Are variations in cuticular hydrocarbons of queens and workers a reliable signal of fertility in the ant *Harpegnathos saltator*?

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One of the key features of insect societies is the division of labor in reproduction between one or a few fertile individuals and many sterile nestmates that function as helpers. The behavioral and physiological mechanisms regulating reproduction in ant societies are still not very well understood, especially in species in which all colony members are reproductively totipotent. In the ponerine ant *Harpegnathos saltator*, queen-worker dimorphism is very limited, and a few mated workers reproduce (''gamergates'') once the founding queen becomes senescent. Worker oviposition is regulated by highly directed aggressive interactions among nestmates, who can recognize different levels of ovarian activity. We show that variations in cuticular hydrocarbons (CHC) correlate with oogenesis, both for queens and workers. 13,23-Dimethylheptatriacontane is present in egg-layers, but not in infertile workers and queens. Proportions of other CHCs vary as well, resulting in clear separation of the ants in a multivariate analysis. Egg-layers are characterized by an elongation of the chain length of CHCs. We used solid-phase microextraction to measure CHCs in live ants that were experimentally induced to start producing eggs. Over a period of 118 days, CHC profiles of infertile workers changed completely to that of reproductives. The effect of age can be excluded in this modification. This striking correlation of ovarian activity with CHC variation and its correspondence with the observed recognition behavior exhibited by the workers toward egg-laying nestmates suggests that CHCs serve as a fertility signal in the ant *H. saltator*, a reliable basis for regulating reproduction.

A striking phenomenon in biology is the evolution of animal societies in which only a minority of individuals transfer their genes to future generations whereas all others give up personal reproduction and help rearing the offspring of relatives. Hamilton's kin selection theory explains why abstaining from reproduction can be evolutionarily stable. As long as the helpers contribute sufficiently to the reproductive success of relatives, they compensate for the loss of their own direct fitness (1). In ant societies, the queens usually produce all of the colony's offspring whereas the workers raise the brood to adulthood. Workers are morphologically divergent and lack a functional sperm reservoir in most species. They have retained functional ovaries and can lay unfertilized eggs that develop into males, but they usually only do this once their queen has died (reviewed in refs. 2 and 3). This worker infertility can result from either self-restraint or mutual control (4), because male production by workers in queenright colonies often has a negative effect on colony productivity.

In situations in which the reproductive interests of queens and workers converge, worker oviposition can be regulated with a simple exchange of information. Many investigations have shown that queens affect the fertility of workers by pheromones (reviews in, e.g., refs. 5 and 6). Indeed, direct contact with the queen's body parts is sufficient for the workers of *Plagiolepis pygmaea* to have undeveloped ovaries (7), and, in *Oecophylla* weaver ants, even queen corpses can prevent workers from producing males (8). It was previously widely accepted that queen pheromones inhibited the workers' reproductive physiology. Alternatively, it was proposed that pheromones inform nestmates about a healthy queen's presence (9–15). Provided that the queen signal is honest, workers can maximize their inclusive fitness by modifying their behavior and refraining from producing sons (15).

What guarantees the honesty of a queen signal? Keller and Nonacs (15) predicted a correlation between the pheromone output of queens and fecundity. Workers benefit from rearing queen offspring because of the queens' greater productivity (16). Most behavioral studies indicate that it is the egg-laying capacity of the queen that is being advertised to the nestmates (e.g., refs. 17–20). For example, only egg-laying queens *of Solenopsis invicta* can effectively suppress fertility in alate nestmate queens (21) with a pheromone that is produced in the poison gland (22).

Further evidence for the chemical signaling of ovarian activity comes from experimentally manipulated colonies in which previously infertile workers were able to start laying eggs. In *Gnamptogenys menadensis*, *Diacamma* sp., and *Harpegnathos saltator*, as well as honeybees, workers with recently developed ovaries were attacked by infertile nestmates (23–26). Similar results were found in *Rhytidoponera confusa* as well as in a single colony of *Aphaenogaster cockerelli* (27, 28). These data suggest that the onset of oogenesis causes a modification of some external characteristics on which recognition is based.

Recent studies suggest that the paradigm of volatile secretions produced in an exocrine gland to mediate reproduction needs re-examination. In the queenless ant *Dinoponera quadriceps*, aggressive interactions among a small number of reproductively totipotent workers lead to hierarchical relationships. The top-ranking worker lays eggs, and its cuticle differs from that of infertile subordinates in the relative amount of one alkene, 9-hentriacontene (29). By using the nondestructive technique of solid-phase microextraction, temporal changes in the cuticular signature were documented in live workers that had been allowed experimentally to gain top rank and start laying eggs (30). Scattered reports in the literature also support a correlation between ovarian activity and an individual's blend of cuticular hydrocarbons: e.g., in the wasp *Polistes dominulus* and the bumble bee *Bombus hypnorum* (31, 32), as well as in various solitary insects (e.g., refs. 33–35). Although it has not yet been demonstrated that these variations in hydrocarbons are used by the ants to recognize egg-layers, such a function is entirely

Abbreviations: CHC, cuticular hydrocarbon; DA, discriminant analysis; diMe-C37, dimethylheptatriacontane; GC, gas chromatography; MS, mass spectrometry; KI, Kovat's Retention Index.

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coherent with the recent findings of Tsuji *et al.* (36) that a signal regulating egg-laying is transmitted by direct contact in the ant *Diacamma*. These and other results (e.g., refs. 23 and 25) suggest that such cuticular chemical signals consist of long-chain hydrocarbons with low volatility.

The primary function of cuticular lipids is protection against desiccation (37), but, given the large variability in their chemistry and dynamics, they would be highly suitable for serving as signals indicating the emitter's social status. They can be used in different contexts, such as species-recognition in insects (reviewed in ref. 38) or colony identification in social insects (e.g., ref. 39; reviewed in refs. 40–43).

We have studied the cuticular hydrocarbons of both queens and workers in the ponerine ant *H. saltator*, in which we have clear behavioral evidence that workers are able to recognize egg-layers (25, 44). Like in most species of the Ponerinae, caste divergence is very limited, and the queen is not very fecund (45). In some species, including *H. saltator*, workers can mate. Although a new colony of *H. saltator* is founded by a young queen, once the foundress is senescent, she is replaced by several gamergates (mated, egg-laying workers) (46) who perpetuate the colony (47). This peculiar life cycle offers the rare opportunity to conduct a comparative study of the putative signals from two categories of reproductives with different larval development. Gamergates' ovaries are only slightly shorter than the queens', and they lay about half as many eggs (44). We first compared the hydrocarbon profiles of individuals differing in morphological castes and reproductive status, then monitored the temporal changes in hydrocarbons of live workers that began to reproduce. We discovered a striking correlation of hydrocarbon patterns with the ovarian activity of the individuals. These findings contribute to a better understanding of signal evolution and reproductive division of labor in social insects.

Methods

Colony Collection and Ant Maintenance. Fourteen colonies of the ponerine ant *H. saltator* were collected in the Western Ghats and near Bangalore, Karnataka State, India between 1992 and 1995. They were cultured in the laboratory at 25°C with 12 h light/12 h dark. The ants lived in plastic boxes with a layer of plaster-of-Paris containing a carved out nest that was covered with a glass plate. No chemicals were used to prevent the ants from escaping. The ants were regularly provided with water and were fed with live crickets.

Gas Chromatography. For extraction of cuticular hydrocarbons (CHCs) from the gaster of the ants, individuals were immobilized on a glass-tube, which was cleaned regularly. CHCs were measured by using the solid-phase microextraction method (48). A fiber (SUPELCO, coated with a 7-μm polydimethylsiloxane film) was directly rubbed on the gaster surface of the ants for 5 min (29). The fiber was then directly inserted in the injection port of a Carlo Erba 8130 gas chromatograph equipped with a DB-1 (J & W Scientific, Folsom, CA) nonpolar capillary column (30 m $\times$ 0.32 mm $\times$ 0.25 μm). Helium was used as carrier gas with a column head pressure of 95 kPa. The fiber was desorbed at 260°C in the splitless mode for 4 min at a column temperature of 60°C. Oven temperature was programmed from 60°C to 250°C at 20°C min^{-1} and from 250°C to 300°C at 2.5°C min^{-1}. Flame ionization detector temperature was set at 310°C.

Gas Chromatography/Mass Spectrometry (GC/MS). GC/MS was performed on a Micromass MasSpec (Micromass, Altrincham, U.K.) double-focusing magnetic sector mass spectrometer (geometry EBE) connected to a Hewlett–Packard HP6890 II gas chromatograph, equipped with an HP-1 (Hewlett–Packard) nonpolar capillary column (30 m $\times$ 0.25 mm $\times$ 0.25 μm). The GC injection port and transfer line were operated at 320°C. For hydrocarbon analysis, the column temperature was held isothermal at 60°C for 5 min before rising to 220°C at 20°C min^{-1}, and then to 320°C at 3°C min^{-1} (isothermal for 10 min). For fatty acid methylester analysis, the column temperature was programmed from 60°C to 280°C at 10°C min^{-1}. Helium was used as a carrier gas at 1 ml min^{-1}, and samples were injected in the splitless mode with a splitless time of 2 min. Electron impact mass spectra were measured at 70 eV, with a source temperature of 200°C, an acceleration voltage of 8 kV, and a resolution of 700. The instrument was set to scan from m/z 45 to m/z 600 at 2 scans sec^{-1}. Kovat's Retention Indices (KIs) were determined for all cuticular lipids. To aid in the identification of branched alkanes, relative retention factors (ΔKI = KI (n-alkane C_n) $-$ KI (isoalkane C_n), on OV-1 phase] were calculated as described in the literature (49, 50). Because determination of ΔKI requires knowledge of the molecular weight of each hydrocarbon, a series of B/E linked scanning experiments were performed to assist molecular ion identification (50). The KIs of branched alkanes were checked against published data (51), where possible, to confirm assignment of the branching positions. GC/chemical ionization/MS was also used to confirm molecular weight. GC/chemical ionization/MS was performed on a Varian gas chromatograph coupled to a Finnigan-MAT (San Jose, CA) Magnum ion-trap mass spectrometer using acetonitrile as the chemical ionization reagent gas. Both linear and methyl branched alkanes gave strong molecular species ($[M + 40]^+$), allowing unambiguous molecular weight assignment (52). Alkenes showed fragmentation characteristic of the double bond positions (53, 54).

Specific analysis for the presence of fatty acids on the cuticular surface of *H. saltator* revealed traces of palmitic, palmitoleic, stearic, and oleic acids, together with their methyl esters. In total, however, they comprised less than 0.1% of the detected cuticular lipids and were therefore not included in the comparison of the different groups of colony members.

Experiments. From 13 colonies, CHCs were measured in reproductively active queens (n = 9, colonies 3, 5, 9–13), callow queens that had eclosed from the cocoons within the last 4 weeks (n = 4, colony 4), older alate queens before mating flight ("dispersing queens," n = 32, colonies 4, 7, 8), gamergates, n = 17, colonies 1–3, 5), and workers active inside ("inside," n = 24, colonies 1–4) and outside the colony ("outside," n = 24, colonies 1–4, 5). The latter two groups of infertile workers reflect a division of labor that is age-correlated; i.e., older workers hunt for prey above ground (J.L., unpublished data). Eighty-two percent of the individuals were sampled twice to control for any contamination of the extracts. When two chromatograms were available, we used the arithmetic mean of the peak areas. Peaks that could not be reproduced were very rare but were always excluded from the analysis.

Colony A was manipulated to study the ontogeny of the CHC profile when individuals start becoming reproductive. It initially contained 9 gamergates and 30 infertile workers, from all of which the CHCs were extracted twice. Then all gamergates were removed from the colony. The workers that subsequently became dominant were sampled again after 11, 22, 40, 60, 76, 98, and 118 days. At these intervals, the individuals were measured only once to keep the sampling interval short and the disturbance of the colony at a minimum. Dominant workers were dissected after the sampling was concluded, to identify their degree of ovarian development.

Data Analysis. There was a high intracolonial variability of CHC profiles of the workers, which has been recognized previously (55). Thus, the number of compounds used in the analysis was reduced: we only included the peaks with a relative peak area of more than 1% occurring in 75% of the members of at least one

Table 1. Hydrocarbons present on the cuticle of *H. saltator*

Number*	Hydrocarbon**	Number*	Hydrocarbon**	Number*	Hydrocarbon**	Number*	Hydrocarbon**
1	*n*-C23†‡		4-MeC30†‡§		*n*-C33†‡§	13	13-MeC35 (45%) +
2	*n*-C25†‡	7	11-C31:1†‡	10	11-MeC33 (10%) +	13	15-MeC35 (30%) +
	n-C26†‡	7	9-C31:1†‡	10	13-MeC33 (30%) +	13	17-MeC35 (25%)†‡§
15¶	*n*-C27:1†‡		*n*-C31†‡§	10	15-MeC33 (50%) +	14	13,17-DimeC35 (20%) +
3	*n*-C27†‡	8	13-MeC31 (50%) +	10	17-MeC33 (10%)†‡§	14	15,19-DimeC35 (80%)†‡§
	n-C28†‡	8	15-MeC31 (50%)†‡§		9-MeC33†‡§	14	13,21-DimeC35 (50%) +
	4-MeC28†‡§		7-MeC31†‡§	11	13,17-DimeC33 (50%) +	14	13,23-DimeC35 (50%)
4	9-C29:1 (25%) +		11,17-DimeC31 (33%) +	11	13,19-DimeC33 (50%)†‡§	14	7,15-DimeC35 (35%) +
4	11-C29:1 (75%)†‡		11,19-DimeC31 (33%) +	11	11,21-DimeC33†‡§	14	7,17-DimeC35 (65%)†‡§
5	*n*-C29†‡§		11,21-DimeC31 (33%)†‡§	11	7,15-DimeC33 (60%) +		14,22-DimeC36 (35%) +
6	13-MeC29 (75%) +		7,11-DimeC31 (40%) +	11	7,17-DimeC33 (40%)†‡§		14,24-DimeC36 (65%)‡§
6	15-MeC29 (25%)†‡§		7,15-DimeC31 (60%)†‡§		12-MeC34 (33%) +		9-C37:1†‡
	7-MeC29†‡§		5,17-DimeC31 (50%) +		14-MeC34 (33%) +		13-MeC37 (20%) +
	4-MeC29†‡§		5,19-DimeC31 (50%)†‡§		16-MeC34 (33%)†‡§		15-MeC37 (40%) +
	3-MeC29†‡§		14-MeC32 (60%) +		14,22-DimeC34 (50%) +		17-MeC37 (20%) +
	n-C30†‡§		16-MeC32 (40%)†‡§		14,24-DimeC34 (50%)†‡§		19-MeC37 (20%)‡§
	12-MeC30 (30%) +	9	11-C33:1†‡	12	11-C35:1†‡	16‖	13,23-DimeC37‡§
	14-MeC30 (70%)†‡§	9	9-C33:1†‡	12	9-C35:1†‡		

The names of the compounds have been abbreviated as follows: C + number, length of the main chain; Me, one methylbranch; Dime, two methylbranches; :1, indicates an alkene.

*Same numbers indicate compounds that were pooled as one area for the analysis.

†Molecular ion present in EI spectrum.

‡Molecular species ($[M + 40]^+$) present in CI spectrum.

§Molecular weight confirmed by B/E linked scanning.

¶The peak was only included for the analysis of colony A.

‖Peak was not included in the discriminant analysis because it is only present in reproductive individuals.

**The analysis was based on gamergates and infertile workers; additional analyses have been performed on dispersing and reproductive queens, which revealed, for example, the pronounced presence of alkadienes in dispersing individuals.

group from the 13 colonies initially investigated. This arbitrary selection reduced the number of peaks considered to 23. All peak areas were then standardized to 100%. Nine compounds or compound classes showed qualitative differences among the groups and were thus compared separately. The remaining 14 peaks were subject to a multivariate analysis after a further standardization to 100%. Because the peak areas represent compositional data, the areas were transformed according to the formula of Reyment (56): $Zi,j = \log[Xi,j/g(Xj)]$, where Xi,j represents the area of peak i for ant j, $g(Xj)$ the geometric mean of the areas of all peaks for ant j, and Zi,j the transformed area of peak i for ant j. To reduce the number of describing variables we performed a principal component analysis. The extracted factors were then used to separate the groups with a discriminant analysis (DA).

In colony A, the 14 peaks previously selected and, additionally, the compound peak of 9- and 11-heptacosene (C27:1) were analyzed, because these substances occurred in all of the investigated groups. For the DA, we divided the colony members into the following groups: gamergates, callow workers, and workers collected inside as well as outside the colony. Furthermore, we separated from the inside workers the callow workers as additional group. The data obtained from the new egg-laying workers remained ungrouped and were added to the two-dimensional representation of the DA for comparison.

For the analysis of chain length and reproductive status, we introduced the following index of relative frequency: The median of the relative areas of the 15 compounds in reproductives divided by the median of the relative peak areas of the 15 selected compounds of reproductives plus the median of the 15 selected compounds of nonreproductive individuals. The same was calculated for both egg-laying queens and gamergates.

Results

Differences in the Hydrocarbon Profiles. GC/MS of the cuticular lipids of *H. saltator* revealed a complex mixture of linear alkanes and alkenes, methyl-branched alkanes, and dimethyl-branched alkanes (Table 1). Gamergates and reproductive queens produced 13,23-dimethylheptatriacontane (diMe-C37), which could not be detected in their infertile nestmates (Fig. 1). In addition, egg-laying and infertile groups differed in their proportions of other substances, which was revealed by a multivariate analysis. A principal component analysis of the standardized and transformed data set produced four principal components that explain 89% of the observed variability. They comprised the basis for the DA in Fig. 2. The reproductives were separated from the nonreproductive groups on the basis of discriminant function 1, which explains 72.5% of the total variation. The classification

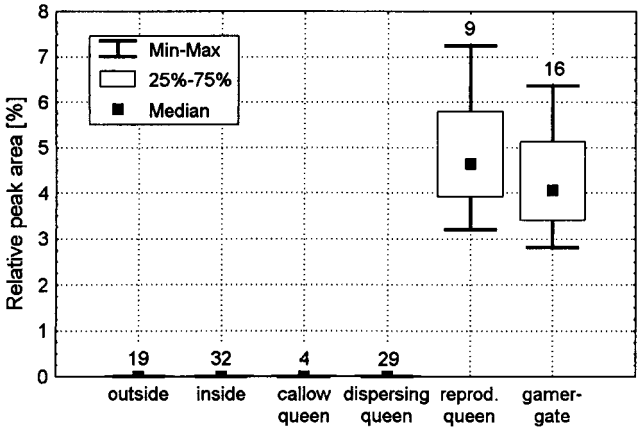

Fig. 1. Qualitative difference in the presence of 13,23-dimethylheptatriacontane between reproductives and nonreproductive individuals. Gamergates and reproductive queens are characterized by the presence of this long-chained dimethylbranched alkane.

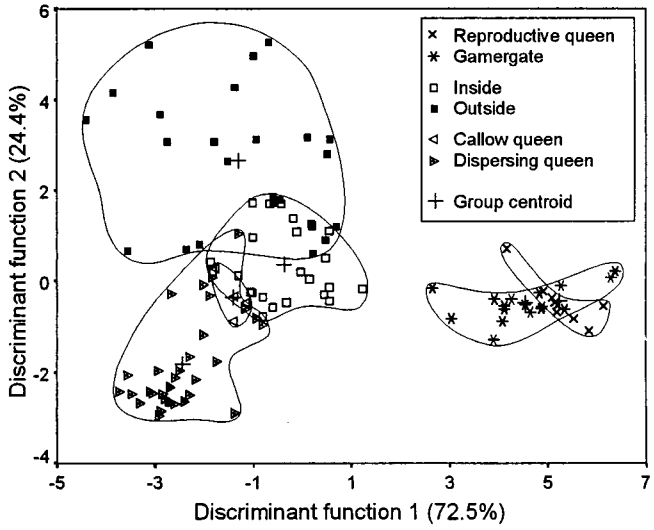

Fig. 2. Discriminant analysis of 6 groups of individuals from 13 colonies. Reproductive and nonreproductive individuals are separated completely (see Table 2) on the basis of the relative areas of 14 compound peaks indicated in Table 1. The groups are encircled arbitrarily.

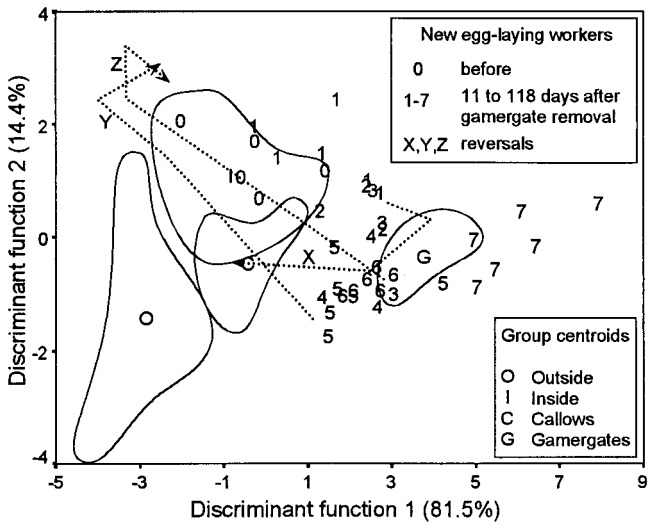

Fig. 3. The change of the hydrocarbon profiles of individuals that activated their ovaries. The discriminant analysis includes all members of colony A classified in four worker groups (gamergates, $n = 9$; callows, $n = 9$; workers from inside, $n = 15$; and from outside, $n = 6$). The differences in the CHC profiles of worker groups are illustrated by the respective encirclements based on the position of the individual group members similar to Fig. 2. Group centroids are marked. The change in the CHC profiles of those workers that started egg-laying after gamergate removal (new egg-laying workers) is indicated by numbers, which represent subsequent sampling periods (for respective sample days and size see Fig. 5). The change of the profiles of three workers that lost their rank (''reversals'') is indicated by dotted lines, whereas the arrows show the directions (worker X sampled at days 48, 52, 58, and 67; Y at days 46, 56, 66, and 74; Z at days 50, 60, 65, and 72).

reveals a 100% separation between these functional groups (Table 2). As shown in Fig. 2, gamergates and reproductive queens cluster together, which is also reflected in the classification results: 22.2–23.5% were classified into the wrong group of reproductives. The reproductives can therefore clearly be separated from nonreproductive individuals on the basis of quantitative differences in some of the CHCs, notwithstanding the characteristic presence of 13,23-diMe-C37 in reproductives.

The nonreproductive groups were ordered along axis 2 with 24.4% of explained variance. Workers from inside and outside the nest were separated despite some overlap of the groups. This overlap is not surprising because these are arbitrary groups in a continuum expressing the behavioral differentiation of workers as they age. When infertile workers get older, they become less active inside the nest, and instead they forage for prey outside.

Dispersing queens are separated from the inside workers, with the callow queens located between both groups. In addition, the pronounced presence of pentatriacontadiene (C35:2; median, 26.6; range, 9.4–34.5) and heptatriacontadiene (C37:2; median, 10.2; range, 1.2–24.6), which were not included in the DA, characterizes the dispersing queens. These alkadienes are only weakly present in callow queens (C35:2; range, 4.5–7.4) (C37:2; range, 0.5–1.6) and inside workers (C35:2; range, 0.5–9.0) (C37:2, range, 0–2.0) but are almost absent in outside workers and completely missing in reproductive queens and gamergates.

Although egg-laying queens and gamergates originated from 13 colonies, they clustered together regardless of colony membership. Therefore, by using the peaks that we selected, differences in profiles were linked with reproductive status, not colony identity.

Table 2. Classification results of the discriminant analysis from 13 colonies

	Predicted group					
Group	RQ	G	CQ	DQ	I	O
Reproductive queen (RQ)	77.8	22.2	0	0	0	0
Gamergate (G)	23.5	76.5	0	0	0	0
Callow queen (CQ)	0	0	100	0	0	0
Dispersing queen (DQ)	0	0	28.1	71.9	0	0
Inside (I)	0	0	37.5	0	54.2	8.3
Outside (O)	0	0	8.3	4.2	25.0	62.5

The Ontogeny of Hydrocarbon Profiles of Egg-Laying Individuals. Although we could show qualitative as well as quantitative differences in the CHC profiles of egg-laying versus infertile individuals, it is important to exclude that this is simply the consequence of different ages. Using a complete colony containing workers only, we induced nonreproductive workers to activate their ovaries by removing the gamergates, and we monitored their CHC profiles at regular intervals. Orphaned workers usually lay eggs after about 14 days (44).

The initial sampling and analysis of all members of colony A confirmed the results about the separation of the groups we found in the other 13 colonies. The gamergates were classified in one group without any error (Fig. 3) whereas all nonreproductive workers were separated either into ''callows,'' inside, or outside workers. The profiles of callow workers did not overlap with those of outside workers whereas both the outside workers and the callows overlapped with inside workers. This is not surprising because inside workers are older than callows but younger than outside workers.

The removal of the gamergates triggered aggressive interactions in the following 2 days that represents the regular pattern observed in several other colonies (25). Before workers establish themselves as new egg-layers in a colony, they change their behavior. They become aggressive and show typical dominance displays that make it easy to identify them (44). In colony A, six inside workers behaved like dominants. Consequently, their CHC profile was measured regularly. Between 22 and 40 days after removal of the gamergates, three of these dominant workers lost their rank, and their behavior reverted to that of

subordinates; i.e., they crouched when being approached by the dominant workers, and they no longer interacted aggressively with the dominants. At the same time, three other individuals started to become aggressive and behaved like dominants. Thus, at the end of the experiment (118 days after orphanage), six dominant workers had established themselves as new egg-layers. These six individuals and those that lost their rank were dissected to confirm their ovarian status. The ovaries of the new egg-layers contained four to five yolky oocytes, which represents an average degree of ovarian activity for gamergates. However, their spermathecae were empty; i.e., they were not gamergates. As soon as males would have been available, these individuals could have mated. Two workers that lost their ranks had no yolky oocyte in their ovaries (one died and could not be dissected).

Correlated with these changes in social status and physiology, there were modifications in the CHC profiles. The individuals that became dominant originated from the inside group, and they initially showed the typical CHC profiles of this group (Fig. 3). Within the first 40 days after removal of the gamergates, the profiles of the dominant individuals became similar to those of the gamergates. From day 40 to day 98, the CHC profiles of the dominant workers changed only slightly. Within the final 20 days, there was again a pronounced change of the profiles in all dominant workers. In contrast to these egg-layers, the CHC profiles of the workers that lost their rank reverted to the profile of their original group (Fig. 3).

The divergent development of the CHC profiles in the dominants and the individuals that lost their rank shows that age does not determine the gamergate profile. The changes in the CHC profiles are, rather, the consequence of changes in the reproductive and social status of the respective individual. This is independent of mating status because all of the newly dominant workers were unmated although showing a similar profile to the gamergates.

Biochemical Basis of the Differences in Hydrocarbon Profiles. The analysis of the qualitative differences between reproductive and nonreproductive individuals showed that one substance, 13,23-diMe-C37, was only present in reproductive queens and gamergates (Fig. 1). On the other hand, the quantitative analysis revealed that the whole pattern of the CHC profiles differs among the groups. In fact, the mechanism underlying the qualitative as well as quantitative differences appears to be the same. Reproductive and nonreproductive individuals differ in the chain length of their CHCs. This was shown by using a relative frequency index. When plotting the equivalent chain length of the 15 compounds against this index, we found a strong positive correlation (Pearson's $r = 0.92$; $P < 0.0001$) (Fig. 4). This means that shorter chained molecules are present in smaller relative amounts in reproductive individuals whereas longer chained hydrocarbons are decreasing in nonreproductives. This pattern is also present at a finer scale. When comparing egg-laying queens with gamergates, we also got a positive but weaker correlation (Pearson's $r = 0.83$; $P < 0.0001$) (Fig. 4).

A similar correlation between reproductive status and chain length of the CHCs was found for the alkenes of the new reproductives in colony A. We followed the change in the relative amounts of alkenes in the CHC profiles with increasing ovarian activity during the 118 days of this experiment (Fig. 5). Heptacosenes (C27:1) as well as the nonacosenes (C29:1) continuously decreased in their relative amounts. On the other hand, the long-chained tritriacontenes (C33:1) and pentatriacontenes (C35:1) showed a continuous increase in their relative amounts.

Discussion

Correlation Between Cuticular Hydrocarbons and Ovarian Activity. The differences in the CHC profiles of both queens and workers are correlated with their respective physiological condition: i.e., the activity of their ovaries. We found qualitative and quantitative differences between reproductive and nonreproductive individuals. Importantly, 13,23-diMe-C37 was only present in egg-laying queens and gamergates. Furthermore, other CHCs also varied according to ovarian activity; e.g., gamergates and reproductive queens were clearly separated from nonreproductive individuals in the DA.

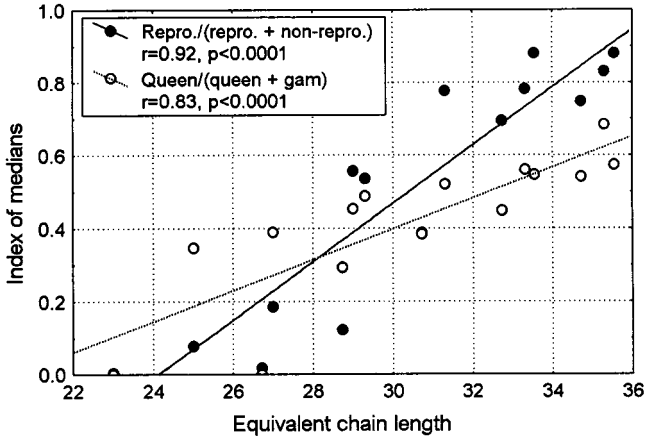

Fig. 4. Comparison of 15 compound peaks (see Table 1) that were present in reproductive queens and gamergates and nonlaying individuals. The equivalent chain length was correlated with a relative peak index (see *Methods*), which represents the relative amount of the compounds in the respective group. The strong correlations suggest that the hydrocarbons are elongated with increasing ovarian activity.

Differences in age cannot explain these results, although queens and gamergates of *H. saltator* can reach greater ages than their nestmates (44), which is generally known from ant queens (57, 58) and has also been shown for gamergates in the ponerine ant *Diacamma* sp. (59). Even if the gamergates of colony A had been the oldest individuals in the colony, their CHC profiles are similar to those of the new egg-layers, although the latter just recently differentiated from the relatively young inside workers. The CHC-profiles of the three other dominant workers that subsequently lost their rank reverted to the original state: i.e., profile of the inside group.

Caste can also be excluded as a factor for the major differences because reproductive queens and gamergates cluster together, and they are completely separated from nonreproductive groups (including infertile queens and workers). Despite their similarities, reproductive queens showed more long-chained CHCs in their profile than gamergates. This difference is most likely attributable to the queen's higher levels of ovarian activity. Indeed, queens can lay more than twice as many eggs as gamergates per day (44), although they do not always reach this maximum.

A striking difference between the queens before mating flight and all other groups in the colony is the prevalence of alkadienes in their CHC profiles. We speculate that these particular compounds play a role during mating. It may also be connected to the specific pattern of ovarian development of dispersing queens, because we have evidence that their ovaries contain eggs even though they do not oviposit in their natal colony (44).

Biochemistry of the Hydrocarbon Variation. Recent investigations of the production of CHC sex pheromones in the house fly *Musca domestica* demonstrate that differential activities of elongase-enzymes regulate the generation of hydrocarbons of different chain length (37). Most likely, a similar biochemical mechanism is involved in *H. saltator*, leading to variations in the CHC profiles

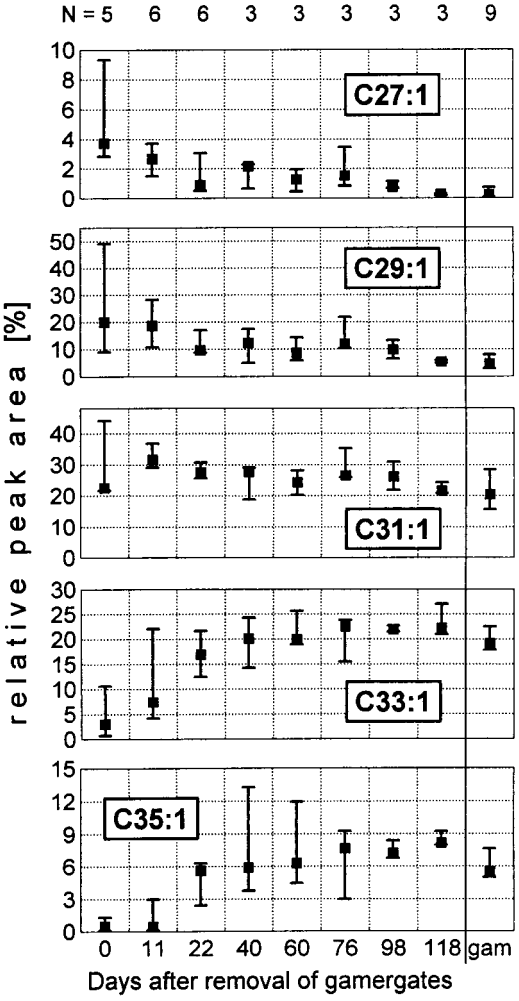

Fig. 5. The relative change of alkenes in the cuticular hydrocarbon profiles of new egg-laying workers after orphanage of the colony. At day 40, the sample size decreased to 3 because of the three workers that lost their rank at that time and were therefore excluded. For comparison, the relative amounts for the gamergates ($n = 9$) that were laying eggs for more than six month were given in the column "gam." The short-chained alkenes show an inverse trend to the larger alkenes.

of reproductive and nonreproductive individuals. With increasing ovarian activity, the fatty acid precursors of CHCs are elongated by one or two acetate-units. This would cause a pronounced shift from short-range to long-range CHCs, and would lead to qualitative differences in the proportions of short-chain as well as long-chain compounds. In fact, egg-laying queens and gamergates are characterized by the exclusive presence of 13,23-diMe-C37, which is the largest compound resolved by the GC/MS-analysis. In addition, this mechanism would also explain our findings that the relative quantities of compounds in reproductive individuals decrease in the short-chain range and increase in the long-chain range.

Recognition of Different Levels of Ovarian Activity. We demonstrated a correlation between ovarian activity and the variation of CHC profiles in *H. saltator* individuals. Thus, information about ovarian activity is encoded in the CHC profiles, which the ants could use to assess each others' fertility. In this way, the ants can recognize the reproductive state of their nestmates solely on the basis of the chemical information, and no other behavioral interactions are needed. For example, workers kept for 22 days without gamergates (colony A) developed CHC profiles that were strikingly different from the profiles they exhibited in the presence of the gamergates (see Figs. 3 and 5). Consistent with this, we previously demonstrated in behavioral experiments that nonreproductive workers attack nestmates that had developed their ovaries during 20 days of isolation from the reproductives (25).

The results from colony A also showed that the CHC profiles of the new egg-layers became similar to those of the gamergates after 40 days (Fig. 5). This is also consistent with the results of another separation experiment. Workers that had been isolated from gamergates for a period of about 90 days and were subsequently returned to their colony were not attacked but were treated like the existing gamergates (44). Any aggression or dominance displays were not necessary to retain their status. This showed clearly that the information about ovarian activity had been perceived by olfaction.

The CHC profiles of gamergates showed small differences in chain length compared with those of reproductive queens (Fig. 4). Therefore, it is possible that the colony members can distinguish between gamergates and reproductive queens. This is exactly what we found. In an additional experiment, workers had been isolated from queenright colonies for about 90 days. When they were returned to their colony, they were attacked by infertile workers in three of four colonies. This is in marked contrast to the results obtained with gamergate colonies (44). Natural colonies have never been found with both a queen and gamergates.

Our data on the correlation with ovarian activity indicate that CHC profiles change gradually, not suddenly, and thus the variation in the CHC profiles reflect the various levels of fertility. The behavioral results about the differential treatment of workers with either partly or well developed ovaries confirm that ovarian activity is not perceived in an all-or-nothing manner, which seems to be a general pattern in ants. In *Myrmecocystus mimicus* (19) and *Lasius niger* (20), workers preferred the most fertile queens in foundress associations. Similarly, when workers could choose among several queens of *Leptothorax* sp. A that were isolated from each other within the same nest, they preferentially stayed with the most fertile queens (18). Workers of the fire ant *Solenopsis invicta* killed less fertile queens from the polygynous colony type, but not more fertile queens from polygynous colonies (17), although genotype discrimination may interfere (60).

The examples of *H. saltator* and from other ant species show that workers can accurately assess the ovarian activity of nestmates and that they adapt their behavior accordingly. Chemical communication must be invoked to explain the observed pattern of reactions to individuals with different levels of ovarian activity. We assume that the ants use either the relative composition or the absolute amounts of CHCs to be informed about each others' ovarian activity because qualitative as well as quantitative differences between the reproductive and nonreproductive individuals exist.

Fertility Signal. The most important feature of a fertility signal is its reliability because workers benefit from helping the queen only when her productivity is sufficiently high (15). When this declines, there is a point when workers benefit more from their own reproduction. Thus, they should either replace her or start reproducing themselves. Because workers are more related to their own offspring than to that of their sister nestmates, they should try to be among the reproductive individuals. Therefore, workers should always carefully monitor the fertility and health of their mother to have the best chances in the competition for reproduction when she dies (16, 61). Because in many ant species workers start reproducing when the queen is removed or has died

(2, 3, 57), this function of a fertility signal may be most important and should be expected in all insect societies. In *H. saltator* this function may be crucial because queens and gamergates are replaced by other workers, which thus represent hopeful reproductives (62).

Our analysis indicates that CHCs have all of the design features needed for a fertility signal. CHCs yield reliable information about rates of egg-laying; i.e., intermediate ovarian development can be perceived. There is only a little time lag between the change of ovarian activity and the change in the CHC profile, both for the development of the reproductive profile and also for its reversion. Because the information about fertility is encoded in the CHCs, and not secreted from a specialized exocrine gland, one could argue that the CHCs are merely a cue that provides information to nestmates (e.g., ref. 9) without being under selection as a fertility signal. We argue, however, that it is a true fertility signal, always selected to be a reliable indicator of the reproductive status of nestmates. In fact, the existence of mutual control of egg-laying among workers (worker policing sensu 4) in *H. saltator* indicates that the chemical recognition of ovarian activity should be under selection. Worker policing is expected when worker reproduction causes costs associated with reduced colony productivity or when the relatedness of the worker offspring to other workers in the colony is too low in comparison to the offspring of the queen or gamergates (4, 63). Worker policing can be expressed by eating eggs (e.g., refs. 64 and 65) or by attacking and inhibiting workers with weakly developed ovaries as found in *H. saltator* (25).

Assuming mating with a single male, relatedness is high in queenright colonies of *H. saltator*, and it remains high in gamergate colonies as a consequence of workers copulating with their brothers (47). Such high intracolonial relatedness does not promote worker policing, and instead costs to colony productivity seem to be the important factor in this species. If any worker starts producing male or female eggs, there would be a surplus production of eggs and, in addition, too many reproductives would interfere with an efficient division of labor, which is the basis for high colony productivity. Although several young workers start to reproduce after the queen's death, once they are established as gamergates, further attempts at reproduction by individual workers are prevented by infertile nestmates because prolonged disputes about reproduction also reduce colony productivity.

Nevertheless, an individual worker may still benefit from own reproduction in the presence of a reproductive queen or gamergates even though her additional eggs lead to a marginal reduction in colony efficiency. However, she can only succeed if she is not detected and consequently policed by her nestmates. This is impossible because she cannot conceal the modifications in her CHCs. On the other hand, the existence of a reliable fertility signal benefits the average worker by allowing her to identify both the most fertile individual(s) whose reproduction she favors as well as cheaters whose reproduction she does not favor. A fertility signal should therefore invade a system in which a cost-free, concealable signal was produced.

In *H. saltator* the production of the CHCs indicating fertility results in indirect costs because a worker with incompletely developed ovaries is attacked and inhibited by nestmates in the presence of an egg-laying queen or gamergates. In contrast, the direct costs of the presumptive fertility signal seem minor because an elongation of hydrocarbons or their differential production are not expensive. The production of CHCs nevertheless appears to be directly linked to fertility by the differential activity of certain enzymes (see "Biochemistry of the Hydrocarbon Variation," above) and thus is part of the direct costs of reproduction. The bearer of such chemicals, however, benefits if she is among the established reproductives in the colony: i.e., if she produces enough eggs to benefit her nestmates. Thus, such chemicals that inform about ovarian activity can be viewed as a fertility signal. It would represent an index (66) or a badge, similar to those discussed in the context of dominance interactions in birds (e.g., refs. 67–69).

Many phenomena have been shown to result from the existence of a "queen pheromone" in social insects. All of these could be explained by our presumptive fertility signal, provided that colonies are small, which enables individual contact with reproductives.

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1. Hamilton, W. D. (1964) *J. Theor. Biol.* **7,** 1–52.
2. Bourke, A. F. G. (1988) *Q. Rev. Biol.* **63,** 291–311.
3. Choe, J. C. (1988) in *Advances in Myrmecology*, ed. Trager, J. C. (Brill, Leiden, the Netherlands), pp. 163–187.
4. Ratnieks, F. L. W. (1988) *Am. Nat.* **132,** 217–236.
5. Fletcher, D. J. C. & Ross, K. G. (1985) *Annu. Rev. Entomol.* **30,** 319–343.
6. Vargo, E. L. (1998) in *Pheromone Communication in Social Insects: Ants, Wasps, Bees, and Termites*, eds. Vander Meer, R. K., Breed, M. D., Espelie, K. E. & Winston, M. L. (Westview, Boulder, CO), pp. 293–313.
7. Passera, L. (1980) *Insect. Soc.* **27,** 212–225.
8. Hölldobler, B. & Wilson, E. O. (1983) *Ann. Entomol. Soc. Am.* **76,** 235–238.
9. Seeley, T. D. (1985) *Honeybee Ecology: a Study of Adaptation in Social Life* (Princeton Univ. Press, Princeton, NJ).
10. Woyciechowski, M. & Lomnicki, A. (1987) *J. Theor. Biol.* **128,** 317–327.
11. Seger, J. (1991) in *Behavioural Ecology*, eds. Krebs, J. R. & Davies, N. B. (Blackwell Scientific, Oxford), pp. 338–373.
12. Seger, J. (1989) *Nature (London)* **342,** 741–742.
13. Crespi, B. J. (1992) in *Cannibalism: Ecology and Evolution among Diverse Taxa*, eds. Elgar, M. A. & Crespi, B. J. (Oxford Univ. Press, Oxford), pp. 176–213.
14. Nonacs, P. (1993) in *Evolution and Diversity of Sex Ratios in Insects and Mites*, eds. Wrensch, D. L. & Ebbert, M. A. (Chapman & Hall, London), pp. 384–401.
15. Keller, L. & Nonacs, P. (1993) *Anim. Behav.* **45,** 787–794.
16. Bourke, A. F. G. & Franks, N. R. (1995) *Social Evolution in Ants* (Princeton Univ. Press, Princeton, NJ).
17. Fletcher, D. J. C. & Blum, M. S. (1983) *Science* **219,** 312–314.
18. Ortius, D. & Heinze, J. (1999) *Behav. Ecol. Sociobiol.* **45,** 151–159.
19. Bartz, S. H. & Hölldobler, B. (1982) *Behav. Ecol. Sociobiol.* **10,** 137–147.
20. Sommer, K. & Hölldobler, B. (1995) *Anim. Behav.* **50,** 287–294.
21. Willer, D. E. & Fletcher, D. J. C. (1986) *Physiol. Entomol.* **11,** 475–482.
22. Vargo, E. L. (1997) *Naturwissenschaften* **84,** 507–510.
23. Gobin, B., Billen, J. & Peeters, C. (1999) *Anim. Behav.* **58,** 1117–1122.
24. Kikuta, N. & Tsuji, K. (1999) *Behav. Ecol. Sociobiol.* **46,** 180–189.
25. Liebig, J., Peeters, C. & Hölldobler, B. (1999) *Proc. R. Soc. London Ser. B* **266,** 1865–1870.
26. Visscher, P. K. & Dukas, R. (1995) *Anim. Behav.* **49,** 542–544.
27. Crosland, M. W. J. (1990) *Anim. Behav.* **39,** 413–425.
28. Hölldobler, B. & Carlin, N. F. (1989) *Psyche* **96,** 131–151.
29. Monnin, T., Malosse, C. & Peeters, C. (1998) *J. Chem. Ecol.* **24,** 473–490.
30. Peeters, C., Monnin, T. & Malosse, C. (1999) *Proc. R. Soc. London Ser. B* **266,** 1323–1327.
31. Bonavita-Cougourdan, A., Theraulaz, G., Bagnères, A. G., Roux, M., Pratte, M., Provost, E. & Clement, J. L. (1991) *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **100,** 667–680.
32. Ayasse, M., Marlovits, T., Tengö, J., Taghizadeh, T. & Francke, W. (1995) *Apidologie* **26,** 163–180.
33. Diehl, P. A. (1975) *J. Insect Physiol.* **21,** 1237–1246.
34. Dillwith, J. W., Adams, T. S. & Blomquist, G. (1983) *J. Insect Physiol.* **29,** 377–386.
35. Ismail, M. T. & Zachary, D. (1984) *J. Chem. Ecol.* **10,** 1385–1398.
36. Tsuji, K., Egashira, K. & Hölldobler, B. (1999) *Anim. Behav.* **58,** 337–343.
37. Blomquist, G. J., Tillman, J. A., Shuping, M. & Seybold, S. J. (1998) in *Pheromone Communication in Social Insects: Ants, Wasps, Bees, and Termites*, eds. Vander Meer, R. K., Breed, M. D., Espelie, K. E. & Winston, M. L. (Westview, Boulder, CO), pp. 34–54.

38. Singer, T. L. (1998) *Am. Zool.* **38,** 394–405.
39. Lahav, S., Soroker, V. & Hefetz, A. (1999) *Naturwissenschaften* **86,** 246–249.
40. Breed, M. D. (1998) in *Pheromone Communication in Social Insects: Ants, Wasps, Bees, and Termites*, eds. Vander Meer, R. K., Breed, M. D., Espelie, K. E. & Winston, M. L. (Westview, Boulder, CO), pp. 57–78.
41. Vander Meer, R. K. & Morel, L. (1998) in *Pheromone Communication in Social Insects*, eds. Vander Meer, R. K., Breed, M., Winston, M. & Espelie, K. E. (Westview, Boulder, CO), pp. 79–103.
42. Singer, T. L., Espelie, K. E. & Gamboa, G. J. (1998) in *Pheromone Communication in Social Insects: Ants, Wasps, Bees, and Termites*, eds. Vander Meer, R. K., Breed, M. D., Espelie, K. E. & Winston, M. L. (Westview, Boulder, CO), pp. 104–125.
43. Clement, J. L. & Bagneres, A. G. (1998) in *Pheromone Communication in Social Insects: Ants, Wasps, Bees, and Termites*, eds. Vander Meer, R. K., Breed, M. D., Espelie, K. E. & Winston, M. L. (Westview, Boulder, CO), pp. 126–155.
44. Liebig, J. (1998) *Eusociality, Female Caste Dimorphism, and Regulation of Reproduction in the Ponerine Ant Harpegnathos saltator Jerdon* (Wissenschaft und Technik Verlag, Berlin).
45. Peeters, C. (1993) in *Queen Number and Sociality in Insects*, ed. Keller, L. (Oxford Univ. Press, New York), pp. 234–261.
46. Peeters, C. & Crozier, R. H. (1988) *Psyche* **95,** 283–288.
47. Peeters, C. & Hölldobler, B. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 10977–10979.
48. Arthur, C. L. & Pawliszyn, J. (1990) *Anal. Chem.* **62,** 2145–2148.
49. Kissin, Y. V., Feulmer, G. P. & Payne, W. B. (1986) *J. Chromatogr. Science* **24,** 164–169.
50. Szafranek, J., Maliñski, E., Dubis, E., Hebanowska, E., Nawrot, J., Oksman, P. & Pihlaja, K. (1994) *J. Chem. Ecol.* **20,** 2197–2212.
51. Carlson, D. A., Bernier, U. R. & Sutton, B. D. (1998) *J. Chem. Ecol.* **24,** 1845–1865.
52. Moneti, G., Pieraccini, G., Dani, F. R., Catinella, S. & Traldi, P. (1996) *Rapid Commun. Mass Spectrom.* **10,** 167–170.
53. Moneti, G., Pieraccini, G., Dani, F. R., Turillazzi, S., Favretto, D. & Traldi, P. (1997) *J. Mass Spectrom.* **32,** 1371–1373.
54. Oldham, N. J. & Svatos!, A. (1999) *Rapid Commun. Mass Spectrom.* **13,** 331–336.
55. Nascimento, R. R. D., Billen, J. & Morgan, E. D. (1993) *Comp. Biochem. Physiol. B* **104,** 505–508.
56. Reyment, R. A. (1989) *Terra Rev.* **1,** 29–34.
57. Hölldobler, B. & Wilson, E. O. (1990) *The Ants* (Belknap, Cambridge, MA).
58. Keller, L. & Genoud, M. (1997) *Nature (London)* **389,** 958–960.
59. Tsuji, K., Nakata, K. & Heinze, J. (1996) *Naturwissenschaften* **83,** 577–578.
60. Ross, K. G. & Keller, L. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 14232–14237.
61. Alexander, R. D., Noonan, K. M. & Crespi, J. (1991) in *The Biology of the Naked Mole-Rat*, eds. Sherman, P. W., Jarvis, J. U. M. & Alexander, R. D. (Princeton Univ. Press, Princeton, NJ), pp. 3–44.
62. West-Eberhard, M. J. (1978) *J. Kansas Entomol. Soc.* **51,** 832–856.
63. Pamilo, P. (1991) *Am. Nat.* **138,** 412–433.
64. Ratnieks, F. L. W. & Visscher, P. K. (1989) *Nature (London)* **342,** 796–797.
65. Ratnieks, F. L. W. (1993) *Behav. Ecol. Sociobiol.* **32,** 191–198.
66. Maynard Smith, J. & Harper, D. G. C. (1995) *J. Theor. Biol.* **177,** 305–311.
67. Rohwer, S. (1975) *Evolution (Lawrence, Kans.)* **29,** 593–610.
68. Møller, A. P. (1987) *Anim. Behav.* **35,** 1637–1644.
69. Johnstone, R. A. & Norris, K. (1993) *Behav. Ecol. Sociobiol.* **32,** 127–134.