavioural phenotype. Our result expands knowledge on the caste differentiation issue in *Polistes* wasps and on the assessment of behavioural phenotypes in a non-colonial context.

Introduction

Variability, the substratum for natural selection to occur, is strikingly present at the behavioural level. Individuals can greatly differ in their behaviour, and we refer to the existence of behavioural phenotypes when different members of the same species systematically perform distinct repertoires of behaviour. The division of labour in insect societies is an outstanding example, with homocolonial and sometimes even clonal individuals performing very different tasks (Wilson 1971, Stern & Foster 1997). Identification of behavioural phenotypes could be however difficult in species lacking morphological castes and in non-colonial contexts.

Polistes dominulus presents a wide spectrum of alternative behavioural phenotypes while lacking morphological castes, thus representing a model species in understanding the social evolution (Pardi 1996, Starks & Turillazzi 2006). In the colonial context, reproductive activity is unequally partitioned between individuals in favour of the dominant female, while foraging and brood-related tasks are performed by subordinate foundresses and workers. However, a small percentage of workers show an alternative behavioural phenotype. Instead of helping their queen these individuals leave their natal nest and try to overwinter to found their own colonies in the following spring (Starks 2001). Moreover, a recent and unexpected finding is that social interactions are maintained in autumnal, pre-hibernating aggregations of these wasps, after the disruption of the colony organization and individual differences in behaviour have been highlighted also in these non-colonial contexts (Dapporto *et al*. 2005, Dapporto *et al*. 2006a). We focused on this recently discovered phenomenon to understand how behavioural phenotypes can be properly assessed in a non-colonial social context.

Pre-hibernation clusters are formed between September and November, just before winter diapause and are composed of dozens to hundreds of wasps originating from different nests (Pardi 1942, Starks 2003), which, at the end of the colony cycle, left the nest, mated, and aggregated in sheltered places. Autumnal aggregations have been long considered devoid of social interactions (Yoshikawa 1963) and composed of inactive wasps just leaving the shelter a few times during rare sunny days to forage (*see* Strassmann 1979, for a similar species, *Polistes fuscatus*). Observations by Dapporto *et al*. (2005) showed that wasps belonging to these clusters, rather than being "sleeping wasps", show a rich repertoire of the characteristic social behaviours usually observed in active colonies. Aggressions (chases, bites, and aggressive mounts), dominance interactions, requests for food and trophallaxis (exchange of liquid food by mouth to mouth contact) are performed at considerable rates and involve almost all the individuals (Dapporto *et al*. 2005, Dapporto *et al*. 2006b). The most relevant finding, however, is that it is possible to discriminate some individuals (helpers) showing high levels of foraging behaviour and food provisioning to other wasps in the cluster. The rest of the wasps spent most of their time in the aggregations, mainly interacting by dominating each other and asking for food from the helpers (Dapporto *et al*. 2005).

The time and place of these observations (pre-hibernating aggregations at the end of the colony cycle), along with the wasps' mating status (all helpers are mated), suggest that helpers are future foundresses (O'Donnell 1998), whereas their altruistic behaviour and their reduced chance to overwinter suggest that they are workers (Dapporto *et al*. 2005, for a detailed review *see* Dapporto & Palagi 2006). The occurrence and the assignment of individuals to phenotypic categories ("castes") is a much debated topic in *Polistes*, because of the slight or even absent morphological diversification and the high castal plasticity (O'Donnell 1998, Hunt 2006). Helpers in clusters may add an important contribution to clarifying the nature of castes in *Polistes* (Hunt & Amdam 2005) and to several other aspects of socioecology of this genus such as behavioural ontogeny (Dapporto *et al*. 2006a, 2006b), host parasite interactions (Dapporto *et al*. 2007) and a facial patterns role as quality signals (Cervo *et al*. 2008). The first aim of this paper is to verify whether helpers actually represent a real behavioural phenotype. The importance of this issue has been highlighted by Nonacs (2006), who explains that some results ascribed to a treatment effect in previous papers (Reeve & Nonacs 1992, 1997) should instead be attributed to a generalised treatment-related increase of wasps' activity. In their 2005 paper, Dapporto and co-workers used a descriptive approach, with results from multivariate analysis (Dapporto *et al*. 2005: fig. 1) showing that some individuals are characterized by high levels of foraging behaviour, giving trophallaxis and responding to requests for food by other individuals. This study however did not rule out the possibility that these wasps may simply be more active individuals, which perform the same set of behaviours as all wasps but at higher frequencies. However, in order to perform any quantitative comparison between helpers and non-helpers' behavioural traits it is necessary to unambiguously classify each individual to one of the two categories.

In order to address these questions, we perform quantitative behavioural observations on four pre-hibernation clusters and we performed novel analyses as compared with those in the Dapporto *et al* (2005) paper trying to (i) assess an operational method based on multivariate statistics to objectively assign each individual to the helper or non-helper category. Such an unambiguous distinction is not possible with the PCA-only method used in the first reports on this issue (Dapporto *et al*. 2005, Dapporto *et al*. 2006b). Following this discrimination between helpers and non helpers, we were finally able to (ii) evaluate if helpers so-defined are merely more active individuals or if they represent a really distinct behavioural phenotype, preferentially performing some behaviours. Finally, (iii) we assessed possible differences between helpers' behaviour in two diametrically opposite contexts, the "cluster area" (where wasps grouped together and spend the night) and the "foraging area" (where wasps, especially helpers, forage for food). If helping behaviour is simply an outcome of a generalised higher activity, one could expect to find no differences between the behaviours performed in the two contexts. On the contrary, we expect that different contexts (like presence of food at foraging site and presence of nestmates to feed in the cluster) could foster specific changes in behavioural patterns.

Materials and methods

Behavioural observations

In September 2005, we collected four aggregations of *P. dominulus* from Reggello (Florence, Italy) (R1, $n = 40$ wasps; R2, $n = 48$ wasps; R3, $n = 51$ wasps; R4, $n = 43$ wasps). We individually marked the wasps and confined them into four containers (50 × 50 × 50 cm) with three glass and two net sides (*see* Dapporto *et al*. 2005 for details). The containers were placed in an open gallery where temperature, humidity, and light exposure were similar to those in natural conditions. We supplied the wasps with water and sugar.

We selected seven types of behaviour according to Dapporto *et al*. (2006a, 2006b): ritualized dominance behaviour (RDB) performed and received, attacks (ATT) performed and received, trophallaxis (TRP) received and given, and foraging on sugar. In ritualized dominance behaviour (RDB), the dominant wasp climbs on and antennates the subordinate wasp, often asking for food by mouth-to-mouth contact. Trophallaxis (TRP) occurs when a wasp gives liquid food to another individual. Attacks (ATT) include lunges, bites, aggressive mounts, chases, falling fights, and stings. We also recorded if the interactions occurred in the vicinity of sugar or inside the aggregations. We recorded behaviour, actor, and receiver using the "all occurrences sampling" method, and we used the "scan sampling" method at 5-min intervals to record individuals foraging on sugar (Altmann 1974). The total time spent in the foraging area by each wasp can be reasonably calculated by multiplying the times a wasp was recorded at the foraging site per 5 min. Similarly the total time of observation minus the time spent in the foraging area gives the time spent in the cluster area. We observed wasps from 10:00 to 16:00 between 5 September and 1 November during warm and sunny days, performing a total of 85 hours of observations of the four aggregations (R1 = 28h, R2 = 21h, R3 = 16h, R4 = 20h).

Statistical analyses

The statistical methods we used to separate helpers from non helpers were as follows:

1. For each aggregation we performed *k*-means clustering on the hourly frequencies of all the behaviour types, imposing the formation of two clusters.
2. We performed a Principal Component Analysis (PCA) on the hourly frequencies of the seven selected behaviours (Dapporto *et al*. 2005) on each aggregation separately. This allowed us to identify the presence of some individuals performing high rates of foraging.
3. In the PCA graphs, we identified the wasps belonging to the two clusters obtained with *k*-means. By comparing the two analyses it was possible to check for the effectiveness of the *k*-means method in classifying the individuals showing high levels of foraging rate, trophallaxis given, and RDB received, which in previous works were considered helpers.

We applied this method on the four aggregations analysed here, and also on three aggregations already studied in previous years (where discrimination was based on the PCA alone) in order to evaluate if the two methods lead to comparable results. The distribution of some variables did not follow a normal distribution, since the presence of a few individuals showing particularly high levels of activity resulted in a highly asymmetric distribution with many outliers when data were transformed. Nonetheless, the Principal Component Analysis and *k*-means clustering can be used as exploratory techniques with descriptive purposes only. Moreover, Dudzinski *et al*. (1975) showed that if there is enough meaningful structure in the population, then PCA of samples with even non-normal distributions may provide answers of comparable reliability to PCA performed on samples with normal distributions. Finally, the high values of Kaiser-Meyer-Olkin (KMO > 0.7 for each aggregation, *see* below), indicated that the four samples are adequate for the analysis. However, since the non-multivariate normality of some samples could affect the precise factor loadings and the precise *p* values, they should be considered here in an indicative way only.

To evaluate differences between helpers and non helpers in activity levels and behavioural patterns we performed: (1) comparisons in hourly frequencies of each behaviour, and (2) comparisons between normalized indices of occurrence for each behaviour. This index was computed by dividing (for each wasp and each behaviour) the frequency of the behaviour type by the sum of the frequencies of all the types. For example, "normalized attack performed" for a certain wasp was calculated as:

$$\frac{\mathrm{ATT_P}}{\mathrm{ATT_P + RDB_P + TRP_R + ATT_R + RDB_R + TRP_G}},$$

where P, R and G respectively denote performed, received, and given.

Table 1. Types of behaviour characteristic of helpers in the four aggregations (R1, R2, R3 and R4) as indicated by *k*-means and PCAs. + = types used as PCs of each aggregation (loading cut-off value = 0.5); # = types used in *k*-means to classify individuals into helpers and non helpers.

Behaviour	R1	R2	R3	R4
Attack performed		+		
Attack received	+		+	+
Trophallaxis received		+		
RDB performed	+			
RDB received	+#	+#	+#	+#
Trophallaxis given	+#	+#	+#	+#
Sugar foraging	+#	+#	+#	+#

To evaluate differences in the behaviour of helpers between the two opposite conditions (foraging and cluster area) we created a similar series of indices for each context, dividing the occurrence of each behaviour performed in a certain context by the sum of the occurrences of all the behaviours performed in the same context. We compared behavioural patterns using a Mann-Whitney *U*-test, and mortality rates using a χ^2-test.

Results

In each aggregation examined, we observed all the behaviour types under study. As shown by Dapporto *et al*. (2005), a few individuals performed many interactions, were often present on sugar, and provisioned food to others as they return to the aggregations. *k*-means performed on the behaviour types classified all wasps into two groups on the basis of the same behaviours (RDB received, trophallaxis given, and food foraging) in all four aggregations (Table 1). For each aggregation PCA gave comparable results for half of the behaviour. From the seven behavioural variables, PCA extracted two PCs for each aggregation (for R1: KMO = 0.832, explained variance = 74.71%; for R2: KMO = 0.777, explained variance = 75.68%; for R3: KMO = 0.725, explained variance = 74.58%; for R4: KMO = 0.760, explained variance = 76.60%). The behaviour types involved in PCs were not exactly the same for different aggregations, but the first PC (explaining the largest amount of variance) always comprised trophallaxis given, RDB received, and sugar foraging (Table 1). Combining the *k*-means results with the PCA graphs, in each aggregation we identified some individuals that can be defined as helpers. Indeed, the PCA confirmed that the cluster so defined by *k*-means, effectively grouped wasps showing high levels of RDB received, sugar foraging, and trophallaxis given. The comparison revealed a high correspondence with few exceptions (Fig. 1). Using this method we classified as helpers about 12% of the wasps in each pre-hibernationg aggregation (R1 = 6 of 43 total; R2 = 10 of 67 total; R3 = 5 of 61 total; R4 = 8 of 68 total). Finally, by re-analyzing with this method the three aggregations already studied by Dapporto *et al*. (2005) we obtain nearly concordant results. Our method classified as helpers three of the four wasps considered helpers in the Cavriglia aggregation ($n = 48$) in the Dapporto *et al*. (2005). In Reggello 1 aggregation ($n = 82$) of the same study, all the four helpers were recognised together with two individuals indicated as uncertain with the preceding method. In the third aggregation (Reggello 3, $n = 87$) the method failed to identify the two helpers highlighted by the PCA-only method. Indeed, *k*-means created two clusters on the basis of dominance and attack performed. Actually, helping behaviour in this cluster was not the main determinant of the behavioural variance among individuals, since the typical behaviours of helpers were involved in the third principal component (PC3) and not in the one explaining the largest amount of variance (PC1) as in all the other aggregations.

Helpers, as identified by *k*-means, were more active than non helpers, showing higher hourly frequencies of all the behaviour types analysed (Table 2). When the frequency of each behaviour was normalized by the total activity of wasps, only some types showed a significant difference between helpers and non helpers. In particular, helpers gave more trophallaxis, performed more attacks, and received more RDB, while non helpers showed higher levels of trophallaxis received and performed more RDB. Proportion of attack received did not differ between the two groups (Table 2).

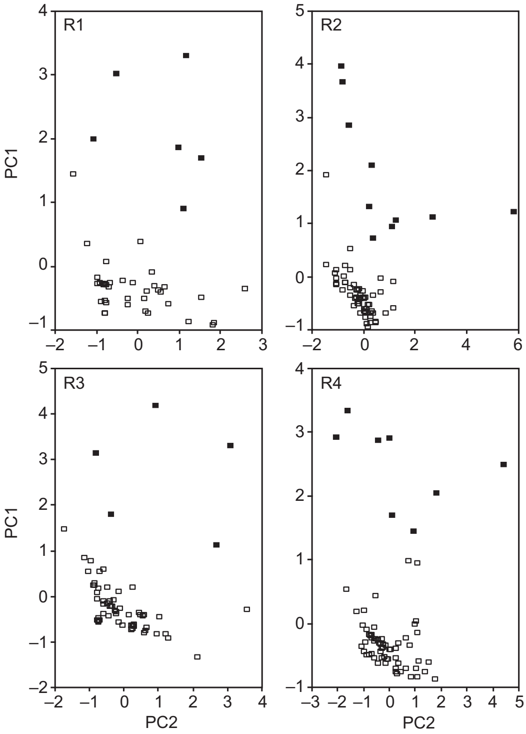

Fig. 1. Principal Component Analyses of the four aggregations (R1, R2, R3, R4). Black and white squares represent the two clusters of wasps identified by *k*-means. By comparing of PCA and *k*-means results "black" individuals can be reasonably classified as helpers.

When helpers' behaviour was compared between the two different situations (foraging and cluster area), differences were found in some

Table 2. Medians and interquartile ranges (in parenthesis) of hourly frequencies of the behaviour types for helper and non-helper wasps of the four aggregations. Wasps were attributed to the helper or non helper group on the basis of *k*-means results. Significantly higher values are set in boldface.

Behaviour	Helpers ($n = 30$)	Non helpers ($n = 207$)	Mann-Whitney test
Trophallaxis given	**0.6190 (0.76)**	0.0000 (0.04)	$U = 1953$, $p < 0.01$
Trophallaxis received	**0.1722 (0.23)**	0.0769 (0.12)	$U = 358.5$, $p < 0.001$
Attack performed	**0.5179 (0.72)**	0.0385 (0.14)	$U = 802.5$, $p < 0.001$
Attack received	**0.4762 (0.45)**	0.0769 (0.15)	$U = 788$, $p < 0.001$
RDB performed	**1.0833 (1.19)**	0.4231 (0.56)	$U = 1393$, $p < 0.001$
RDB received	**2.3036 (0.23)**	0.3077 (0.40)	$U = 342$, $p < 0.001$
Sugar foraging	**4.4119 (2.63)**	0.1768 (0.81)	$U = 4$, $p < 0.001$
Normalized trophallaxis given	**0.1116 (0.08)**	0.0000 (0.02)	$U = 580$, $p < 0.001$
Normalized trophallaxis received	0.0269 (0.05)	**0.0690 (0.11)**	$U = 2121.5$, $p < 0.01$
Normalized attack performed	**0.1096 (0.11)**	0.0333 (0.10)	$U = 1722$, $p < 0.001$
Normalized attack received	0.0797 (0.09)	0.0690 (0.11)	$U = 2745$, $p > 0.05$
Normalized RDB performed	0.2474 (0.17)	**0.3750 (0.23)**	$U = 1477.5$, $p < 0.001$
Normalized RDB received	**0.4158 (0.18)**	0.3030 (0.30)	$U = 2298$, $p < 0.05$

types (Table 3). Namely, when at the foraging site, helpers gave and received relatively more attacks than when in the cluster area. On the contrary, while in the cluster area, helpers showed higher numbers of trophallaxis given and RDB-received as compared with those at the foraging site. Trophallaxis-received and RDB-given levels did not differ between the two contexts (Table 3).

At the end the winter about 52% of wasps in the four aggregations died. The mortality rate strongly differed between helpers and non helpers ($\chi^2 = 7.203$, df = 1, $p < 0.01$), with the survival rates of the former being around 14% and that of the latter being around 59%.

Discussion

Our results show that some wasps inside the aggregations can be univocally classified as helpers by using a proper statistical approach. The *k*-means clustering largely agrees with the PCA results, thus indicating that this method actually captures the phenomenon described by Dapporto *et al*. (2005). Wasps recognised as helpers through this classification show higher overall levels of activity than non helpers, but at the same time they perform certain behaviours at particularly higher or lower levels as compared with other individuals. They thus cannot be considered just particularly active wasps, since they preferentially perform a particular set of actions that are exactly those expected to be the idiosyncratic behaviours of the helpers as first described by Dapporto *et al*. (2005) (e.g., higher levels of dominance received and trophallaxis given, lower levels of dominance performed and trophallaxis obtained).

This specificity seems to be evident not only by differences at the inter-individual level (helpers *vs*. non helpers) but also at the intra-individual one. Indeed, helpers show a different pattern of social behaviour in two different contexts (the foraging and the cluster area). In the foraging area, helpers mainly interact with each other, as non helpers rarely leave the cluster area. Here the helpers show a higher relative frequency of aggressions as compared with that in the cluster area, where instead dominance received and trophallaxis given are more frequent. The rise in aggressions in the foraging area is probably due to competition for the resources, while, when helpers meet their cluster mates in the aggregation, the altruistic traits prevail. This is a further evidence that helpers posses a distinct and context-dependent behavioural phenotype. Therefore, the classification method we assessed, actually permitted the separation of wasps in aggregations into two different phenotypes roughly represented by selfish and altruistic individuals. Analytical methods used previously (the "PCA only" method), leave some room for uncertainty and did not allow obtaining a clear binary discrimination using an objective criterion.

Taking into account that helpers rarely survive through winter, this classification seems to mirror the separation between workers and queens of the nesting phase. Indeed, helpers show a worker-like behaviour, since they leave the cluster to forage, and provide cluster mates with liquid food upon return to the aggregation. This study thus adds to the much-debated topic of occurrence, differentiation degree, and discrimination of a worker caste in *Polistes* wasps (Strassmann 1985, Mead & Gabouriaut 1993, O'Donnell 1998, Hunt & Amdam 2005). Indeed,

Table 3. Median and interquartile range (in parenthesis) of hourly normalized frequencies of the behaviour types showed by helpers ($n = 30$) of the four aggregations in the two different contexts: foraging area and cluster area. Significantly higher values are set in boldface.

Behaviour	Foraging area	Cluster area	Wilcoxon signed rank test
Trophallaxis given	0.0000 (0.06)	**0.1376 (0.10)**	$Z = -4.054$, $p < 0.001$
Trophallaxis received	0.0000 (0.04)	0.0277 (0.05)	$Z = -1.490$, $p > 0.05$
Attack performed	**0.1390 (0.21)**	0.0783 (0.11)	$Z = -3.189$, $p < 0.01$
Attack received	**0.1581 (0.16)**	0.0614 (0.07)	$Z = -4.021$, $p < 0.001$
RDB performed	0.1999 (0.30)	0.1978 (0.50)	$Z = -0.504$, $p > 0.05$
RDB received	0.3333 (0.36)	**0.4296 (0.15)**	$Z = -2.952$, $p < 0.01$

Polistes wasps do not present a clear morphological characterization, and often size differences between queens and workers gradually disappear as the season progresses and larger "late" workers emerge (West-Eberhard 1969, Turillazzi 1980, Miyano 1990). However, some reliable differences seem to occur in physiological traits. Hunt *et al*. (2003) found that *P. fuscatus* females emerging in August (and thus presumably future foundresses) posses a storage protein of the hexamerin family that is not found in females emerging earlier in the colony cycle (workers). The development time for the two larval categories also differs, and the switch between these two possible developmental pathways is probably triggered by differences in larval nourishment (Hunt *et al*. 2007). This evidence seems to support the hypothesis of the evolution of two different castes in *Polistes* from the physiological ground plan that would have been apparent in a bivoltine ancestor as proposed by Hunt and Amdam (2005). They presented an individual-based model that intriguingly predicted the existence of a small number of "late workers" (having characteristics of the first generation of a bivoltine solitary wasp) emerging at the end of the season together with future foundresses (with characteristics of the second generation of a bivoltine solitary wasp), and the authors suggested that these "late workers" might become helpers in the aggregations.

In conclusion, the present study provides a definitive evidence that two distinctly different behavioural phenotypes occur in the autumnal aggregations. Similarly to the nest situation, where queens and workers do not greatly differ from a morphological perspective while behaving very differently, helpers and non helpers in autumnal clusters of *Polistes dominulus* show a marked behavioural allometry even though no evident morphological differences can be found. Based on these results, the nature and the real meaning of this just unravelled phenomenon should be further investigated, which could give researchers useful and innovative insights on fundamental topics such as the basis of social behaviour and caste differentiation in *Polistes* wasps.

Moreover, this finding raises the question of the evolutionary significance of one of the many alternative behavioural phenotypes showed by *Polistes dominulus*. The "helper" phenotype is indeed present in a non-colonial context, where wasps coming from different colonies coexist and melt their recognition cues (Dapporto *et al*. 2004). It follows that the help could also benefit unrelated individuals (Dapporto & Palagi 2006). A deep investigation of physical and physiological traits related to the "helper" phenotype and of the social determinants of helpers' acts is thus necessary to evaluate proximate and ultimate causes of this behavioural phenotype.

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