The look of royalty: visual and odour signals of reproductive status in a paper wasp

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Reproductive conflicts within animal societies occur when all females can potentially reproduce. In social insects, these conflicts are regulated largely by behaviour and chemical signalling. There is evidence that presence of signals, which provide direct information about the quality of the reproductive females would increase the fitness of all parties. In this study, we present an association between visual and chemical signals in the paper wasp *Polistes satan*. Our results showed that in nest-founding phase colonies, variation of visual signals is linked to relative fertility, while chemical signals are related to dominance status. In addition, experiments revealed that higher hierarchical positions were occupied by subordinates with distinct proportions of cuticular hydrocarbons and distinct visual marks. Therefore, these wasps present cues that convey reliable information of their reproductive status.

Keywords: cuticular hydrocarbons; social dominance; nest-mate discrimination reproductive conflict; visual signals

1. INTRODUCTION

Animals living in societies should present some degree of organization, which is based on behavioural interactions and kinship among group members. In the most eusocial hymenopterans, reproductive conflicts occur because all females can potentially lay eggs, but the extent to which they do is regulated largely by dominance hierarchy (Pardi 1946, 1948), policing (Ratnieks 1988) and chemical signalling (Wilson 1971). The presence of signals that provide direct information about the quality of the reproductive females in terms of productivity would increase the fitness of all parties. Moreover, it would be expected that social behaviour could select for distinct mechanisms of nest-mate recognition. In fact, this recognition ability has been extensively documented in social insects (Hölldobler & Michener 1980; Howard *et al.* 1982; Gamboa *et al.* 1986). Understanding how individuals detect and use these characteristics are the central questions to highlight the behavioural bases of sociality.

In many social insects, queens are not able to inhibit worker reproduction through physical intimidation (West-Eberhard 1967; Wilson 1971; Keller & Nonacs 1993, but see Nascimento *et al.* 2004). Rather, the queen informs the colony of her presence through olfactory signals, which can be assessed by all members of the colony (Liebig *et al.* 1999; Endler *et al.* 2004). These signals should provide reliable information by which the reproductive status of an individual can be assessed without recourse to aggressive interactions (Beekman 2004). On the other hand, simple insect societies present a high reproductive totipotency and queens should suppress worker reproduction through constant intimidation leading to dominance hierarchies rather than using

indirect signals (West-Eberhard 1969; Ratnieks & Reeve 1992; Keller & Nonacs 1993).

Polistes paper wasps have a relatively flexible system of castes in which queen replacement is possible since most females are able to reproduce (West-Eberhard 1967, 1986; Strassmann 1981; Reeve 1991; O'Donnell 1996). Interestingly, the queen's reproductive quality is reflected by reliable cues of fertility, phenotypic traits that may be used in the resolution of conflicts (reviewed in Dani 2006; Tibbetts 2006). Analyses of epicuticular and egg compounds of *Polistes dominulus* revealed that a wide range of hydrocarbons may be involved in the queen's signal (Bonavita-Cougourdan *et al.* 1991; Sledge *et al.* 2001, 2004; Dapporto *et al.* 2007). Moreover, behavioural dominance seems to be more important than egg-laying activity to determine the epicuticular chemical blends of *P. dominulus* females (Dapporto *et al.* 2007).

In this species, studies have proposed that distinct facial patterns of *P. dominulus* foundresses from North America could predict quality signal during the foundation phase (Tibbetts & Dale 2004; Tibbetts 2006; Tibbetts & Curtis 2007). A recent report did not find evidence that visual marks found in *P. dominulus* foundresses from Italy were linked to any signal of dominance (Cervo *et al.* 2008). The variable black patterns, however, seem to be more important within the social context of *P. dominulus* wasps than previously thought (Tibbetts & Lindsay 2008). So far, there have been no studies examining whether visual marks and cuticular hydrocarbons concurrently can be used as predictors of reproductive status or fertility signal within the nests.

Here, we present the first test, to our knowledge, of the association between visual and chemical signalling. In *Polistes satan*, colonies are usually founded after the end of winter (dry season). New foundations are initiated by 1–18 associated females, and satellite nests are built nearby (Tannure-Nascimento *et al.* 2005). Usually, a

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Figure 1. (a) Portraits of *P. satan* showing the variation of heads in four females. (b) Distribution of brown cephalic areas of dominant (blue bars) and subordinate (red bars) in 23 pre-emergence colonies. (c) Aggressive behaviours directed towards returning females with the painted (experimental) and unpainted face (group).

single female becomes dominant and assumes the queen role, while associated foundresses act like workers. *Polistes satan* females have distinct marks on the face (figure 1a) and variable cuticular hydrocarbon profiles, suggesting that these signals could be recognized by nest-mates. In this paper, we show that (i) the visual variation and chemical profiles are linked to the relative fertility and social dominance during the founding phase, (ii) nest-mates use information from experimental alterations of visual patterns to recognize the dominant female, and (iii) after removal experiments, the alpha position in the ranking was occupied by inferior quality subordinates. Therefore, our results strongly support the idea that these wasps have distinct signals that convey information of reproductive and dominance status.

2. MATERIAL AND METHODS

(a) Protocol of observations and dissections

The observations and experiments were carried out in Cajuru, São Paulo State, Brazil between September and November 2004. Twenty-three colonies in pre-emergence phase were numbered and all females marked with enamel paint on the thorax. Observations and video recordings (Sony MiniDV HC43) were made before and after each experiment. We registered approximately 310.83 hours of interactions between individuals (13.5 ± 7.18 hours per colony, $\bar{x} \pm$ s.d.). To increase egg-laying activity and facilitate the identification of the dominance status, we removed several laid eggs from the nest (West-Eberhard 1986). Dominance-subordination

matrices were built using only very aggressive acts, such as biting, mounting, pushing and falling fights (Pardi 1948; West-Eberhard 1969; Strassmann 1981). The ranking was constructed dividing the most aggressive behaviours by the total number of acts. Foundresses were classified either as dominants or subordinates based on their positions in the matrices, functional roles and egg laying. In pleometrotic foundations of *P. satan*, only subordinate females perform foraging activities while dominants lay the most eggs in the colonies (Tannure-Nascimento 2002). Behavioural interactions were analysed before and after the experiments to verify changes in the hierarchy of studied colonies. After the observations, all females were dissected and the results were compared with the variation of head marks. We analysed the ovary development, counted and measured mature eggs in the ovarioles (Strassmann 1983; Nascimento & Cruz-Landim 1997). We also examined the presence of sperm in the spermathecae.

(b) Visual marks and body size

Heads of 123 observed females were removed and placed on white modelling clay. Images were taken under a Leica MZ75 stereomicroscope equipped with a digitalizing system and an automated program for PC (IM50 v. 4.0). Four measurements in each head were used: (i) total area of head (except compound eyes and mandibles), (ii) brown-pigmented area (mm^2), (iii) black-pigmented area (mm^2), and (iv) maximum width used as a body size indicator (mm). The measurements were made blind in relation to the individual ranking, and each image file was doubled, reanalysed and compared

in order to minimize errors. The pigmented areas (black or brown) were converted to their percentage relative to the total head area.

(c) Experiments

To test whether brown marks are used as signals of dominance, we selected pre-emergence colonies according to their size (12 ± 5 individuals; 10 colonies). Before each trial, a single wasp was held with forceps, put in a small plastic vial and anaesthetized in an ice chest cooler. We used two experimental designs: in group 1, females with a large brown marked area were captured with forceps, anaesthetized and had their black face marks covered with black paint spots (control trials). Later, we took the same females and changed their brown face marks with black paint and then released them after warming up completely (experimental trials). In group 2, females with small pigmented areas or with no brown marks were also painted with black on the clypeus, interorbital area and gena. Control group 2 consisted of black faced females with no paint alterations which were released after the complete anaesthetizing and warming process. In the laboratory, a third person analysed blind and randomly the video recordings, so that the observer was unaware of the identity, ranking and sequence (experimental or control group).

In the first experimental set-up, we tested whether nest-mates use brown marks as dominance signals because we expected that wasps would react aggressively towards unfamiliar painted females. On the other hand, dominant females with no changed brown marks would not be rejected as they have their dominance marks unaltered. In the second set-up, we predicted that if black-faced females did not occupy the higher rankings, they would be received indifferently by nest-mates. Therefore, the effect of paint alterations on the behaviour of nest-mates was also checked. The observations started at the moment the wasp returned to her natal nest, and the reactions of nest-mate females to the focal wasp were recorded. Later, the results of analyses were compared with the observations of dominance-subordinate relationships to verify the position of each paint changed female in the hierarchy and ovary development.

In a third experiment, dominant foundresses were removed and stored in the freezer for chemical analyses. Agonistic interactions among the remaining females were registered to verify the process of queens' succession as described above. After 20 days, all nests and individuals were collected for dissections, morphological and chemical analysis. The face pattern of substitutes was also analysed. Relative fertility between control dominants and substitutes was compared by the number and size of mature oocytes present in the ovarioles.

(d) Chemical analyses and statistics

In order to test whether cuticular hydrocarbon profiles predict dominance ranking, the compounds were extracted by washing each individual for 1 min in hexane in a numbered vial. The extracts were dried under a stream of nitrogen. The remaining compounds were suspended in 50 μ l of hexane and analysed on a Shimadzu GCMS (model QP2010), equipped with automated injector, and a DB-5MS capillary column, using helium as the carrier gas. The temperature protocol was as follows: 70–150°C at a rate of 30°C min⁻¹ (held for 5 min) and 150–320°C at 5°C min⁻¹ (held for 13 min). Analyses were performed in splitless mode and individual mass spectra were compared with Wiley

library data and standard compounds. The areas of 23 peaks, representing one or more cuticular compounds, were transformed to relative proportions and subjected to principal component analysis; the main peaks (representing more than 5% of variation) were directly analysed with stepwise discriminant analysis. The discriminant analysis was used to determine whether the predefined colony groups could be discriminated according to their profiles, and Wilk's λ values were used to infer the contribution of each variable to the discrimination. All statistical analyses were performed using STATISTICA software (Statsoft, Inc.).

3. RESULTS

(a) Dominance hierarchy and visual marks

Of the 53 females examined from 23 pre-emergence colonies, 24 females were classified as dominants and 29 females as associated subordinates in accordance with aggressive interactions and ovarian condition. As expected, dominant foundresses had more developed ovaries, laid all eggs during the observations and performed the most aggressive behaviours ($46 \pm 12\%$ of acts; Kruskal-Wallis test: $H=13.15$; $p<0.01$; $n=53$).

The brown-pigmented area in these females varied between 3.44 and 93.22% of the total cephalic area (figure 1a). Differences of these brown patterns were strongly correlated with dominance status (figure 1b). Females with more brown facial pigmentation were more dominant than those with less pigmentation. Although an overlap of 14 per cent females with intermediate pigmented areas was observed, the distribution of brown area of alpha females was between 40 and 95% of their total area ($\bar{x}=69.85\%$, s.d. = 17.99), whereas in subordinates, this pattern ranged between 3 and 60% ($\bar{x}=17.20$, s.d. = 17.87; $t=10.11$; $p<0.001$; $n=53$). Brown marks were weak predictors of body size. Although dominant females had larger heads than subordinates, this difference was not significant (dominants = $4.81 \text{ mm} \pm 0.22$ versus subordinates = $4.77 \text{ mm} \pm 0.15$; $\bar{x} \pm \text{s.d.}$; $t=0.76$; $p=0.45$; $n=47$).

(b) Visual signalling

Figure 1c shows that alterations of the facial patterns significantly affected the behaviour of nest-mate females towards the black-painted wasps. The removal of brown cephalic marks resulted in more hostile acts performed by workers towards the altered colour wasp than when the same unmanipulated wasps returned (Wilcoxon signed-rank test: $Z=2.02$, $p<0.05$; $n=5$ colonies). In all cases, the black-painted wasp had the highest position in the hierarchy. Dominant females responded very aggressively to threatening individuals, and these encounters with aggressive nest-mates resulted in falling fights in two out of five trials. Dominants were accepted only after two or three returns. Black-painted females with no brown marks did not significantly receive more aggressive acts than before the manipulation (Wilcoxon signed-rank test: $Z=1.02$, $p>0.05$; $n=5$ colonies). A distinct pattern of responses between the experimental groups was that queens never responded submissively to aggressive nest-mates while altered low-ranked females did in all trials.

Figure 2. (a) Discrimination of dominant females (blue circles), substitutes (red squares) and subordinates (green diamonds) based on the cuticular hydrocarbons profiles. All individuals were classified according to their groups. Gas chromatograms of a (b) dominant and a (c) subordinate showing the most important compounds: 1, C_{25} ; 2, C_{27} ; 3, C_{29} ; 4, 3-Me C_{29} ; 5, C_{31} . (d) Ovary of a dominant female; (e) ovary of a substitute female after 20 days of the dominant removal.

(c) Chemical and visual marks after removal experiments

Dominant foundresses, substitutes and subordinates differed in relation to their cuticular hydrocarbon profiles (figure 2a–c). Linear and long-chained alkanes were the most important compounds separating females (figure 2b–c). Discriminant analysis separated 100 per cent of females in to their predicted groups (table 1). One particular alkane, nC_{29} , was significantly more abundant on the surface of dominant females ($Z=2.30$, $p<0.05$). Substitute dominants had a lower proportion of brown facial marks than did the control queens (Mann–Whitney test: $Z=2.29$, $p<0.05$). The dissections after 15 days showed that substitute queens had lower relative fertility than removed control queens, as verified by their mature oocytes (dominant foundresses = $9.8 \text{ oocytes} \pm 1.48$ versus substitutes = $5.42 \text{ oocytes} \pm 1.9$; $\bar{x} \pm \text{s.d.}$; $t=4.27$; $p<0.01$; $n=12$; figure 2d–e).

4. DISCUSSION

Our results indicate that the social dominance in nest-founding phase of *P. satan* is reflected by a brown facial pattern of pigmentation and distinct cuticular hydrocarbons profiles that signal aspects of reproductive status and relative fertility of foundresses. Dominant foundresses had a higher proportion of brown facial marks and distinct chemical profile compared with other nest-mates.

Table 1. Discriminant analysis of the main cuticular compounds of dominants, subordinates and workers.

	Wilk's λ	partial λ	F -remove (2,32)	p -level
C_{31}	0.252	0.470	12.402	0.001
C_{27}	0.232	0.510	10.575	0.001
3-Me C_{29}	0.178	0.666	5.525	0.01
C_{29}	0.170	0.698	4.760	0.05
C_{25}	0.166	0.714	4.397	0.05

Subordinates responded aggressively to the experimental alteration of the brownish facial patterns of dominants, therefore confirming the visual recognition of these brown marks. Moreover, alpha females did not respond submissively after paint alteration while paint altered females occupying lower positions were subordinate with respect to aggressive co-foundresses. Behaviour, visual marks and odour, therefore, are multi-modal signals that allow the subordinates to distinguish unequivocally their alphas (see Hebets & Papaj 2005).

In social insects, it is known that social sanctions reduce the number of egg-laying workers (paper wasps: Gervet 1964; honeybees: Ratnieks 1988; reviewed in Wenseleers & Ratnieks 2006). In small social insect colonies, reproductive conflicts are resolved by queens or dominant females that directly prevent subordinate females from reproduction

through physical aggression (Pardi 1948; West-Eberhard 1969; Heinze *et al.* 1996; Monnin *et al.* 2002). Satellite nests provide a chance for workers to escape from queen control and to lay unfertilized male eggs, but we did not observe workers laying eggs even in large associations of nests. This confirms that either queens of *P. satan* are able to prevent worker egg laying, or that workers give up reproduction under a dominant's presence. In fact, the present analysis of cuticular hydrocarbons showed that the dominant foundresses unequivocally present a distinct chemical blend, which can be correlated with relative fertility and dominance status. Indeed, workers assess a queen's fertility by monitoring directly her reproductive output; therefore, all individuals must gain fitness by abdicating direct reproduction (Liebig *et al.* 2005).

Previous studies report a sophisticated system of communication in these societies, where non-breeders may assess breeder quality (honest signal *sensu* Keller & Nonacs 1993). Chemical variations in cuticular hydrocarbons have been shown to be reliable indicators of reproductive activity in several ants and wasps (Liebig *et al.* 2000; Dani *et al.* 2001; Sledge *et al.* 2001; Cuvillier-Hot *et al.* 2002; reviewed in Monnin 2006). Furthermore, another mechanism of signalling, such as facial marks, reflects quality in social wasps as well (Tibbetts & Lindsay 2008). Our results concur that both signals have evolved in some paper wasps. The present data corroborated with previous results that females occupying higher hierarchical positions present distinct chemical cuticular profiles (Bonavita-Cougourdan *et al.* 1991; Sledge *et al.* 2001).

Although our results regarding to GC-MS analysis are concordant with those obtained in *P. dominulus*, we observed that substitute females presented distinct chemical proportions compared with dominant foundresses and other subordinates. This is a significant difference between the present data and previous results found in the temperate *P. dominulus* (Sledge *et al.* 2001; reviewed in Monnin 2006). Heavier linear and methyl-branched alkanes seem to be the most important compounds to separate alphas, betas and low-ranked females (Sledge *et al.* 2001; Dapporto *et al.* 2004), even though only the methyl-branched alkanes played a role as a recognition pheromone (Dani *et al.* 2001). In *P. satan*, one methyl-branched and three methyl-branched alkanes were the most important compounds to separate dominant foundresses, substitutes and other subordinates. Other linear and methyl-branched alkanes were important to distinguish colony membership (Tannure-Nascimento *et al.* 2007). Interestingly, a recent study showed that 11 out of 17 chemical compounds discriminating social ranking in *P. dominulus* were also linear and methyl-branched alkanes (Dapporto *et al.* 2007).

Phenotypic variation in paper wasps seems to be related to ecological constraints (Hunt & Dove 2002; Seal & Hunt 2004). In a previous study, we showed that seasonality affected reproductive conditions in *P. satan*. Inseminated females had larger body sizes than uninseminated females (Tannure-Nascimento *et al.* 2005). However, our present data showed that dominant females were not larger than subordinates. In another tropical paper wasp, *Polistes versicolor*, the new queens produced at the end of colonial cycle that passed through the winter were also larger than other females (Gobbi *et al.* 2006).

Although the distribution of facial patterns of alpha foundresses and subordinates *P. satan* overlaps, dominant females presented heads with larger brown areas, while in subordinates, these brown areas were significantly smaller. However, the visual marks of *P. dominulus* are quite distinct from those presented by *P. satan*. Tibbetts & Curtis (2007) showed that food supplementation affected the facial patterns of quality between queens and workers. In Italian *P. dominulus*, these visual patterns were not associated with quality or size (Cervo *et al.* 2008).

Visual signals have evolved in distinct modalities of communication in paper wasps (Tibbetts & Dale 2004). Flexible founding strategies selected for high variability between nest-mates that provide individual recognition in *Polistes fuscatus* (Tibbetts 2002). Our results show that visual marks in *P. satan* were strongly associated with social dominance of foundresses (aggressiveness and number of mature oocytes), but it is also evident that nest-mates are able to recognize these signals. In fact, nest-mates were able to reject experimentally black-painted females, but also showed that black-faced females experimentally painted did not receive more physical aggression than those unmanipulated.

Phenotypic variation of social castes perhaps is reflected by different selective pressures that individuals experience during their developmental period (West-Eberhard 1986; Giray *et al.* 2005; Hunt & Amdam 2005). Evolving from a solitary ancestor, social castes in paper wasps have been presented as a mechanism of reciprocal deletion, in which queen and workers are complementary morphs that cooperate as alternatives strategies (West-Eberhard 1979, 1996; see also O'Donnell 1996). In fact, the cost of queen loss in established paper wasps colonies is high because workers take several days to start lay eggs (Strassmann *et al.* 2004). However, theory predicts that individuals should try to become reproductives even if the outcome decreases colony productivity (reviewed in Ratnieks *et al.* 2006). Therefore, potential conflict among totipotent females is resolved through dominance hierarchies and policing (Cuvillier-Hot *et al.* 2004; Saigo & Tsuchida 2004; Tsuchida & Suzuki 2006; reviewed in Wenseleers & Ratnieks 2006). Chemical and visual signals evolved in some paper wasps because they facilitate recognition of the most productive female and, therefore, provide stability during the behavioural interactions.

The existence of different signals of recognition in paper wasps, coupled with social regulation, appears to be unique for a primitively eusocial insect. These results support that visual and chemical signals should be complementary features of social dominance. In fact, selection for multiple messages conveying different aspects of a signaller's quality may also account for multi-modal displays (reviewed in Hebets & Papaj 2005). We conclude that both visual marks and cuticular hydrocarbons are signals that convey information on the dominance and/or reproductive status of each female within the dominance hierarchy.

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