# Workers of a *Polistes* Paper Wasp Detect the Presence of Their Queen by Chemical Cues

## Leonardo Dapporto[1], Antonio Santini[1], Francesca R. Dani[2] and Stefano Turillazzi[1,2]

[1]Dipartimento di Biologia Animale e Genetica ''Leo Pardi,'' Università di Firenze, via Romana 17, 50125 Firenze, Italy and [2]Centro Interdipartimentale di Spettrometria di Massa, Università di Firenze, Vle Pieraccini 6, 50139 Firenze, Italy

*Correspondence to be sent to: Leonardo Dapporto, Dipartimento di Biologia Animale e Genetica "Leo Pardi," Università di Firenze, via Romana 17, 50125 Firenze, Italy. e-mail: leondap@gmail.com*

## Abstract

Differences in long-chain hydrocarbon mixtures among reproductive and nonreproductive individuals have been often revealed in social insects. However, very few papers demonstrated that these signatures actually act as contact pheromones used by nonreproductive to recognize the presence of a related queen in the colony. Cuticular and glandular hydrocarbons of *Polistes* paper wasps have been extensively studied, but, until now, the perception and recognition of such cues was not demonstrated. In this paper, we show, for the first time in Vespidae, that *Polistes gallicus* workers distinguish nestmates from alien individuals and queens from workers by the hydrocarbon mixtures of the Van der Vecht organ secretion (VVS). We also demonstrated that stroking behavior (a peculiar behavior of *Polistes* by which queens probably lay VVS on the nest) acts as an inhibitor of ovarian development in workers.

**Key words:** bioassay, chemical communication, gas chromatography/mass spectrometry, hydrocarbons, queen recognition, Van der Vecht organ

## Introduction

Reproductive division of labor is one of the key characteristics of eusociality. Typically, when one or more queens are present in a eusocial insect colony, workers refrain from reproduction, but when queens disappear, workers usually start to lay their own eggs (Wilson 1971). In queenright colonies, reproductive division of labor is also a matter of communication between individuals: in primitively eusocial insects, with small societies, it is generally assumed that worker ovarian suppression is regulated by queen behavior, whereas in the enormous colonies of highly eusocial species, this regulation is based on pheromones emitted by the queen (Wilson 1971; Vandermeer et al. 1998). In many social insects, chemical differences between queens and workers in potential pheromonal secretion have been found; however, the definitive demonstration of their actual perception and functional importance as caste signals has been demonstrated in only very few species (Keller and Nonacs 1993; Monnin 2006). The hypothesis of queen suppression of worker reproduction by pheromones seems to suffer from some flaws; in fact, it can be predicted that, without any indirect fitness benefit, workers should develop immunity to queen pheromones

over evolutionary time (Keller and Nonacs 1993). According to kin selection theory, the queen's pheromones could instead act as honest signals of dominance or fertility that keep workers informed of how they can maximize their fitness by helping the related queens (Hamilton 1964a, 1964b; Keller and Nonacs 1993; Monnin 2006; Dapporto, Dani, and Turillazzi 2007). That is, workers (or subordinate individuals) can assess if it is more rewarding for them to gain indirect fitness or to attempt direct reproduction.

In *Polistes* paper wasps, the simple presence of the dominant female (and thus of her pheromones) is not sufficient to suppress worker and subordinate reproduction (Liebig et al. 2005; Dapporto, Dani, and Turillazzi 2007), and several factors probably interact together in determining worker decisions. Among these we note that behavioral aggression by dominant, egg-laying individuals (Pardi 1942, 1946), the presence of eggs in the nest cells (Liebig et al. 2005; Dapporto, Dani, and Turillazzi 2007), and individual recognition mediated by visual cues (Tibbetts 2002; Tibbetts and Dale 2004) have been demonstrated to play a role in molding the colonial reproductive skew.

Moreover, great attention has been given, in the last 20 years, to the pheromonal role of the hydrocarbon blends on the cuticle surface (cuticular hydrocarbons, CHCs) and of some glandular secretions in social insects. The complex mixtures of these compounds have been often demonstrated to act as contact pheromones involved in recognition processes (Howard 1993; Lorenzi et al. 1996; Vandermeer et al. 1998; Howard and Blomquist 2005; Dani 2006; Monnin 2006). In fact, insects are able to perceive slight qualitative and quantitative differences in hydrocarbon mixtures, and these, due to their complexity, potentially retain a large amount of information for the recognition of species, kin, reproductive and health status, colony membership, etc. (Breed 1998; Dani et al. 2001, 2005; Howard and Blomquist 2005; Dani 2006; Dapporto, Cini, et al. 2007; Dapporto, Dani, and Turillazzi 2007).

The hydrocarbon mixtures present on the cuticle and in some glandular secretions of paper wasps (in particular those belonging to the genus *Polistes*) have been extensively studied (reviewed in Dani 2006); however, even if several researches have demonstrated the occurrence of neat differences in hydrocarbon composition between queens and workers and between dominants and subordinates in the common species *Polistes dominulus* (Bonavita-Cougourdan et al. 1991; Sledge et al. 2001; Dapporto et al. 2004, 2005; Dani 2006; Dapporto, Dani, and Turillazzi 2007), there is still no evidence that the wasps are actually able to perceive these differences and no information regarding their actual function in the social economy of the colony.

*Polistes* paper wasps present a particular behavior called "stroking": females (mostly the dominant ones) "stroke" their abdomen on the nest paper, probably leaving some glandular secretion on it (Dani et al. 1992; Van Hooser et al. 2002). In accord with this hypothesis, the frequency of stroking by subordinates increases after dominant females have been removed from the colony, and, similarly, the frequency of stroking of the dominants increases when they are reintroduced into the colony (Dani et al. 1992; Van Hooser et al. 2002). These authors agree in suggesting that the nest is marked with colony- and queen-specific pheromones through stroking behavior.

Due to its anatomical position and to its structure, the Van der Vecht organ (VdV) is the best candidate to be the source and dispenser of the secretion deposited during nest marking by stroking. The VdV organ is a hairy, slightly sclerotized cuticular area placed at the anterior edge of the last gastral sternite of the females in species belonging to the vespid subfamilies Polistinae and Vespinae. The ducts of numerous tegumental glands are clustered in 2 lateral masses and open on the cuticle of this area (Turillazzi 1979). VdV organs have been demonstrated to be involved in the production and application of ant-repellent secretions on the nest pedicel in various species (London and Jeanne 2000). Post et al. (1984) found methyl hexadecanoate in the VdV organ secretion (VVS) of the North American *Polistes fuscatus*, whereas Espelie and Hermann (1990) found hexadecanoic and octa-

decenoic acid in the VVS of *Polistes annularis*. Dani et al. (1995) analyzed the tegumental glands of the sixth (VdV) and fifth gastral sternite of the European *P. dominulus* and of its social parasite *Polistes sulcifer*. They found only 7 long-chain carboxylic acids (4 of which were also present in the social parasite), but in a more recent study, performed with a different sampling technique (the same as that used in the present paper), they found that the secretion collected directly from the VdV organ presented hydrocarbons similar to the cuticular ones and only very small amounts of palmitic acid in some samples; moreover, they found the same carboxylic acids to be present in a variety of tissues other than the glands (Dani et al. 2003). From their studies, these authors concluded that the secretions produced by the glands associated with the sixth and fifth sternite are substantially the same and are constituted by a mixture of hydrocarbons. Moreover, they demonstrated that any compound containing an aliphatic long chain and being liquid at room temperature deters ants from walking on it, so that the presence of a specific functional group seems irrelevant (Dani et al. 2003).

To verify whether the VVS is also implied in nestmate and queen recognition by workers, we choose *Polistes gallicus* as a model species. This species, quite common in Mediterranean countries, is always characterized by solitary nest foundation and by a clearly identifiable worker caste, at least for a large part of the postemergence phase (Dani 1994). If the VVS is deposited, during stroking behavior, on the nest paper to leave information about colony ownership and about the presence of the queen, we expect that 1) chemical differences occur between the VVS of wasps belonging to different colonies and between the VVS of queens and workers of the same colony, 2) workers should distinguish between VVS of nestmate and alien individuals and of queens and workers, and 3) the frequency of the stroking behavior performed by the queen should influence the ovarian development of workers. In order to verify these predictions, we coupled behavioral experiments and gas chromatography/mass spectrometry (GC/MS) analyses of the secretions collected from the VdV organs of the foundresses and workers from different colonies.

## Materials and methods

### Species studied

*Polistes gallicus* is a quite common countryside species in Mediterranean regions. It forms nests preferentially on low shrubs or on man-made structures, and its colonies are initiated in March–April by single foundresses. Pleometrosis is quite rare in this species in comparison with the sympatric *P. dominulus*. First workers to emerge are small in comparison with the foundress, and size differences between workers and reproductives continue to be evident even toward the end of the colony cycle (Dani 1994).

## Chemical analyses

As a first step in demonstrating the effectiveness of VVS in the chemical communication of paper wasps, we searched for chemical differences in their composition between queens and workers (Breed 1998). We collected 7 preemergence colonies of *P. gallicus* in Tuscan countryside (Central Italy). The colonies were housed in glass cages ($15 \times 15 \times 15$ cm) in the laboratory in Florence and provided with honey, water, paper (as nest-building material), and fly maggots. After the emergence of the fifth worker, all foundresses and one worker per nest (14 individuals in total) were killed in a freezer and sampled for VVS by rubbing a fiber (7 μm polydimethylsiloxane by Supelco, Bellefonte, PA) for solid-phase micro extraction (SPME) on their VdV organ with a procedure similar to that used by Dani et al. (2003). Orphaned colonies were then left undisturbed for a 5-day period; afterward all workers were removed and frozen and their VVS sampled by SPME. We finally dissected all workers (28 individuals in total) to determine the state of their ovarian development. We divided workers into 3 categories depending on the size of their ovaries (undeveloped, slightly developed, and markedly developed).

SPME fiber was directly injected into a Hewlett Packard (Palo Alto, CA) 5890A gas chromatograph coupled with an HP 5971A mass selective detector. A fused silica capillary column coated with 5% diphenyl to 95% dimethyl polysiloxane (Rtx-5MS, 30 m $\times$ 0.25 mm $\times$ 0.5 μm; Restek, Bellefonte, PA) was used in the GC analysis. The injector port and transfer line were set at 280 °C, and the carrier gas was helium (at 12 psi). The temperature protocol was 70–150 °C at a rate of 30 °C/min (held for 5 min) and 150–310 °C at 5 °C/min (held for 11.3 min). Analyses were performed in splitless mode. Identification of cuticular compounds was performed on the basis of their mass spectra produced by electron impact ionization (70 eV).

In order to avoid the complications inherent in compositional data analysis, the relative abundance of each compound to be considered in the analysis was calculated according to the formula (Aitchison 1986)

$$Z = \ln[\text{Ap}/g(\text{Ap})],$$

where Ap is the area given by each compound (or by more compounds if they coeluted) and $g(\text{Ap})$ is the geometric average of all considered chromatographic peaks.

First, we directly compared the magnitude of influence of caste, ovarian development, and colony membership on the single VVS hydrocarbons by general linear model (GLM). Further, we performed principal components analysis (PCA) that allows the identification of groups of covarying compounds and to transform their percentages in new variables (principal component [PC]). The PCA allowed us to reduce the number of variables subsequently considered in a discriminant function analysis (DFA), thus increasing the reliability of the discriminant functions (Sledge et al. 2004). Moreover, it highlights any instance where some compounds vary together according to wasp characteristics (caste, ovarian development, and colony membership) by comparison with other analyses (GLM and DFA; Sledge et al. 2004). We retained PCs showing eigenvalues higher than 1. Bartlett's test was used to check for homogeneity of variances, and Kaiser–Meyer–Olkin (KMO) test was used to measure sampling adequacy. Obtained components were varimax rotated. DFA was applied to PCs to determine whether the predefined groups of wasps (foundresses, workers before removal of foundresses, and fertile and unfertile workers after removal) could be discriminated on the basis of their cuticular profiles, as well as which PCs were relevant to discrimination. The significance of Wilks' λ and the percentage of correct assignments were used to evaluate the validity of the DFA. To understand if the absolute quantity of VVS collected by SPME fibers from queens and workers differs, we compared by Mann–Whitney test the sum of the peak areas obtained by GC/MS.

## Perception of VVS differences

To verify if workers actually perceive differences in chemical mixtures, we performed simple experiments involving VVS presentation. In a queenright colony, however, workers are not expected to react differently to the nestmate queen compared with nestmate workers as they generally live together peacefully. However, Röseler (1991) demonstrated that if *P. dominulus* queens are removed from their nests for a few days, and then reintroduced into their colony, nestmate workers and former subordinates react aggressively trying to drive them off the colony. Thus, we conducted our experiments in colonies both when the queen had been absent for few minutes and when the queen had been absent for 5 days in order to enhance worker reaction.

We collected 30 preemergence foundations of *P. gallicus*, 10 colonies in May 2004 and 20 colonies in May 2005. We performed experiments only after at least 5 workers were present in the colonies. We removed the queen and one worker from each colony. During each experiment, we presented pairs of glass capillaries previously rubbed on the VdV organ of the previously removed queens and workers. The 2 capillaries were mounted on a *Y*-shaped rod and presented contemporaneously to colony members. Presentations lasted 6 min in total, after 3 min from the beginning of the experiment, the positions of the 2 capillaries were inverted with a 180° rotation of the rod stem to avoid position effects. During presentation, the rod was gently vibrated because we observed that this stimulated more attention by the wasps. We performed 2 kinds of experiments: 1) nestmate recognition and 2) caste recognition. For nestmate recognition experiments, we presented to 15 colonies pairs of capillaries that had been rubbed on a nestmate and on an alien worker. For caste recognition experiments, we performed 30 presentations with 2 capillaries applied with VVS from the queen and from the worker belonging to the tested colony and 30 presentations with

capillaries applied with VVS obtained from individuals belonging to alien colonies a few hours after queen removal. On 27 colonies, we repeated the same experiments 5 days after the queen and the worker had been removed when the wasps to which the stimuli were presented should be considered as orphaned workers.

All experiments were performed blind and video recorded in order to ensure standardized interpretations of the wasps' responses. The responses of host individuals were scored as the number of acts of biting and stinging toward the 2 different capillaries and normalized by the number of individuals present on the nest. We used the Wilcoxon signed-rank test to search for differences in the responses toward the pairs of presented stimuli.

### Function of the stroking behavior in workers ovary suppression

To verify if queen stroking behavior influences the ovarian development of workers, we collected 37 postemergence colonies of *P. gallicus* in July 2005. We recognized queens by their body size (Dani 1994) and by the high frequency of dominance behavior. After removing all the eggs from the combs, we cut each nest into 2 parts and we placed the 2 halves into separate glass cages ($15 \times 15 \times 15$ cm). We placed the queen into a half nest cage and the workers into the other one, obtaining therefore a solitary queen colony and an orphaned colony. Every morning between 900 and 1100 h, we removed workers from all the orphaned colonies for 2 h and reintroduced them afterward. During this timespan, we replaced workers with their former queens in 16 experimental orphaned colonies, whereas we left uninhabited the nests of 21 control colonies. We removed the eggs laid by queens in this timespan. The queen stroking frequency was registered, and after 2 h, the queens were returned to their own cages. Every day at 8:00 AM, we checked the nest cells of experimental and control orphaned colonies for the presence of worker-laid eggs.

For experimental and control orphaned colonies, we compared the influence of 2 different factors on the number of days from colony division and the day when workers started to lay their first eggs: 1) the presence or absence of queens, for 2 h a day, in experimental versus control colonies and 2) the queen stroking frequency. We tested the relative importance of these 2 factors through a GLM. To fit the assumption of GLM, we log transformed the frequencies of queen stroking behavior according to the formula $\log Sf = \log (Sf + 1)$, where $Sf$ = queen hourly stroking frequency.

## Results

### Cuticular compounds

The total amount of compound detected in the VVS of queens ($n = 7$) and workers ($n = 7$) by GC/MS did not differ significantly (Mann–Whitney $U$-test $U = 17.00$, $P = 0.383$).

This indicates that in the bioassays, we presented a similar quantity of VVS from the 2 castes.

The chemical mixture secreted by the *P. gallicus* VdV organ was dominated by a series of linear, monomethyl- and dimethyl-branched alkanes with the main chain ranging from 25 to 33 carbon atoms; we did not detect alkenes. The compounds present in greatest quantities were linear and monomethyl-branched alkanes with odd-numbered carbon chains. Dimethyl-branched alkanes were present in much lower quantities.

GLM revealed that 14 out of the 21 considered compounds showed quantitative differences among colonies, 9 showed differences between queens and workers, whereas no compounds showed a correlation with ovarian development (Table 1). Moreover, among the compounds showing significant differences between queens and workers, only one (*n*-C27) revealed caste/colony interactions, thus demonstrating that the differences between queens and workers are similar throughout the colonies.

PCA extracted 5 PCs with eigenvalues higher than 1 (Table 1) that explained 82.92% of the total variance with a KMO of 0.563. PC1 was negatively represented by 2 light linear alkanes and positively by one alkane, one methyl-branched alkane, and 3 heavy dimethyl-branched alkanes. Five of these 7 compounds showed individual differences for caste membership in GLM (Table 1), suggesting that this PC (explaining 30.93% of the total variance) grouped together most of the caste-dependent compounds; PC2 was negatively represented by 2 light linear alkanes and positively by 2 monomethyl alkanes and by 4 dimethyl alkanes. Five of these compounds showed significant differences among colonies and only one to caste, thus suggesting that this PC (explaining 23.67% of variance) grouped compounds implied in nestmate recognition. PC3 (explaining 13.23% of variance) is positively represented by 3 peaks formed by a mix of central-branched monomethyl alkanes and by one peak represented by one dimethyl alkane. Two of these 4 peaks vary between castes in GLM. PC4 is positively represented by one central-branched methyl alkane and by one dimethyl alkane both differing among colonies. PC5 is negatively represented by one linear alkane and one methyl-branched alkane and positively by one methyl-branched alkane; the linear alkane differs among colonies. To understand the dynamics of worker chemical profiles, we performed DFA on 5 groups of females (queens $n = 7$, workers in queenright colonies $n = 7$, and orphaned workers with 1) well-developed ovaries $n = 5$, 2) slightly developed ovaries $n = 11$, and 3) undeveloped ovaries $n = 12$) using the 5 PCs as predictors. The chemical profiles of queens and workers are clearly distinguishable before the removal of the queens on the basis of their CHC profile (100% correct classification, function 1: Wilks' $\lambda = 0.081$ $P < 0.001$, explaining 100% of variance) (Figure 1). As predicted by the previous GLM and PCA analyses, queens and workers have been discriminated on the basis of their PC1, PC2, and PC3 regression scores. Using

**Table 1** Analysis of variance *F* and *P* values obtained by GLM in searching for influences of caste, ovarian development, and colony membership on the percentages of each peak (representing one or more hydrocarbons) detected by GC/MS in VVS

| | Compound | GLM *F* for caste | GLM *F* for ovarian development | GLM *F* for colony | PC1 e.v. 30.93% | PC2 e.v. 23.67% | PC3 e.v. 13.23% | PC4 e.v. 8.42% | PC5 e.v. 6.66% |
|---|---|---|---|---|---|---|---|---|---|
| 1 | *n*-C25 | 8.984** | 0.327 | 9.871*** | −0.891 | | | | |
| 2 | *n*-C27 | 12.331** | 0.890 | 12.081*** | −0.768 | | | | |
| 3 | c-meC27 | 2.497 | 0.344 | 7.315*** | | −0.866 | | | |
| 4 | 5-meC27 | 1.799 | 0.167 | 5.596** | | −0.642 | | | |
| 5 | 3-meC27 | 10.156** | 0.718 | 2.020 | | | | | 0.540 |
| 6 | 2-meC28 | 0.572 | 1.702 | 1.606 | | | | | −0.607 |
| 7 | *n*-C29 | 0.382 | 2.384 | 2.884* | | | | | −0.777 |
| 8 | c-meC29 | 0.096 | 0.152 | 1.187 | | | 0.864 | | |
| 9 | 5-meC29 | 3.448 | 0.058 | 2.251 | 0.783 | | | | |
| 10 | 9,19-dimeC29 | 0.098 | 0.472 | 7.250*** | | 0.833 | | | |
| 11 | *n*-C30 | 54.153*** | 0.111 | 11.775*** | 0.877 | | | | |
| 12 | c-meC30 | 0.033 | 0.215 | 1.831 | | | 0.736 | | |
| 13 | 9,19-dimeC30 | 3.790 | 0.204 | 2.665* | | 0.955 | | | |
| 14 | c-meC31 | 11.460** | 0.613 | 2.699* | | | 0.900 | | |
| 15 | 11,17/9,19-dimeC31 | 9.145** | 0.497 | 2.378 | | 0.906 | | | |
| 16 | 5,y-dimeC31 | 1.869 | 0.049 | 2.417 | 0.696 | | | | |
| 17 | 3,13-dimeC31 | 4.871* | 0.596 | 8.291*** | 0.699 | | | | |
| 18 | c-meC32 | 2.294 | 1.009 | 9.880*** | | | | 0.820 | |
| 19 | 11,19-dimeC32 | 0.015 | 0.065 | 5.284** | | 0.708 | | | |
| 20 | 13,15/9,11-dimeC33 | 10.411** | 0.388 | 8.812*** | | | 0.697 | 0.617 | |
| 21 | 11,21-dimeC33 | 11.883** | 0.173 | 5.477** | 0.805 | | | | |

Factor loadings (with varimax rotation) for each of the PC. Only loadings >0.5 are shown; e.v., explained variance. The peaks indicated as "c-me" (central-methyl) represent a mix of branched alkanes with methyl in positions from 9 to the center of the chain.
*$P < 0.05$.
**$P < 0.01$.
***$P < 0.001$.

the same discriminant function that separated these groups, we then included orphaned workers in the analysis. All the orphaned workers were classified to the worker group, independently from their ovarian development, with the exception of one single fertile worker who was assigned to the queen group (Figure 1).

## Recognition of chemical signatures of VdV organ secretions

### Nestmate recognition experiment

*Polistes gallicus* workers reacted more aggressively toward glass capillaries applied with VVS belonging to alien individuals than to those belonging to nestmates (Wilcoxon signed-rank test: $n = 15$, positive ranks = 13, ties = 1, $Z = −3.235$, $P = 0.001$).

### Caste recognition experiments

*Polistes gallicus* workers reacted with comparable aggressiveness toward glass capillaries applied with VVS belonging to their queen and worker nestmates when queen had been removed from the nest only a few hours in advance (Wilcoxon signed-rank test: $n = 30$, positive ranks = 18, ties = 1, $Z = −1.027$, $P = 0.304$). However, 5 days after queen removal, former nestmate workers reacted more aggressively toward queen VVS with respect to worker VVS (Wilcoxon signed-rank test: $n = 27$, positive ranks = 19, ties = 3, $Z = −2.843$, $P = 0.004$). When VVS belonging to alien queens and workers are presented, both queenright workers and orphaned workers reacted more aggressively toward alien queens demonstrating that they are able to distinguish not only between nestmate queens and workers but also between
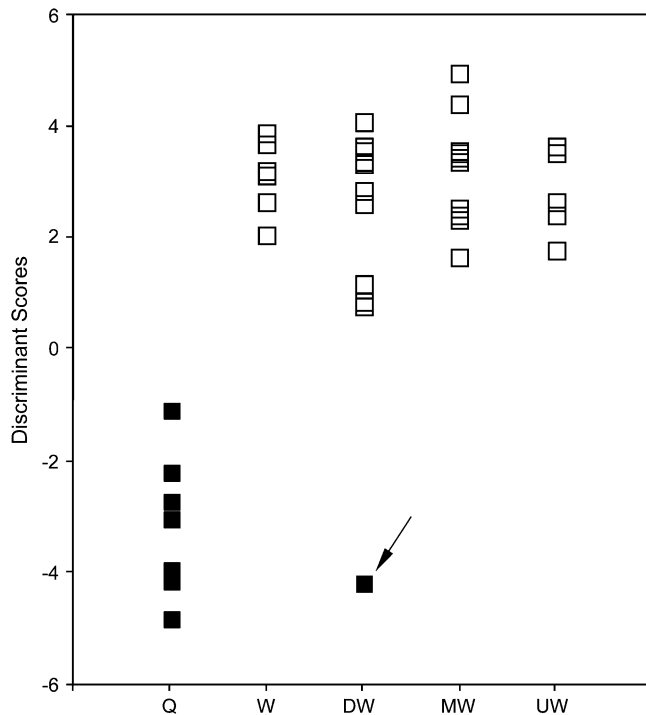
**Figure 1** Discriminant scores from the function separating queens (Q) and queenright workers (W). Orphaned workers with well-developed ovaries (DW), medium ovaries (MW), and undeveloped ovaries (UW) revealed to possess a signature similar to queenright workers. Black squares indicate individuals predicted as queens by DA, whereas white squares indicate individuals predicted as queenright workers. Only one orphaned worker (indicated by the arrow) was incorrectly predicted as a queen.

unfamiliar individuals (Wilcoxon signed-rank test: $n = 30$, positive ranks = 22, ties = 2, $Z = -2.843$, $P = 0.011$ for queenright colonies and $n = 27$, positive ranks = 27, ties = 0, $Z = -3.269$, $P = 0.001$ for orphaned colonies).

*Stroking behavior and ovarian suppression*

GLM (corrected model $n = 37$, degrees of freedom = 2, $F = 5.180$, $P = 0.011$) revealed that the presence of the queen did not influence the time before workers laid the first egg ($F = 1.751$, $P = 0.195$). Conversely, the frequency of stroking during the daily period the queen was put back on its nest did influenced the "latency" time before workers started laying the first eggs ($F = 5.313$, $P = 0.027$) (Figure 2).

## Discussion

Several papers have demonstrated that in independent-founding wasps, the secretion produced by the VdV organ is used as a repellent to defend the nest from the attacks of ants (London and Jeanne 2000). Dani et al. (2003) showed that the secretion of the VdV organ of *P. dominulus* is composed by a hydrocarbon mixture, which is similar in composition to that found on the cuticle. In a study on *P. gallicus* (cited as *Polistes foederatus*), Delfino et al. (1982) observed
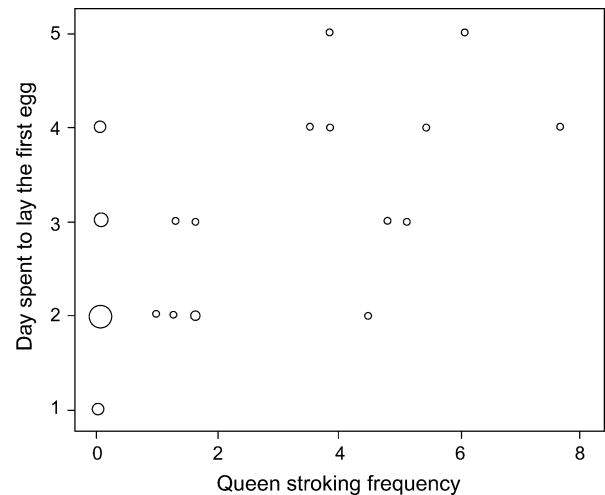


**Figure 2** Correlation between queen stroking frequency and the time before workers laid the first egg. Circle area is proportional to the number of colonies.

that the unicellular glands associated with the VdV organ of foundresses present a secretory cycle that has its activity peak just around the emergence of the workers. These authors observed that this finding could fit well with the necessity of the solitary foundress to produce a relevant amount of ant-repellent secretion to defend the still worker-less nest. However, this could be related also with the deposition activity of a sort of mark on the nest by the foundress, which could function as a signal for emerging workers (Cervo and Turillazzi 1989).

Our results clearly show that VVS is a hydrocarbon mixture that vary among colonies and between queens and workers. Intriguingly, the chemical differences between queens and workers in *P. gallicus* are not dependent on the social situation and/or fertility status. Indeed, the average VVS profile of orphaned workers showing developed ovaries does not match that of the queens but maintains a worker specificity. Also in the sympatric, congeneric *P. dominulus*, it has been demonstrated that fertility status does not determine cuticular hydrocarbon composition (Dapporto, Dani, and Turillazzi 2007), but in this species worker blends are clearly dependent on social context, and orphaned workers rapidly change their cuticular hydrocarbon profiles to resemble that of their lost queen (Dapporto et al. 2005). We actually do not know if the differences in the dynamic of cuticular hydrocarbons between these species are due to different physiological mechanisms or different dynamics of cuticular hydrocarbons (analyzed in *P. dominulus*) and VVS (analyzed in *P. gallicus*). Dani et al. (2003) demonstrated in *P. dominulus* a substantial identity between cuticular hydrocarbons and VVS profiles, and this suggests that changes in the composition of the 2 mixtures are synchronized. Workers are able to perceive such differences and react to different mixtures with different degrees of aggressiveness. These

responses are concordant with the expected ones. The presence of a colonial badge cue in VVS is probably important in worker reproductive "decisions." Indeed in *P. gallicus*, it has been demonstrated that in colonies usurped by an alien foundress, the worker daughters of the original queen develop significantly larger ovaries compared with those of workers in control, nonusurped colonies (Cervo and Turillazzi 1989). Workers may perceive some differences among chemicals present on the natal nest with respect to those of the unrelated queen and this anomaly may induce them to attempt direct reproduction. Workers discriminated VVS belonging to queens and workers not only when odors belonging to nestmates were presented but also when secretions of alien individuals were used in the experiments. This result clearly demonstrated that caste recognition is not based on the familiarity with the "peculiar odor" of their own queen but on a generalized queen signal (revealed by the lack of interactions between colony and caste in GLM) present in the whole population (Dapporto et al. 2004). The differences that *P. gallicus* presents with respect to *P. dominulus* in worker–queen cuticular signature dynamics can account for important differences in their social biology. In fact, a preimaginal component in caste differentiation (Hunt et al. 2003; Hunt 2006) seems quite evident in the first species judging also from the data, which Dani (1994) reports on body size differences of emerged individuals registered in the course of the colonial cycle.

Finally, the frequency of queen stroking behavior and not the "simple" presence of the queen on the nest resulted in a delay of worker reproduction. However, the presence of the queen on the nest for 2 h per day did not prevent workers from developing ovaries because in all the nests workers laid eggs after 4 days. The inefficacy of stroking in fully inhibiting worker reproduction is not surprising as Liebig et al. (2005) recently demonstrated in *P. dominulus* that if empty cells are present in the nest workers start to lay eggs after 20 days also in the presence of the queen. However, Liebig et al. (2005) and our findings indicate that the absence of empty cells, the presence of queen, and the queen signature on the nest concur to inhibit the ovarian development of workers.

In conclusion, the difficulty of obtaining reproductive success by indirect fitness in social insects adds challenging for direct reproduction by workers and subordinates to the other processes such as cheating, interspecific social parasitism, and intraspecific colony usurpation that are well represented in insect societies (Reeve 1991; Cervo and Dani 1996; Lenoir et al. 2001). Moreover, complex mechanisms to optimize the fitness of reproductive individuals and helpers have been hypothesized (Keller and Reeve 1994; but see also Queller et al. 2000; Nonacs 2006). According to the presence of such a "weak equilibrium," helpers probably assess their possibility of obtaining the largest benefit by a combination of indirect and direct fitness on the basis of a large variety of cues. In small societies, where queens can directly police the workers challenging for direct reproduction, direct physical interactions and visual cues (revealing the presence of queen and eggs) are probably the most important inhibitors of worker direct reproduction (Reeve 1991; Tibbetts 2002; Tibbetts and Dale 2004; Liebig et al. 2005). However, we demonstrated that also in these "simple" societies, chemical signaling plays a role in worker "decisions." This role is probably more important when "colonial elements" bearing no visual or behavioral cues (such as the nest paper) need to be marked to give supplementary information about the presence in the colony of a related queen.

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