

SHORT COMMUNICATION

Onset of fights and mutual assessment in ant founding queens

Kévin Berthelot^{1,*}, Felipe Ramon Portugal² and Raphaël Jeanson¹**ABSTRACT**

In animals, the progress and outcome of contests can be influenced by an individual's own condition, their opponent's condition or a combination of the two. The use of chemical information to assess the quality of rivals has been underestimated despite its central role in the regulation of social interactions in many taxa. Here, we studied pairwise contests between founding queens of the ant *Lasius niger* to investigate whether the decision to engage in agonistic interactions relies on self-assessment or mutual assessment. Queens modulated their aggressive behaviours depending on both their own status and their opponent's status. We found no influence of lipid stores or size on the onset of fights. However, differences in cuticular chemical signatures linked to fertility status accurately predicted the probability of behaving aggressively in pairs. Our study thus suggests that ant queens could rely on mutual assessment via chemical cues to make informed decisions about fight initiation.

KEY WORDS: Aggression, Contests, Cuticular hydrocarbons, Fertility cues

INTRODUCTION

Animal contests occur when individuals attempt to monopolise a resource such as a mate, prey or shelter. To limit costly fights, individuals can estimate their own condition (self-assessment) or compare their condition with that of their rivals (mutual assessment) based on a variety of traits including body size, weaponry, metabolic rate or energetic stores (Arnott and Elwood, 2009; Hardy and Briffa, 2013). Research on arthropod contests has largely focused on visual, auditory and/or vibratory cues, to the detriment of understanding the role of chemical cues, despite their importance in the regulation of interactions (Hardy and Briffa, 2013). Although some studies have examined how chemicals released during a fight impact the progress and outcome of contests (Goubault et al., 2006), the constitutive expression of chemical cues and their putative role in the decision to engage in aggressive interactions remains an open question.

Social insects are relevant models to address this issue as their behaviours heavily rely on chemical communication, which conveys a diversity of information (Blomquist and Bagnères, 2010). Many studies have highlighted that breeders advertise their fertility status with cuticular hydrocarbons and that these compounds are involved in the regulation of the reproductive division of labour within colonies (Monnin, 2006; Liebig, 2010). Colony foundation represents a critical stage where conflicts are

intense among females aiming to monopolise reproduction. Competition leading to queen selection can notably arise in species reproducing by colony fission and in associations of founding queens (Cronin and Monnin, 2009). In several ant species, young mated queens can found their colony either alone or cooperatively with other queens (pleometrosis) (Bernasconi and Strassmann, 1999). Upon the emergence of the first workers, queens in most pleometrotic associations engage in fatal fights and all but one queen perishes. In *Lasius niger*, aggression between reproductives is believed to be decisive for determining which queen survives, and the involvement of workers in fights may only have a marginal influence on the outcome of queen selection (Sommer and Hölldobler, 1995; Aron et al., 2009). Across species, earlier work showed that several factors including queen initial mass, mass loss during foundation, body size, productivity and proximity to the brood influence survival (Aron et al., 2009; Balas and Adams, 1996; Bernasconi and Keller, 1996, 1998; Sommer and Hölldobler, 1995). Although these studies provided insights into the mechanisms driving the loss of cooperation in pleometrotic associations, they did not inform on the behavioural rules and information used by queens to start fighting.

The benefit of cooperative foundation lies mostly in the production of a larger worker force that allows incipient colonies to be more efficient at brood raiding and to better resist attacks (Bernasconi and Strassmann, 1999). In pleometrotic associations, highly fertile queens are predicted to attack queens with lower fertility as the latter represent potential competitors that do not pay the costs of cooperation and increase their chances of winning fights by retaining their resources.

Here, we paired founding queens of *L. niger* differing in fertility to investigate which factors best predict the onset of aggressive interactions. We asked whether the decision to behave aggressively relies on energetic stores, on an assessment of morphological traits and chemical signatures of participants, or on a combination of these factors.

MATERIALS AND METHODS

Lasius niger (Linnaeus 1758) queens were collected after nuptial flight on July 2015 at two locations separated by 40 km (43°17'N, 1°13'E and 43°33'N, 1°28'E, Haute-Garonne, France) and were housed individually in glass tubes. We aimed to obtain queens differing in fertility status by exposing them to two temperatures: half of the queens were placed into a climate-controlled dark room (25°C; high fertility queens, $N=80$) and the other half were maintained in a cooled incubator at 5°C to delay ovarian activation (low fertility queens, $N=80$). No queens laid eggs during incubation at 5°C.

After 30 days, low fertility queens were removed from the incubator and placed into a dark room at 25°C for 72 h. Upon their removal from the 5°C incubator, these queens laid eggs. Forty-eight hours before the experiments were begun, the number of eggs produced over 24 h was counted in a random subset of 25 low and 25 high fertility queens. We also counted the total number of brood

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items (eggs, larvae and cocoons) produced by each queen. We compared the number of brood items using *t*-tests on square root-transformed data.

Behavioural trials consisted of dyadic encounters between a dead (hereafter, stimulus) and a living (hereafter, focal) queen that were conducted during the daytime under red light in a semi-circular arena (height: 0.5 cm, diameter: 3 cm). Confronting a live queen with a dead queen is the only way to evaluate a queen's decision to attack without the confounding effect of her rival fighting. Half of the queens in each treatment were haphazardly selected and killed by freezing. A maximal delay of 30 min was permitted between the death of a queen and her introduction in the arena. The assignment of queens between treatments (low or high fertility, focal or stimulus) was balanced between populations and each pair comprised queens from each population. The focal queen was introduced first into the arena and after 5 min of acclimation the stimulus queen was gently added. All queens were tested once. Arenas were video-recorded with a camera (Sony® Handycam HDR-XR350). All video-recordings were analysed blind to treatment. We recorded the behaviour of the focal queen for 5 min after the first contact with the stimulus queen. A binary score of aggression was used with 0=no aggressive interactions and 1=presence of aggressive interactions (biting and gaster flexion). Aggression between treatments was compared with a χ^2 -test followed by a multiple comparison test (Zar, 1999).

We then assessed body size by measuring head width (minimum width across the eyes) to the nearest micrometre using a stereomicroscope Nikon SMZ1000 at a magnification of $\times 40$. We assessed energy stores by measuring internal lipids in the thorax and abdomen using chloroform baths (the difference between dry mass before and after chloroform baths gives the amount of lipids; Bazazi et al., 2016). Lipid stores and head size were compared using a two-way ANOVA on square root-transformed data.

Testing our hypotheses required characterisation of the chemical signature of each queen. Ant bodies were individually soaked in 0.5 ml of pentane (Merck®, Darmstadt, Germany) for 10 min (Blomquist, 2010). GC-MS analysis was performed on a mass spectrometer (ISQ™QD Single Quadrupole GC-MS System, Thermo Fisher Scientific), fitted with a capillary column (Restek RTX-5MS 30 m \times 0.25 mm, 0.25 μ m film thickness, 5% diphenylpolysiloxane and 95% dimethylpolysiloxane) and a splitless injector (280°C). Oven temperature was maintained at 70°C after sample injection (2 μ l), then programmed to 20°C min⁻¹ to 180°C, then 5°C min⁻¹ to 320°C and held for 5 min. Peak areas were calculated by manual integration using Xcalibur 2.2 SP1 48 software. A total of 42 hydrocarbons, identified by mass spectra and retention indices, were used in the analysis (Table S1, Fig. S1). For each individual, we calculated the relative abundance of each compound by dividing the area of each peak by the total area of all peaks. Ten pairs for which analysis of the cuticular hydrocarbons of one queen failed were discarded. Peak areas were log normalised after adding a constant value of 0.001 (Aitchison, 1986). We ran a first discriminant analysis (partial least squares discriminant analysis, PLSDA) (Hervé, 2014) to identify which cuticular compounds contributed most towards partitioning low and high fertility queens. Queen fertility signature was obtained by selecting compounds with VIP (variable importance in projection) scores above 1 (i.e. compounds highly influential in discrimination) to run an additional PLSDA. For each queen, we calculated the Cartesian distance between her coordinates on the PLSDA and the coordinates of the centroid for all highly fertile queens (Fig. S2). A large distance reveals an important difference between the chemical profile of a queen and the typical

signature associated with high fertility. We next conducted a binary logistic regression to predict aggression patterns. Predictors were head size, lipid stores (normalised by head size), the distance of each queen to the typical high fertility signature and the relevant interactions. The response variable was presence or absence of aggression. We then ran an additional logistic regression using only significant predictors to calculate the odds ratio.

We performed an additional experiment to determine the influence of cold treatment by comparing low fertility queens that did or did not experience cold exposure. We collected queens after nuptial flights in June 2016 at the same locations as queens collected in 2015. Upon collection, queens were placed individually into glass tubes for 72 h in the climate-controlled room (25°C). We counted egg production over 24 h prior to the experiment and tested 21 pairs of these low fertility queens. The cuticular profiles of a random subset of 12 queens from each population were analysed. All analyses were performed with R 3.2.1 (R Development Core Team, 2015). Results are reported as means \pm s.d.

RESULTS AND DISCUSSION

We verified that queens assigned to low and high fertility treatments differed in fertility. Over 24 h, low fertility queens laid fewer eggs than high fertility queens (low fertility: 4.2 \pm 2.7, *N*=25, high fertility: 12.9 \pm 8.8, *N*=25; *t*-test: t_{48} =4.75, *P*<0.001). In total, low fertility queens produced fewer brood items (8.5 \pm 4.8, *N*=66) than high fertility queens (76.6 \pm 18.3, *N*=72; *t*-test: t_{136} =33.33, *P*<0.001). We next determined whether aggression varied between treatments. In our study, queens started to lay eggs alone, before being paired. This absence of early social experience contrasts with natural conditions where queens associate immediately after mating and we can speculate that this explains the high level of aggression. Nevertheless, we found that queens modulated their aggressive behaviours depending on both their own status and that of their rival (χ^2 =9.89, d.f.=3, *P*=0.019), with highly fertile queens being twice as aggressive towards stimulus queens of low fertility as low fertility queens were towards highly fertile stimulus queens (multiple comparison test: q =4.80, k =4, *P*<0.01) (Fig. 1). Lipid stores differed between low (7.13 \pm 1.25 mg, *N*=66) and high (2.67 \pm 0.82 mg, *N*=72) fertility queens ($F_{1,134}$ =602.62, *P*<0.001) but not between focal (4.73 \pm 2.39 mg, *N*=69) and stimulus (4.87 \pm 2.56 mg, *N*=69) queens ($F_{1,134}$ =0.30, *P*=0.58). Head size did not vary with fertility status ($F_{1,134}$ =0.39, *P*=0.53) or between focal and stimulus queens ($F_{1,134}$ =0.51, *P*=0.47).

Because differences between low and high fertility queens could result from cold exposure and not from a difference in fertility, we performed an additional experiment. We found no difference in the number of eggs produced by queens with low fertility that experienced cold (8.5 \pm 4.8, *N*=66) or not (9.9 \pm 4.83, *N*=42; *t*-test: t_{106} =1.51, *P*=0.13). Also, aggression did not differ between pairs of queens of low fertility exposed to cold (60%, *N*=16) or not (71%, *N*=21; χ^2 =0.13, d.f.=1, *P*=0.72). Finally, the discriminant analysis on cuticular profiles separated queens depending on their fertility status and we detected no difference between low fertility queens that were exposed or not to cold (Fig. 2). This indicates that the differences in behavioural patterns and cuticular profiles observed between low and high fertility queens resulted from differences in fertility, not from cold. Overall, our results showed that queens used information about their own condition and their rival's status to engage in aggression, thus providing support for the hypothesis that mutual assessment was involved in their decision. Using a logistic regression, we then examined which factors best predicted focal queen aggressiveness. We found no influence of lipid stores or head

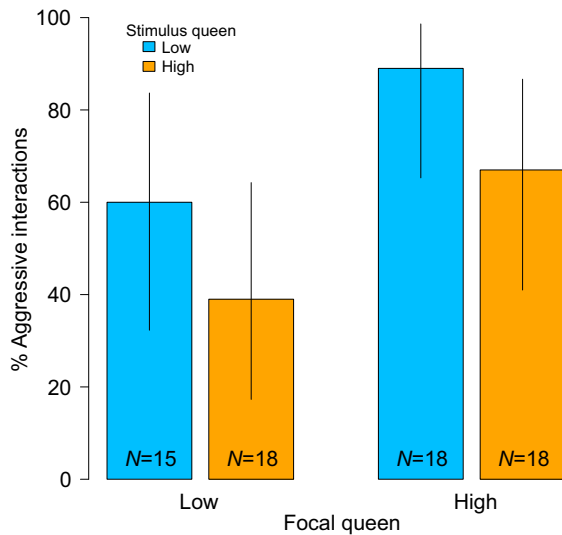


Fig. 1. Proportion of aggressive contests between focal and stimulus queens as function of their fertility. Focal and stimulus queens of either low or high fertility were paired and responses of focal queens were recorded. Data are presented \pm 95% confidence interval.

size on the likelihood of engaging in aggression (Table 1). Fertility distances of queens were highly significant predictors of aggression: the probability of focal queens behaving aggressively decreased with their chemical distance to the highly fertile signature [$\beta = -0.53 \pm 0.22$, odds-ratio (95% CI): 0.59 (0.37, 0.88)]; and, reciprocally, the probability of stimulus queens being attacked increased with their chemical distance to the highly fertile signature [$\beta = 0.54 \pm 0.25$, odds-ratio (95% CI): 1.72 (1.08, 2.95)].

In ants, the traditional view is that nestmate recognition involves a comparison between the chemical profile of an intruder and the internal representation of an individual's own odour, any mismatch leading to rejection (Blomquist and Bagnères, 2010). In this context, aggression in workers generally increases with chemical

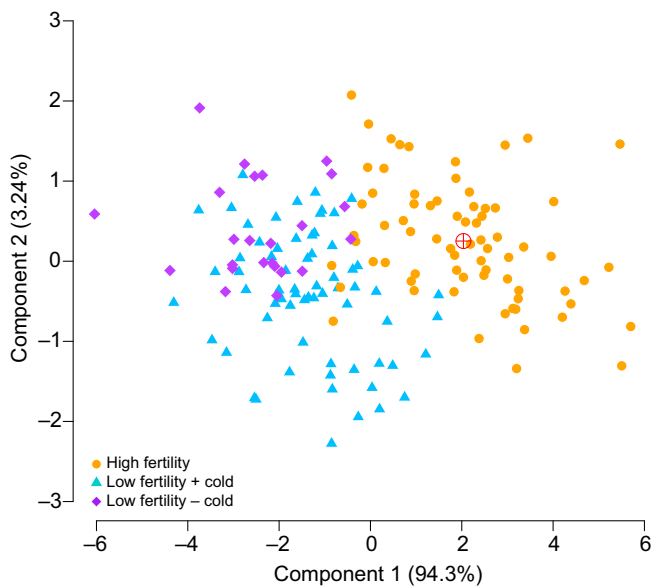


Fig. 2. Discriminant analysis of cuticular hydrocarbon profiles of low and high fertility queens. Data are shown for low fertility queens that were or were not exposed to cold. The circled red cross is the centroid of highly fertile queens and represents the typical signature of a high fertility status.

Table 1. Binary logistic regression of the relative influence of lipid stores, size and chemical profiles of queens on the proportion of aggressive interactions

Predictors	Deviance	d.f.	Residual deviance	P
Size of focal queens	1.27	67	89.09	0.26
Size of stimulus queens	0.01	66	89.08	0.91
Profile of focal queens	9.65	65	79.43	0.002
Profile of stimulus queens	7.39	64	72.03	0.007
Lipid stores of focal queens	0.02	63	72.01	0.89
Lipid stores of stimulus queens	0.19	62	71.82	0.67
Size of focal queens \times size of stimulus queens	0.67	61	71.16	0.41
Profile of focal queens \times profile of stimulus queens	0.08	60	71.08	0.78
Lipid stores of focal queens \times lipid stores of stimulus queens	0.06	59	71.02	0.81
Size of focal queens \times profile of stimulus queens	0.29	58	70.73	0.59
Size of focal queens \times lipid stores of stimulus queens	0.68	57	70.04	0.41
Size of stimulus queens \times profile of focal queens	0.06	56	69.98	0.81
Size of stimulus queens \times lipid stores of focal queens	0.90	55	69.08	0.34
Profile of focal queens \times lipid stores of stimulus queens	0.64	54	68.44	0.43
Profile of stimulus queens \times lipid stores of focal queens	0.09	53	68.35	0.76

Bold indicates significance.

distance (Martin et al., 2012). If queens based their decision on an 'absolute value' of chemical distance, similar aggressiveness would have been predicted in pairwise contests involving low and high fertility queens, irrespective of the actual fertility status of the focal and stimulus individuals. Such a pattern was not found in our study, where aggression differed markedly when queens of low and high fertility were tested as stimulus and focal individuals. This provides an indirect line of evidence that queens could use chemical cues and compare their fertility status to initiate aggression.

Our study provides additional support to earlier findings that hydrocarbons could be used as reproductive signals in *L. niger*. However, the putative compounds likely to be responsible for mutual assessment between queens were not the same as those experimentally identified previously (Holman et al., 2010a,b). One major difference concerns 3-MeC31 that was considered to advertise queen quality but was found here to only have a minor influence in comparison to other more explanatory compounds (Table S1). Such discrepancy opens interesting avenues for future research to increase our understanding of reproductive signalling in *L. niger* and, more generally, to investigate whether similar compounds play similar roles across contexts.

In pleometrotic associations, queens are usually unrelated and overt fighting is expected because they cannot gain inclusive fitness by ceding the contest (Cronin and Monnin, 2010). The fact that the cuticular profiles of queens are associated with their fertility and, possibly, with their decision to behave aggressively suggests that queens could use chemical signals advertising reproductive state to adopt strategies maximising their fitness. Under an ultimate perspective, we can speculate that the onset of fights between queens differing in fertility results from reduced benefits of accepting individuals of low fertility as they produce few or no brood but still represent a potential competitor during the

elimination phase. Similar patterns were reported in wasps, where individuals advertising high quality using visual signals were more likely to challenge rivals while individuals of low advertised quality were more likely to be confronted with aggressive behaviour (Tibbetts et al., 2010).

In conclusion, our study suggests that cuticular signatures conveying information about fertility status could be involved in mutual assessment. This opens interesting avenues for future research aimed at understanding how chemical cues contribute to the regulation of interactions between reproductives and, eventually, to queen selection in social insects.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

K.B. and R.J. conceived the study, K.B. and F.R.P. conducted the experiments. All authors analysed the data, wrote the manuscript and approved the final version.

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Data availability

Data from this study have been deposited in the Dryad Digital Repository (Berthelot et al., 2017): <http://dx.doi.org/10.5061/dryad.t1sg6>

Supplementary information

Supplementary information available online at <http://jeb.biologists.org/lookup/doi/10.1242/jeb.150375.supplemental>

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SUPPLEMENTARY MATERIALS

Table S1. Compounds in the cuticular extracts of queens. VIP (Variable Importance in Projection) scores (1st and 2nd component) of each compound on each component of the discriminant analysis (PLS-DA). Compounds highlighted in bold were retained in the analysis (a VIP score >1 denotes a highly influential compound in the discrimination between lowly and highly fertile queens). KI: Kovat's index.

Peak	Compound	KI	VIP score	
			Comp.1	Comp.2
1	n-C ₂₇	2695	1.52	1.44
2	3-MeC ₂₇	2769	1.86	1.75
3	5,9-diMeC ₂₇	2777	1.45	1.4
4	n-C ₂₈	2794	0.04	0.36
5	5,9-diMeC ₂₈	2875	2.06	1.97
6	n-C ₂₉	2895	0.32	0.55
7	7-/ 9-/ 11-/ 13- and 15-MeC ₂₉	2926	0.12	0.15
8	11-MeC ₂₉	2946	1.41	1.33
9	7,11-/ 9,15- and 11,15-diMeC ₂₉	2959	0.12	0.48
10	7-MeC ₂₉	2964	0.21	0.2
11	3-MeC ₂₉	2969	0.75	0.73
12	5,15-diMeC ₂₉	2975	0.63	0.6
13	x,15-diMeC ₂₉	2987	0.91	0.89
14	n-C ₃₀	3002	1.11	1.05
15	12,14-diMeC ₃₀	3025	0.33	0.32
16	12,14-diMeC ₃₀	3029	0.54	0.65
17	10,14-diMeC ₃₀	3055	0.18	0.57
18	C _{31:1}	3074	1.47	1.41
19	n-C ₃₁	3095	2.16	2.09
20	9-/ 11-/ 13- and 15-MeC ₃₁	3127	0.45	0.61
21	9,x-diMeC ₃₁	3152	0.29	0.27
22	7,17-/ 9,15-/ 9,17-/ 11,15- and 13,17-diMeC ₃₁	3160	0.72	0.85
23	7-MeC ₃₁	3164	0.1	0.32
24	3-MeC ₃₁	3171	0.9	0.96
25	5,13- and 5,17-diMeC ₃₁	3176	0.59	0.56
26	7,13,17-triMeC ₃₁	3189	0.7	0.75
27	3,9-/ 3,11-/ 3,13-/ 3,15- and 3,19-diMeC ₃₁	3200	0.47	0.62
28	10-/ 14- and 15-MeC ₃₂	3224	1.11	1.17
29	4-MeC ₃₂ and 10,14-diMeC ₃₂	3253	1.39	1.41
30	7-/ 9-/ 11-/ 13-/ 15-/ 17- and 19-MeC ₃₃	3326	1.43	1.42
31	11,15-/ 13,17- and 15,19-diMeC ₃₃	3352	0.65	0.64
32	9,15- and 11,17-diMeC ₃₃	3357	2	1.89
33	5,13- 5,17-diMeC ₃₃	3373	0.04	0.18
34	2-MeC ₃₃	3386	0.78	0.74
35	n-C ₃₄	3399	0.1	0.58
36	17-MeC ₃₄	3422	1.02	0.98
37	10-MeC ₃₄	3453	0.89	0.97
38	13-MeC ₃₅	3523	0.68	0.88
39	12-MeC ₃₅	3548	0.03	0.12
40	9-/ 11-MeC ₃₅	3555	0.88	0.88
41	13,x-diMeC ₃₅	3566	0.51	0.5
42	9-/ 11-MeC ₃₇	3749	1.05	1

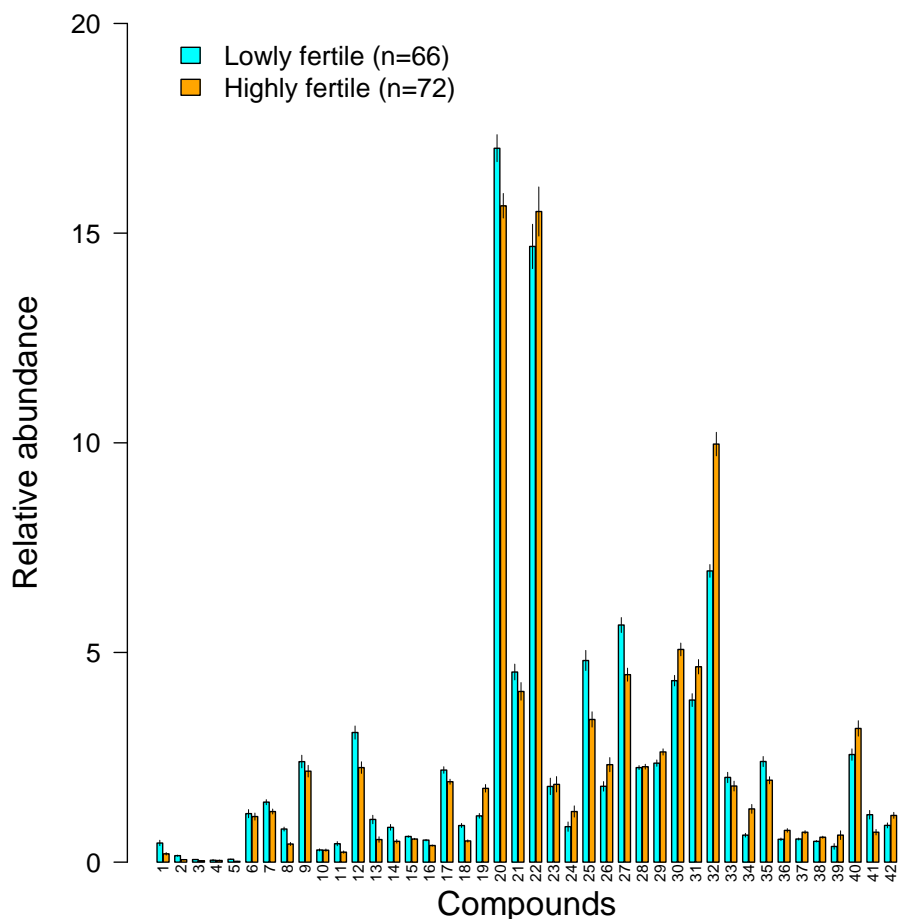


Figure S1. Relative abundance (mean ± SE) of hydrocarbons for lowly and highly fertile queens. The identity of each compound is reported in Table S1.

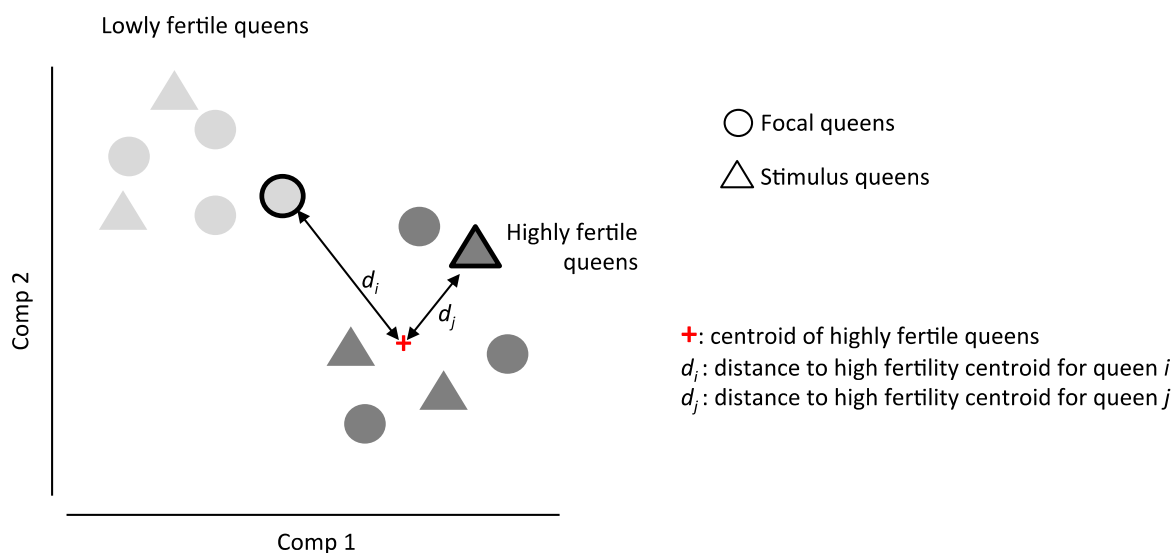


Figure S2. Schematic explanation of how the distance of each queen to high fertility signature was determined. The distance d_i is the distance of the focal queen i to the centroid of highly fertile queens and the distance d_j is the distance of the stimulus queen j to the centroid of highly fertile queens.