Onset of fights and mutual assessment in ant founding queens

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Abstract

In animals, the progress and outcomes of contests can be influenced by an individual's own condition, its opponent's condition or a combination of both. 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 or mutual assessment. Queens modulated their aggressive behaviours depending both on their own status and their opponent's status. We showed 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.

Keywords: aggression, contests, cuticular hydrocarbons, fertility cues

Summary Statement

Lasius niger ant queens compare their own status of fertility to their rivals' one before deciding or not to engage in lethal interactions.

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 relatively to their rivals' one (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 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). Though some studies examined how chemicals released during fight impact the progress and outcomes of contests (Goubault et al., 2006), the constitutive expression of chemical cues and their putative role in the decision to engage in aggressive interactions still remains an open question.

Social insects are relevant models to address this issue since their behaviours heavily rely on chemical communication, which conveys a diversity of information (Blomquist and Bagnères, 2010). Many studies highlighted that breeders advertise their status of fertility with cuticular hydrocarbons and that these compounds are involved in the regulation of reproductive division of labor within colonies (Monnin, 2006; Liebig, 2010). Colony foundation represents a critical stage where conflicts are intense among females aiming at monopolising 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 either found their colony 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 perish. 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 queens' initial mass, weight 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). If 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 better resist attacks (Bernasconi and Strassmann, 1999). In pleometrotic associations, highly fertile queens are predicted to attack lowly fertile queens as they 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 predicted 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

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 at obtaining queens differing in their fertility status by exposing them at two temperatures: half of the queens were placed into a climate dark room (25°C) (highly fertile queens, N=80) and the other half were maintained in a cooled incubator at 5°C to delay ovarian activation (lowly fertile queens, N=80). No queens laid eggs during their stay at 5°C.

After 30 days, lowly fertile queens were removed from the incubator and placed into a dark room at 25°C for 72 hours. Upon their removal from incubator, lowly fertile queens laid eggs. Forty-eight hours before starting experiments, the number of eggs produced over 24 h was counted in a random subset of 25 lowly and 25 highly fertile queens. We also counted the total number of brood 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 in dyadic encounters between a dead (hereafter, stimulus) and a living queen (hereafter, focal) that were conducted during daytime under red light in a half-circular arena (height: 0.5 cm, diameter: 3 cm). Confronting a live to 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 minutes was permitted between queen's sacrifice and her introduction in the arena. The assignment of queens between treatments (low or high fertility, focal or stimulus queens) 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 blindly to treatment. We recorded the behaviour of the focal queen for 5 minutes 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 10^{-3}mm using a stereomicroscope Nikon SMZ1000 at a magnification of 40X. We assessed energetic stores by measuring internal lipids in thorax and abdomen using chloroform baths (difference between dry weight 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 to characterise the chemical signature of each queen. Ant bodies were individually soaked in 0.5 ml of pentane (Merck®, Darmstadt, Germany) for 10 minutes (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 × 0.25 mm, 0.25 μm film thickness, 5% diphenyl and 95% dimethylpolysiloxane) and a splitless injector (280°C). Oven temperature was maintained at 70°C after sample injection (2 μl), then programmed at 20°C/min to 180°C, then 5°C/min 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 total area of all peaks. Ten pairs for which the analysis of the cuticular hydrocarbons of one queen failed were discarded. Peak areas were log normalized after adding a constant value of 0.001 (Aitchison, 1986). We ran a first discriminant analysis (PLSDA) (Hervé, 2014) to identify which cuticular compounds contributed most towards partitioning lowly and highly fertile queens. Queen's fertility signature was obtained by selecting compounds with VIP (Variable Important 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 next ran an additional logistic regression using only significant predictors to calculate odds-ratio.

We performed an additional experiment to determine the influence of cold treatment by comparing lowly fertile queens that experienced or not cold exposure. We collected queens after nuptial flights on June 2016 at the same locations than queens collected in 2015. Upon collection, queens were placed individually in glass tubes for 72 hours in the climate room (25°C). We counted egg production over 24 hours prior experiment and we tested 21 pairs of these lowly fertile queens that did not experience cold exposure to assess aggression. 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 mean ± SD.

Results and discussion

We verified that queens assigned to lowly and highly fertility treatments differed in fertility. Over 24 hours, lowly fertile queens laid fewer eggs than highly fertile queens (lowly fertile queens: 4.2±2.7, N=25, highly fertile queens: 12.9±8.8, N=25; t-test: t_{48}=4.75, P<0.001). In total, lowly fertile queens produced less brood items (8.5±4.8, N=66) than highly fertile queens (76.6±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 their rival's status (χ^2=9.89, df=3, P=0.019) with highly fertile queens being twice more aggressive toward lowly fertile stimulus queens than lowly fertile queens toward highly fertile stimulus queens (multiple comparison test: q=4.80, k=4, P<0.01) (Fig. 1). Lipid stores differed between lowly (7.13±1.25 mg, N=66) and highly (2.67±0.82 mg, N=72) fertile queens ($F_{1,134}$=602.62, P<0.001) but not between focal (4.73±2.39 mg, N=69) and stimulus (4.87±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 lowly and highly fertile queens could result from cold exposure and not from difference in fertility, we performed an additional experiment. We found no difference in egg number produced by lowly fertile queens that experienced cold (8.5 ± 4.8, N=66) or not (9.9 ± 4.83, N=42) (t-test: $t_{106}=1.51$, $P=0.13$). Also, aggression did not differ between pairs of lowly fertile queens exposed to cold (60%, N=16) or not (71%, N=21) ($\chi^2=0.13$, $df=1$, P=0.72). Finally, the discriminant analysis on cuticular profiles separated queens depending on their fertility status and we detected no difference between lowly fertile queens that were exposed or not to cold (Fig. 2). This indicates that the differences in behavioural patterns and cuticular profiles observed between lowly and highly fertile queens resulted from differences in fertility, not from cold. Overall, our results showed that queens used information about their own condition and their rivals' status to engage in aggression, thus providing support for the hypothesis that mutual assessment participated to their decision. Using a logistic regression, we then examined which factors best predicted focal queens' aggressiveness. We found no influence of lipid stores or head size on the likelihood to engage in aggression (Table 1). Fertility distances of queens were highly significant predictors of aggression: the probability of focal queens of behaving aggressively decreased with their chemical distance to highly fertile signature ($\beta=-0.53\pm0.22$, odds-ratio [95% CI]: 0.59 [0.37, 0.88]), and reciprocally, the probability of stimulus queens of being attacked increased with their chemical distance to highly fertile signature ($\beta=0.54\pm0.25$, 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 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 lowly and highly fertile queens, irrespective of the actual fertility status of the focal and stimulus individuals. Such pattern was not found in our study where aggression differed markedly when lowly and highly fertile queens were tested as stimulus and focal individuals. This provides indirect lines of evidence that queens could use chemical cues and compare their fertility status to initiate aggression.

Our study provided additional support to earlier findings that hydrocarbons could be used as reproductive signals in *L. niger*. However, the putative compounds likely 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's quality but that 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 maximizing 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 lowly fertile individuals since 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 and individuals of low advertised quality were more likely to be aggressed (Tibbetts et al., 2010).

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

Author contributions. KB and RJ conceived the study, KB and FRP conducted the experiments. All authors analysed the data, wrote the manuscript and approved the final version.

Data accessibility. All data are deposited in the Dryad repository: http://dx.doi.org/10.5061/dryad.t1sg6

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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	df	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
Profiles of focal queens	**9.65**	**65**	**79.43**	**0.002**
Profiles 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 X Size of stimulus queens	0.67	61	71.16	0.41
Profiles of focal queens X Profiles of stimulus queens	0.08	60	71.08	0.78
Lipid stores of focal queens X Lipid stores of stimulus queens	0.06	59	71.02	0.81
Size of focal queens X Profiles of stimulus queens	0.29	58	70.73	0.59
Size of focal queens X Lipid stores of stimulus queens	0.68	57	70.04	0.41
Size of stimulus queens X Profiles of focal queens	0.06	56	69.98	0.81
Size of stimulus queens X Lipid stores of focal queens	0.90	55	69.08	0.34
Profiles of focal queens X Lipid stores of stimulus queens	0.64	54	68.44	0.43
Profiles of stimulus queens X Lipid stores of focal queens	0.09	53	68.35	0.76

Figures
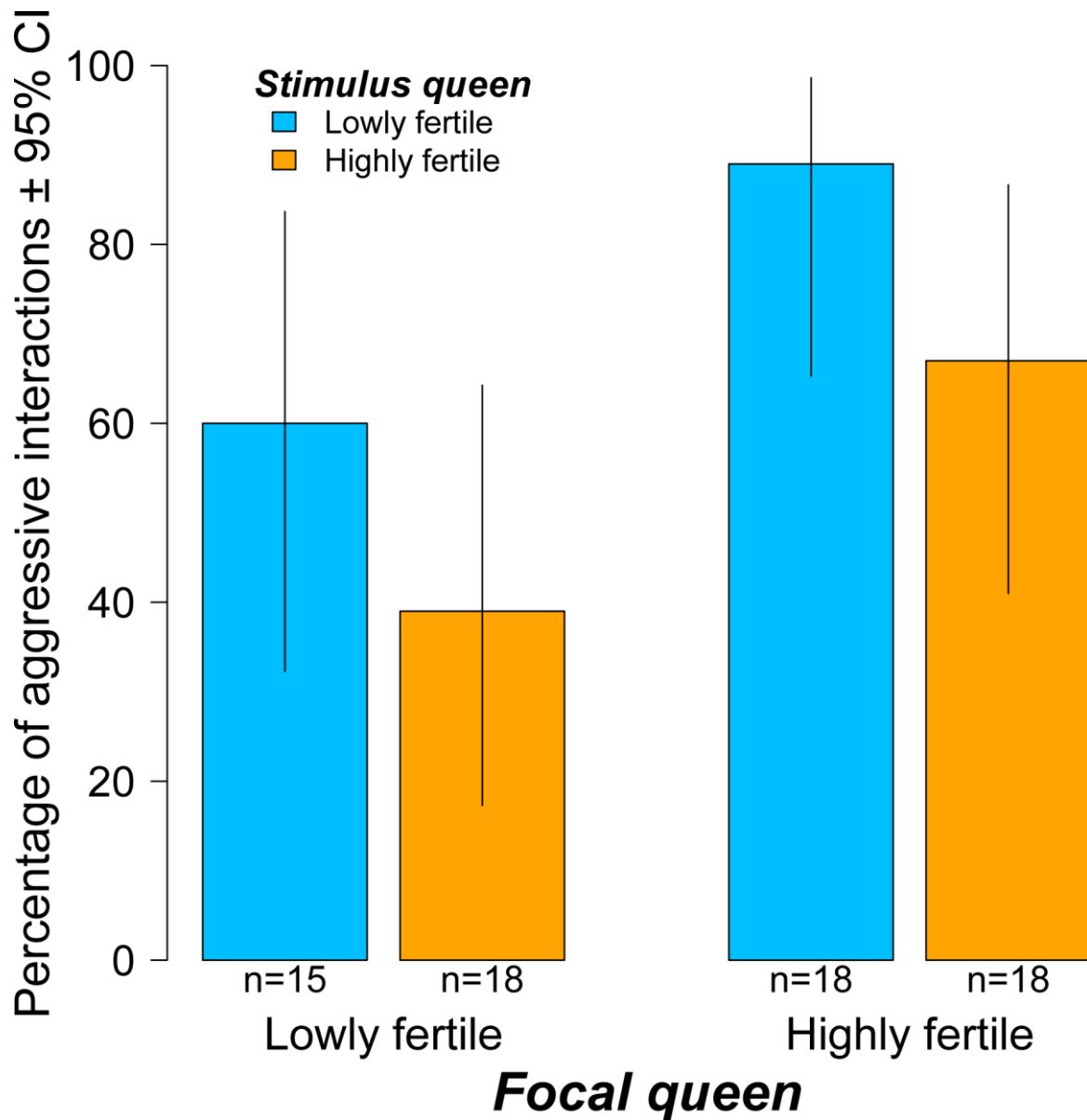
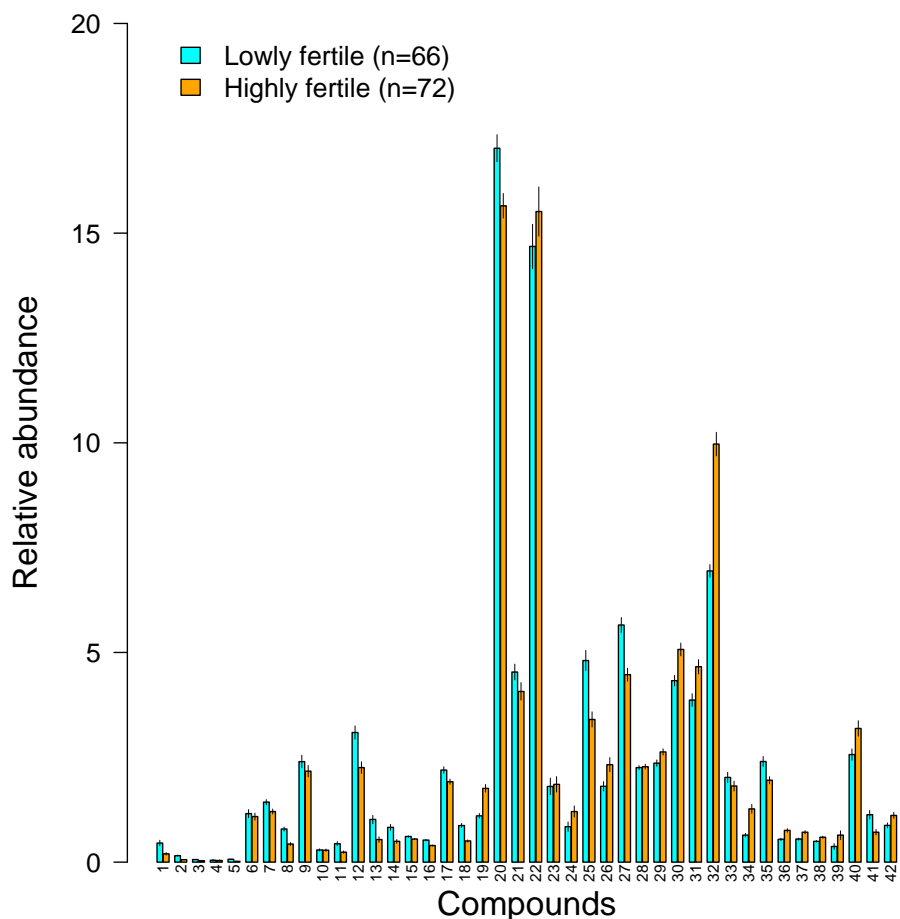Figure 1. Proportion of aggressive contests between focal and stimulus queens as function of their fertility.
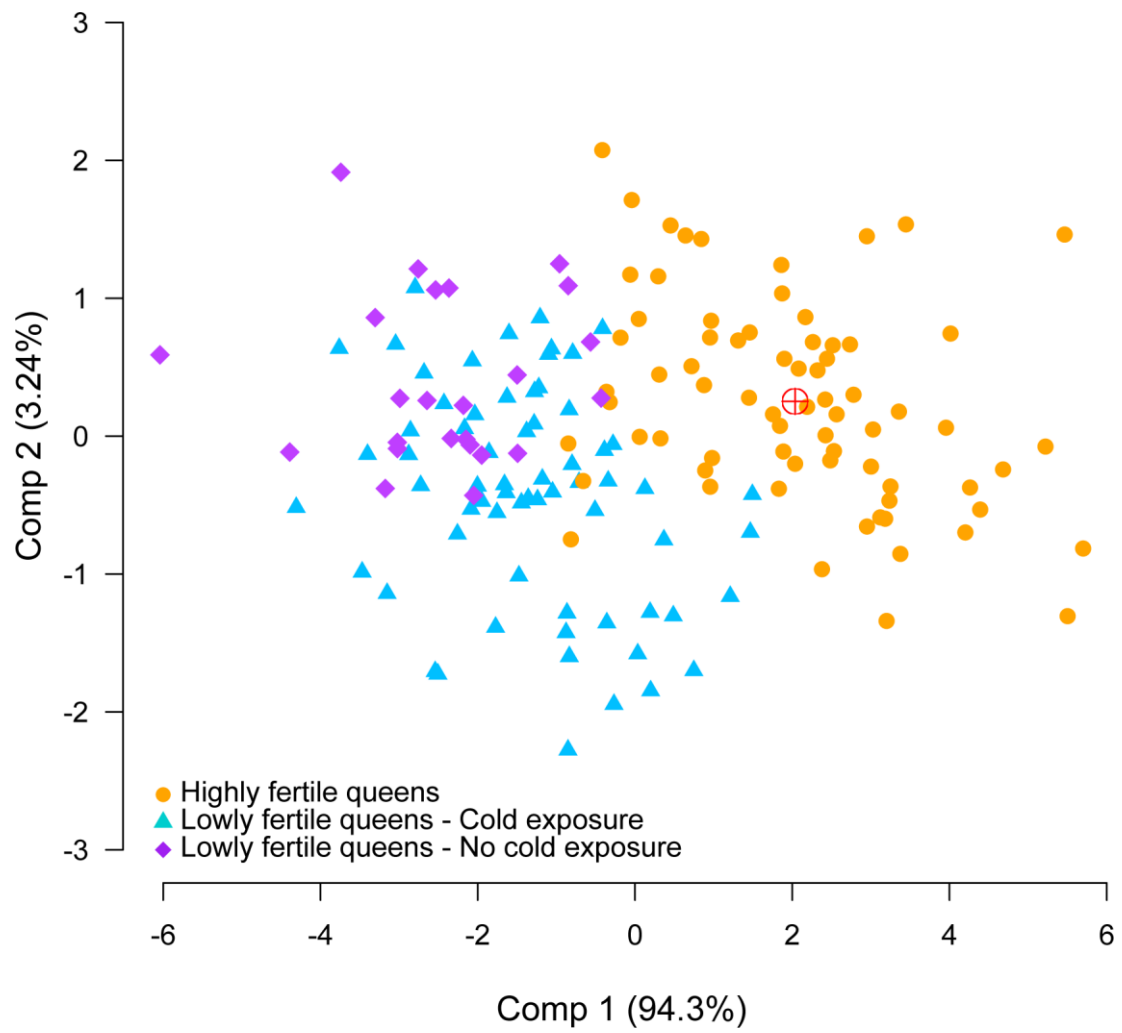
Figure 2. Discriminant analysis of cuticular profiles of lowly and highly fertile queens. The circle red cross is the centroid of highly fertile queens and represents the typical signature of a high fertility status.
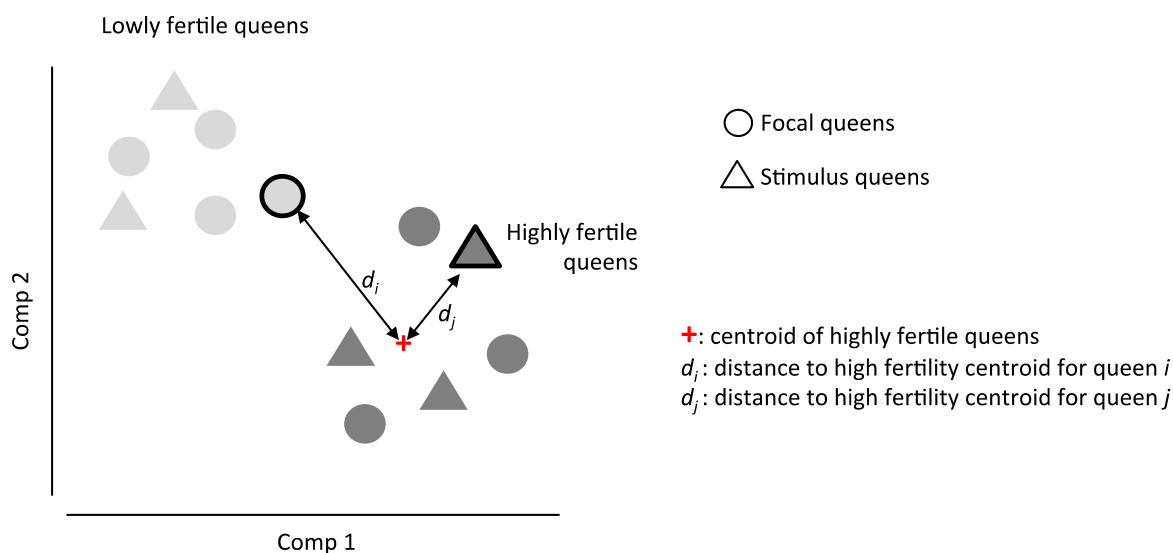
SUPPLEMENTARY MATERIALS

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.

			VIP score	
Peak	Compound	KI	Comp.1	Comp.2
1	$n\text{-}C_{27}$	2695	**1.52**	**1.44**
2	3-MeC_{27}	2769	**1.86**	**1.75**
3	$5,9\text{-diMeC}_{27}$	2777	**1.45**	**1.4**
4	$n\text{-}C_{28}$	2794	0.04	0.36
5	$5,9\text{-diMeC}_{28}$	2875	**2.06**	**1.97**
6	$n\text{-}C_{29}$	2895	0.32	0.55
7	$7\text{-/ }9\text{-/ }11\text{-/ }13\text{- and }15\text{-MeC}_{29}$	2926	0.12	0.15
8	11-MeC_{29}	2946	**1.41**	**1.33**
9	$7,11\text{-/ }9,15\text{- and }11,15\text{-diMeC}_{29}$	2959	0.12	0.48
10	7-MeC_{29}	2964	0.21	0.2
11	3-MeC_{29}	2969	0.75	0.73
12	$5,15\text{-diMeC}_{29}$	2975	0.63	0.6
13	$x,15\text{-diMeC}_{29}$	2987	0.91	0.89
14	$n\text{-}C_{30}$	3002	**1.11**	**1.05**
15	$12,14\text{-diMeC}_{30}$	3025	0.33	0.32
16	$12,14\text{-diMeC}_{30}$	3029	0.54	0.65
17	$10,14\text{-diMeC}_{30}$	3055	0.18	0.57
18	$C_{31:1}$	3074	**1.47**	**1.41**
19	$n\text{-}C_{31}$	3095	**2.16**	**2.09**
20	$9\text{-/ }11\text{-/ }13\text{- and }15\text{-MeC}_{31}$	3127	0.45	0.61
21	$9,x\text{-diMeC}_{31}$	3152	0.29	0.27
22	$7,17\text{-/ }9,15\text{-/ }9,17\text{-/ }11,15\text{- and }13,17\text{-diMeC}_{31}$	3160	0.72	0.85
23	7-MeC_{31}	3164	0.1	0.32
24	3-MeC_{31}	3171	0.9	0.96
25	$5,13\text{- and }5,17\text{-diMeC}_{31}$	3176	0.59	0.56
26	$7,13,17\text{-triMeC}_{31}$	3189	0.7	0.75
27	$3,9\text{-/ }3,11\text{-/ }3,13\text{-/ }3,15\text{- and }3,19\text{-diMeC}_{31}$	3200	0.47	0.62
28	$10\text{-/ }14\text{- and }15\text{-MeC}_{32}$	3224	**1.11**	**1.17**
29	$4\text{-MeC32 and }10,14\text{-diMeC}_{32}$	3253	**1.39**	**1.41**
30	$7\text{-/ }9\text{-/ }11\text{-/ }13\text{-/ }15\text{-/ }17\text{- and }19\text{-MeC}_{33}$	3326	**1.43**	**1.42**
31	$11,15\text{-/ }13,17\text{- and }15,19\text{-diMeC}_{33}$	3352	0.65	0.64
32	$9,15\text{- and }11,17\text{-diMeC}_{33}$	3357	**2**	**1.89**
33	$5,13\text{- }5,17\text{-diMeC}_{33}$	3373	0.04	0.18
34	2-MeC_{33}	3386	0.78	0.74
35	$n\text{-}C_{34}$	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	$9\text{-/ }11\text{-MeC}_{35}$	3555	0.88	0.88
41	$13,x\text{-diMeC}_{35}$	3566	0.51	0.5
42	$9\text{-/ }11\text{-MeC}_{37}$	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.

SUPPLEMENTARY MATERIALS

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14	$n\text{-}C_{30}$	3002	**1.11**	**1.05**
15	$12,14\text{-diMeC}_{30}$	3025	0.33	0.32
16	$12,14\text{-diMeC}_{30}$	3029	0.54	0.65
17	$10,14\text{-diMeC}_{30}$	3055	0.18	0.57
18	$C_{31:1}$	3074	**1.47**	**1.41**
19	$n\text{-}C_{31}$	3095	**2.16**	**2.09**
20	$9\text{-/ }11\text{-/ }13\text{- and }15\text{-MeC}_{31}$	3127	0.45	0.61
21	$9,x\text{-diMeC}_{31}$	3152	0.29	0.27
22	$7,17\text{-/ }9,15\text{-/ }9,17\text{-/ }11,15\text{- and }13,17\text{-diMeC}_{31}$	3160	0.72	0.85
23	7-MeC_{31}	3164	0.1	0.32
24	3-MeC_{31}	3171	0.9	0.96
25	$5,13\text{- and }5,17\text{-diMeC}_{31}$	3176	0.59	0.56
26	$7,13,17\text{-triMeC}_{31}$	3189	0.7	0.75
27	$3,9\text{-/ }3,11\text{-/ }3,13\text{-/ }3,15\text{- and }3,19\text{-diMeC}_{31}$	3200	0.47	0.62
28	$10\text{-/ }14\text{- and }15\text{-MeC}_{32}$	3224	**1.11**	**1.17**
29	$4\text{-MeC}32\text{ and }10,14\text{-diMeC}_{32}$	3253	**1.39**	**1.41**
30	$7\text{-/ }9\text{-/ }11\text{-/ }13\text{-/ }15\text{-/ }17\text{- and }19\text{-MeC}_{33}$	3326	**1.43**	**1.42**
31	$11,15\text{-/ }13,17\text{- and }15,19\text{-diMeC}_{33}$	3352	0.65	0.64
32	$9,15\text{- and }11,17\text{-diMeC}_{33}$	3357	**2**	**1.89**
33	$5,13\text{- }5,17\text{-diMeC}_{33}$	3373	0.04	0.18
34	2-MeC_{33}	3386	0.78	0.74
35	$n\text{-}C_{34}$	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	$9\text{-/ }11\text{-MeC}_{35}$	3555	0.88	0.88
41	$13,x\text{-diMeC}_{35}$	3566	0.51	0.5
42	$9\text{-/ }11\text{-MeC}_{37}$	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.
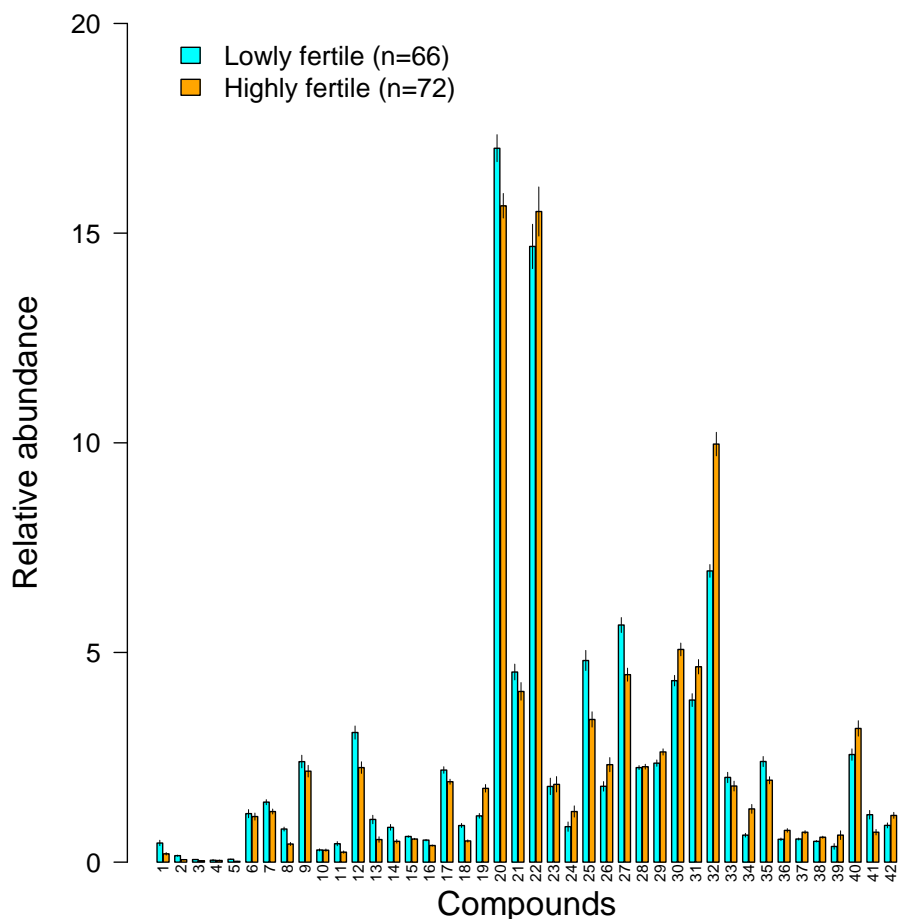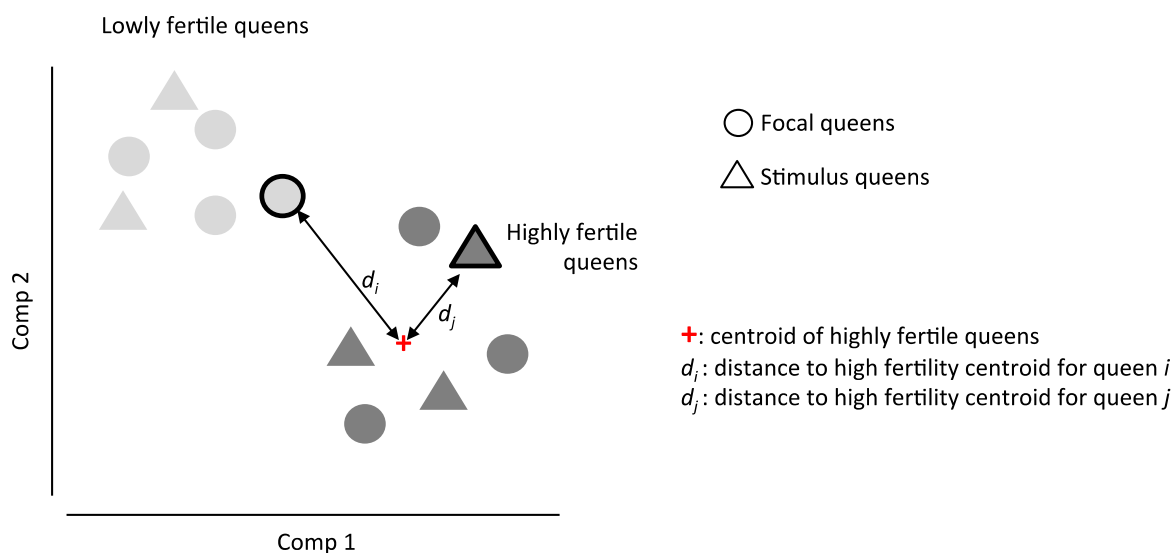

Figure S2. Schematic explanation of how the distance of each queen to high fertility signature was determined. The distance d_i is the distance of the focal queen i to the centroid of highly fertile queens and the distance d_j is the distance of the stimulus queen j to the centroid of highly fertile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