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Queen Ants Make Distinctive Sounds That Are Mimicked by a Butterfly Social Parasite

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Published 6 February 2009, *Science* **323**, 782 (2009)
DOI: 10.1126/science.1163583

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Queen ants make distinctive sounds that are mimicked by a butterfly social parasite

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Materials and Methods

Materials. *Myrmica schencki* colonies (N = 12) and *Maculinea rebeli* larvae (N = 14) were collected in May 2006 at Colle di Tenda, Piedmont, northern Italy following protocols that ensure no damage to the populations (*S1*). Laboratory ant colonies of >100 workers each were established in 28cm x 15cm x 10cm perspex containers and maintained on a diet of sugar and *Drosophila* larvae (*S1*). Eight additional unparasitised colonies which had not encountered *Maculinea* larvae or pupae during the previous year were collected in July for the behavioural play-back experiments, supplemented by four colonies which had had no contact with *Maculinea* for a minimum of 6 weeks. It is highly unlikely that ants from any of the former eight colonies had experienced *Maculinea rebeli* during their life-times because: (i) only about 10% of host ant nests were 'infected' on the main study site at any one time; (ii) the average life-span of a worker is c 2 years, so 50% workers, in a nest that contained *Maculinea* >12 months earlier, would not have experienced the parasite; (iii) most

nests were excavated several metres away from the initial foodplant *Gentiana cruciata*, and could only have contained caterpillars in the past if a flowering plant of this long-lived perennial had existed in their territories >12 months earlier, but had since died.

Although we found no difference in responses to *Maculinea* sounds between naïve ants and others that had experienced *M. rebeli* weeks or months earlier, in a future study it would be interesting to compare responses of naïve and recently experienced ants to parasite sounds, including the former from populations well beyond the known range of *M. rebeli*. We predict that distant naïve *Myrmica schencki* workers would respond similarly to experienced ants, since they also tend, feed and carry caterpillars in preference to their own kin brood, and appear to treat them more like adult queens than workers.

Stridulation organ morphology. Three *M. schencki* queens and three workers from two colonies were kept in 70% ethanol and dissected between the post-petiolum and the abdomen to expose the *pars stridens* and the plectrum. The two ant parts were mounted on the same steel stub, coated with gold, and the length of the *pars stridens* and the distance between adjacent ridges were measured automatically (10 measurements per individual) using a Cambridge Stereoscan S360 SEM. *Maculinea rebeli* pupae and larvae were dried in hexamethyldisilazane to avoid cell structure disruption (*S2*) before coating. The SEM operated at 20-25 kV.

Sound recording and analysis. Recordings were made of individual workers (N = 13) and queens (N = 11) of *Myrmica schencki* and of *Maculinea rebeli* larvae (N = 10) and pupae (N = 4). The recording equipment consisted of a 12.5cm x 8cm x 2cm

recording chamber with a moving-coil miniature microphone attached through the centre. A second microphone of the same type was used to record ambient noise but in anti-phase. An amplifier was attached to each microphone and calibrated to maximise the noise cancellation of ambient noise from the two microphones leaving the signal from the recording chamber. The resulting signal was processed through a two-stage low-noise amplification before being recorded digitally on a laptop computer using Audacity v. 1.2.4 (http://audacity.sourceforge.net/). To further reduce ambient noise and interference, the equipment was powered by a 12V gel cell battery, and the recording chamber and microphones were placed inside an anechoic chamber. Pulse length, pulse repetition frequency, and dominant frequency (*S3*) were measured for 15 min periods starting 5 min after an insect was introduced and had calmed, providing ∑285 sound sequence measurements averaged within individuals for queens, ∑345 for workers, ∑115 for pupae, ∑292 for larvae. Recordings were analysed using Audacity 1.2.4 and tested by ANalysis Of SIMilarity (ANOSIM); individual sound parameters were analysed using linear mixed effect models (see below).

Play-back experiments. Behavioural assays were carried out in six 7cm x 7cm x 5cm perspex arenas each containing a central 0.4 cm^3 moist sponge to maintain humidity and three kin ant larvae placed on the sponge to simulate nest conditions for the test worker ants. The sponge was equidistant from the box edges so did not bias where the ants settled with respect to the edges. Speakers were attached through a hole in the side wall, sealed on the outside with Blu-tac™.

Ten workers from the same colony were placed in each arena and allowed to settle for 10 minutes before being played one of the six test sounds. The sounds were produced

by MP3 players playing loops of the original recordings, with each volume adjusted to the natural level by attaching the speaker to the microphone of the recording equipment and by calibrating to the same levels reached during the recording. Each trial lasted 60 minutes: counts were made of all instances of repellent or attractive (antennation, aggregation, on guard attendance) behaviours during periods of one minute for each box in sequence between the six treatments, i.e. ∑10 minutes for each sound per trial. The source of sound for each arena was randomly assigned before each trial was replicated, to control for possible positional effects. Between each trial, a fresh sponge was introduced and all equipment, including speakers and arenas, was cleaned with absolute alcohol and rinsed with distilled water. These play-back experiments were repeated 24 times (twice for each of 12 different *M. schencki* colonies) and the average responses per colony were used for the final analysis (Fig. 3).

Statistical analyses. Differences between the mean length of the *pars stridens* and the distance between its ridges on the stridulation organs of *M. schencki* queens and workers were compared using Student's t-test.

The sound parameters used by DeVries et al (*S3*), dominant frequency, pulse repetition frequency (the reciprocal of the duration of one pulse) and pulse frequency, were measured using Audacity 1.2.4. We obtained 292 sound fragments from 10 larvae, 115 from 4 pupae, 345 from 13 workers and 285 from 11 queens. We analysed each sound parameter separately with mixed effect models (library nlme; http://www.r-project.org/) to account for the hierarchical data structure using 'Individual' as a random factor and 'Group', i.e. larvae, pupae, workers, queens, as a fixed factor (*S4*). Pairwise differences among groups were tested using Likelihood

Ratios while merging factor levels in 'Group'. To test whether sound as a multivariate construct differs between groups we calculated the Pairwise normalized Euklidean distance for all data and used a nested ('Individual' within 'Group') ANOSIM (Primer-e 5.2.8). To further test whether the overall sounds produced by butterfly pupae and larvae were more similar to one caste of ant or another, we estimated mean Euklidean distances between all individuals and used Student's t-test to estimate the significance of the differences.

The counts of worker behavioural responses to different sounds were log(x+1) transformed. We fitted both a factor reflecting the sound sources and the identity of trialled colonies in Generalized Linear Models (normal errors, identity link; SPSS v. 15.0), where the second factor was fitted as a caveat to control for the typical overall differences in activity among individual colonies. Pairwise differences among groups were established using a measure of Least Significant Difference (Table S1). All models were inspected for heteroscadascity and the normality of errors (*S4*).

SOM Text: Queen ant attacks on *Maculinea rebeli* pupae and larvae

Although we did not play recordings of *Maculinea rebeli* sounds to queen ants in the play-back experiments (Fig. 3), we performed a small pseudo-replicated experiment that corroborates the hypothesis that, whereas *M. rebeli*'s mimicry of queen ant acoustics provokes enhanced benevolent behaviours (and no aggression) when played to low caste *Myrmica schencki* workers (Fig. 3), it should provoke rivalry and hostility if played to queens. We established a small (38 cm^2) one-chamber laboratory culture (*S1*) containing 25 workers, 4 adult queens and a few brood taken from the

same original nest, to which, after 1 week's acclimatization, we introduced four live *Maculinea* pupae and two recently eclosed pupal cases as single items for up to 20 minutes each on successive days. It should be noted that, due to their spatial segregation within ant nests, queen-pupal encounters are unlikely to occur in nature (most pupae are located in the outer surface chambers of *Myrmica* nests while queen ants inhabit the deeper brood chambers).

Each of the four test pupae was violently attacked, bitten and stung by all 4 queens, before several workers attacked their own queens with bites and stings, pulling them away to the furthest corner leaving their sister workers to peacefully antennate the pupa (Fig S1: the few occasions when a worker attacked the butterfly pupa occurred when it had already been provoked into fighting its own queen, and was sufficiently aroused to bite any object in its vicinity, including sister workers). In contrast, the recently shed pupal cases of eclosed *Maculinea rebeli* provoked no hostility from queens and greatly reduced antennation by workers. The few instances when workers attacked pupal cases occurred when these light objects rolled onto them during antennation, provoking a brief display of aggression. Since immature *Maculinea* appear not to mimic queen semio-chemicals (*S5*), and since recent pupal cases are likely to retain their hydrocarbon profiles, we presume that the queens mistook the live pupae for intruding rivals from their sounds. It is important to note that in a real nest, with many cells, the queens are well segregated from *Maculinea*, the former generally living in the deeper chambers while the latter usually pupate in empty cells near the surface . Similar, but much less violent, adult queen hostility was exhibited towards living *M. rebeli* larvae, whereas the workers always tended them benevolently and with priority (*S5,6,*), but this was not scored accurately enough to include in Fig. S1.

Future experiments are required to establish whether the results of this pseudo-replicated treatment, in which all observations were based on a single *Myrmica schencki* nest, are reproducible in a larger sample.

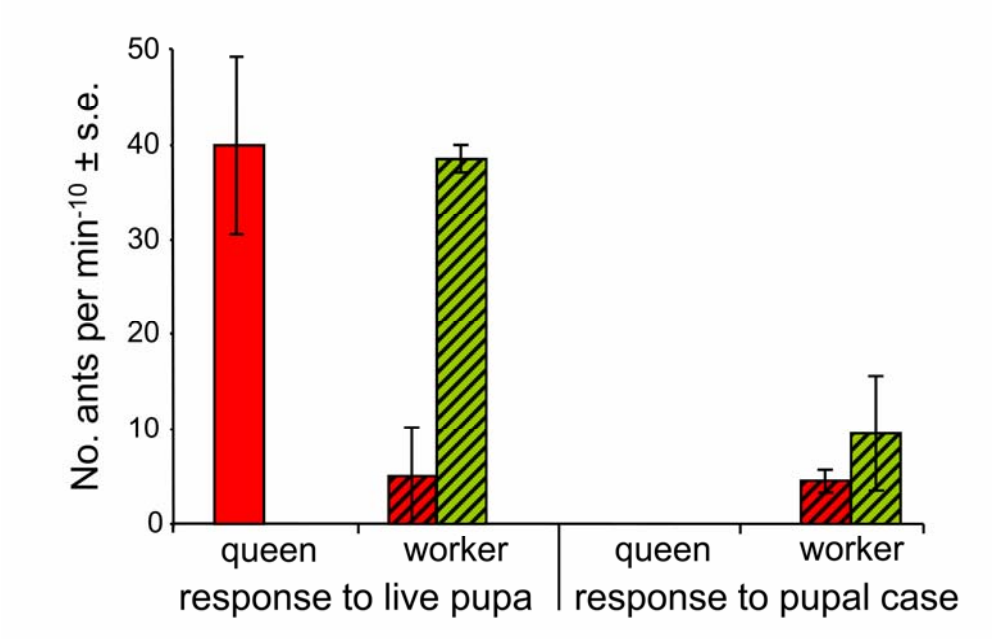
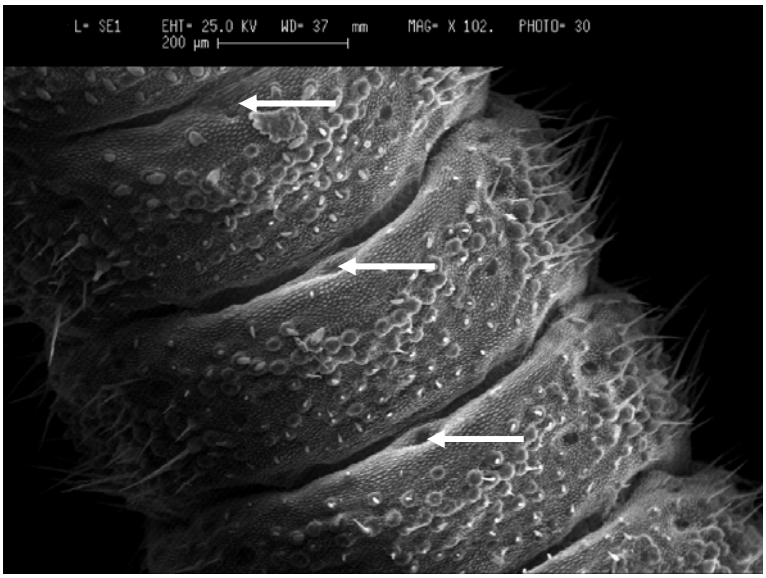


Figure S1. Reaction of *Myrmica schencki* ants to the introduction of a *Maculinea rebeli* pupa or empty pupal case to a single-celled laboratory colony. Red, aggression by 4 queen ants (bite, sting *Maculinea*); hatched red, aggression by worker ants; green hatch, benevolent tending of *Maculinea* by workers (no queens tended the *Maculinea).* $N_{ant\ colony}$ =1, $N_{live\ pupae}$ = 4, $N_{pupal\ case}$ = 2.

A



B

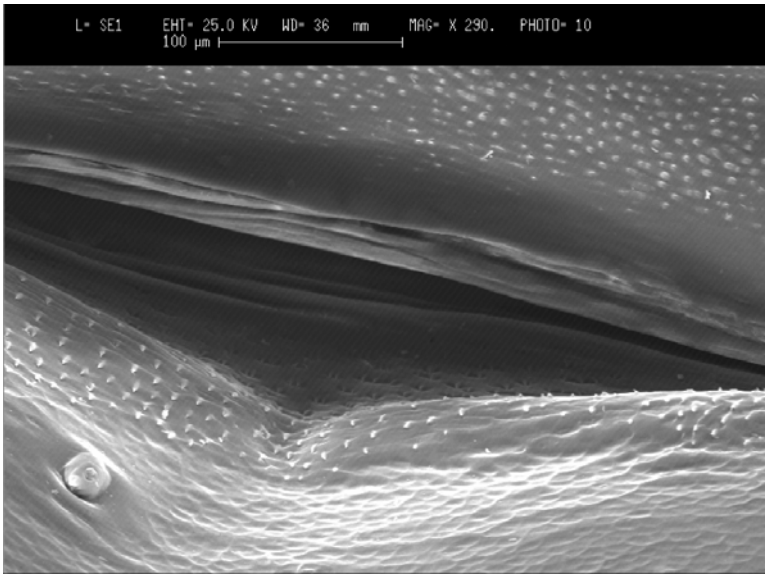


Figure S2. Scanning Electron Micrographs of (A) the final (IV^{th}) instar larva and (B) the pupa of *Maculinea rebeli*. (A) The arrows on the dorsal view of a *M. rebeli* larvae indicate the location of putative acoustic organs between tergites 4–7. (B) The pupal tooth-and-comb stridulation organ. For a better view of the organ, the gap between the tergites was artificially increased.

SOURCES OF SOUNDS		DIFFERENCES IN WORKER RESPONSES TO SOUNDS			
		Repellence	Aggregation	Antennation	On guard behaviour
WORKERS	QUEEN	0.00	0.13	0.07	**0.39**
WORKERS	LARVAE	0.00	0.02	0.21	0.13
WORKERS	PUPAE	0.03	0.41	0.04	**0.40**
WORKERS	SILENCE	0.08	**0.49**	**1.25**	0.06
WORKERS	WHITE	**0.27**	**0.49**	**1.11**	0.09
QUEEN	LARVAE	0.00	0.10	0.28	**0.27**
QUEEN	PUPAE	0.03	0.29	0.11	0.01
QUEEN	SILENCE	0.08	**0.61**	**1.32**	**0.45**
QUEEN	WHITE	**0.27**	**0.61**	**1.18**	**0.48**
LARVAE	PUPAE	0.03	0.39	0.18	**0.28**
LARVAE	SILENCE	0.08	**0.51**	**1.03**	0.18
LARVAE	WHITE	**0.27**	**0.51**	**0.90**	**0.22**
PUPAE	SILENCE	0.04	**0.90**	**1.21**	**0.46**
PUPAE	WHITE	**0.24**	**0.90**	**1.07**	**0.50**
SILENCE	WHITE	**0.19**	0.00	0.14	0.03

Table S1. Post hoc tests for pairwise differences in the behavioural responses of *Myrmica schencki* workers to the respective sources of sound. Fisher's least significant difference (LSD) was used to test for the equality of means. Values in bold indicate differences at $P < 0.05$.

Acoustic tracks

We provide samples of stridulations made by *Myrmica schencki* workers and queens and by *Maculinea rebeli* larvae and pupae. While these recordings formed part of the data analysed for this study, the short individual tracks presented here do not display the full variation of calls within each group.

Audio S1. Sample of stridulations by a *Myrmica schencki* worker

Audio S2. Sample of stridulations by a *Myrmica schencki* queen

Audio S3. Sample of stridulations by a *Maculinea rebeli* larva

Audio S4. Sample of stridulations by a *Maculinea rebeli* pupae

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