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Animal choruses emerge from receiver psychology

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Synchrony and alternation in large animal choruses are often viewed as adaptations by which cooperating males increase their attractiveness to females or evade predators. Alternatively, these seemingly composed productions may simply emerge by default from the receiver psychology of mate choice. This second, emergent property hypothesis has been inferred from findings that females in various acoustic species ignore male calls that follow a neighbor's by a brief interval, that males often adjust the timing of their call rhythm and reduce the incidence of ineffective, following calls, and from simulations modeling the collective outcome of male adjustments. However, the purported connection between male song timing and female preference has never been tested experimentally, and the emergent property hypothesis has remained speculative. Studying a distinctive katydid species genetically structured as isolated populations, we conducted a comparative phylogenetic analysis of the correlation between male call timing and female preference. We report that across 17 sampled populations male adjustments match the interval over which females prefer leading calls; moreover, this correlation holds after correction for phylogenetic signal. Our study is the first demonstration that male adjustments coevolved with female preferences and thereby confirms the critical link in the emergent property model of chorus evolution.

Choruses of acoustic species are among the more spectacular displays of animal behavior in the natural world¹. Beyond the sheer number of participating individuals and the sound intensity of their collective broadcast, animal choruses may also feature striking harmonization between individual singers². Such precision is especially evident in species where individuals maintain a particular call rhythm when singing in solo and then adjust the phase and/or rate of their rhythm when accompanied by neighbors. The overall result of these individual adjustments may be in-phase synchrony, out-of-phase alternation, or an elaborate combination of both³. Analogous cases of synchrony are also reported in species using visual signals^{4–6}.

Although the neuroethological mechanisms by which individuals may adjust their song are rather well-known^{3,7,8}, understanding why animals generate choruses that feature synchrony and alternation has evaded rigorous testing and is largely conjectural. Most of the conjectures propose that synchrony and alternation are specialized adaptations through which cooperating males directly or indirectly increase their mating success or reduce their vulnerability to natural enemies attracted to the songs^{9,10}. For examples, both types of chorusing patterns may reduce signal interference and thereby offer females improved perception of call properties, and they may also increase the combined sound intensity perceived from a group of singers^{11,12}. Additionally, synchrony may prevent predators from localizing any one singer within a dense chorus. Support for the reduced signal interference^{12–14} and increased group intensity^{12,15} hypotheses has been found in a few species, but in general the purported benefits of the displays are difficult to examine and have not been checked.

An alternative to the adaptationist paradigm above, and a key to its thorough examination, is the null hypothesis that the collective singing patterns in choruses, so conspicuous to human observers, simply emerge from the 'receiver psychology'¹⁶ of female perception and preference^{10,15,17}. For want of explicit experiments conducted on appropriate chorusing species, the null hypothesis has been especially conjectural. But recent neuroethological studies of female preference point toward a specific 3-step pathway along which basic perception and sexual selection could ultimately lead to chorusing: In various acoustic species females prefer male calls that precede a neighbor's call by a brief interval¹⁸, a variant of the precedence effects known from psychoacoustic research¹⁹. The next step in the pathway is the finding in many species which sing rhythmically that when a male hears a song

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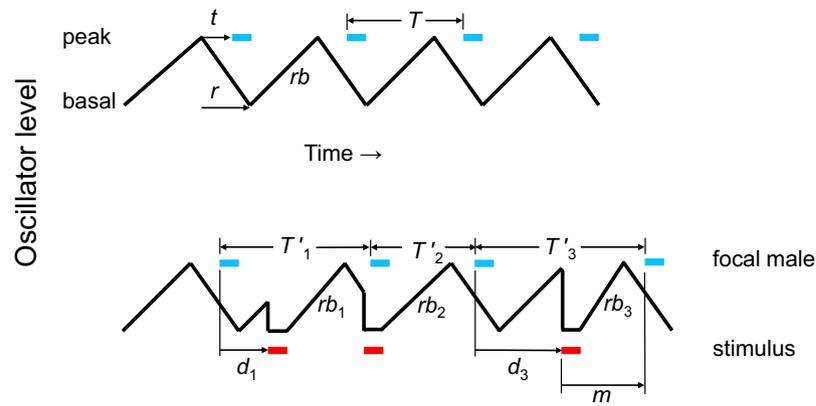


Figure 1. Inhibitory-resetting model for signal interactions between male neighbors in rhythmic acoustic species. Black sawtooth line in upper trace shows the periodic ascent (rb , rebound) and descent of the free-running central rhythm generator. After an effector delay t following ascent to the peak level, a call (thick blue dash) is broadcast; meanwhile the generator descends to its basal level over interval r . Calls are repeated rhythmically with a period T . Lower trace shows the same central rhythm generator as it is repeatedly inhibited and reset by a stimulus (male neighbor or acoustic playback; thick red dash). T' is the modified call period following a stimulus. The rebound rb from inhibition following a stimulus steepens when the stimulus occurs after a longer post-call delay d ; rb is steepest (rb_3) when the stimulus occurs just as the rhythm generator has ascended to its peak level (following post-call delay d_3), yielding the shortest post-stimulus call delay for the focal male's next call. This minimum post-stimulus call delay is designated m . The model is adapted from ref. 21 and was derived from results in extensive playback experiments with various species, including *E. diurnus*.

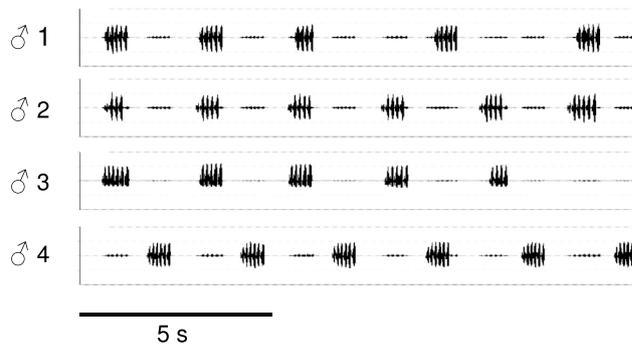


Figure 2. Elaborate chorusing in *E. diurnus*. Multi-channel oscillogram of a representative 15-s sample of calling recorded from a 4-male chorus in the Peyriac de Mer population (Fig. 4A). See Supplementary note S1 on the occurrence of both synchrony and alternation.

stimulus, he delays his subsequent call via a mechanism involving momentary inhibition of his central rhythm generator and resetting of his phase relative to that stimulus^{9,10} (Fig. 1). Finally, when multiple males use equivalent mechanisms an expansive chorus comprised of synchrony and/or alternation may arise²⁰ (Supplementary note S1). These collective outcomes are predicted by Monte Carlo simulation, and they are consistent with observations of synchrony and alternation in various acoustic insects and anurans²¹ (Fig. 2). Importantly, the display can be generated in the absence of any selection expressly favoring synchrony or alternation.

The 3-step pathway along which chorusing may emerge assumes that male resetting is a competitive mechanism favored by the selection imposed by female preference for leading calls. Simulations showing that males who inhibit and reset their call rhythms broadcast more leading calls and fewer following ones, and attract more females, than males with comparable song but who do not reset²¹ are in agreement with this assumption. Nonetheless, the inferred coevolution between the sensorimotor activity of male resetting and the female preference for relative call timing has not been established empirically in any acoustic species. Owing to this uncertainty, reflecting the lack of experiments probing the critical relationship between male and female timing parameters, the emergence of chorusing has remained speculative.

To test the null, or emergent property, hypothesis we chose to study *Ephippiger diurnus*, a European katydid that offered a special opportunity to analyze the apparent relationship between male resetting adjustments and female preference. *E. diurnus* are distributed in genetically distinct, geographically isolated populations²² to which comparative phylogenetic methods could be applied. The various populations exhibit divergent male songs, chorusing patterns (Fig. 2), and female preferences for male song^{23,24}. Male *E. diurnus* effect rhythm adjustments by inhibitory-resetting in response to the playback of song stimuli or to neighbors²¹, and females have moderate to

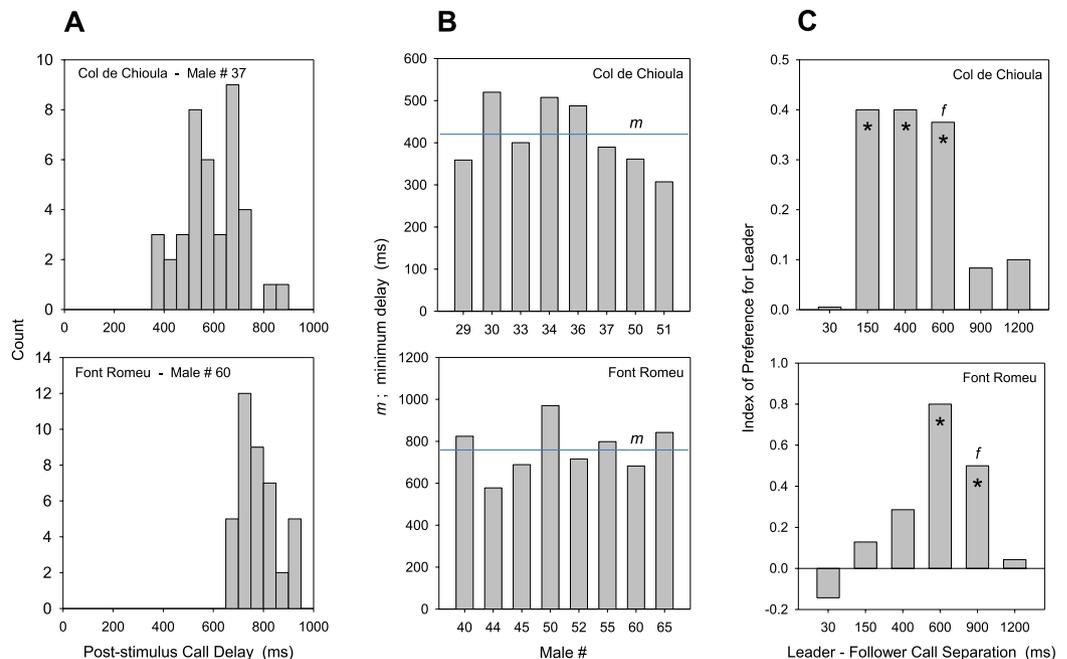


Figure 3. Timing parameters in male chorusing and female preference. (A) Call delay histograms of representative males from 2 populations (Col de Chioula, Font Romeu; Fig. 4A). The numbers of calls produced during 50-ms bins beginning at the onset of a synthetic call stimulus broadcast at random intervals are shown. The post-stimulus delay of the earliest of the male's 40 calls following inhibition is equivalent to m in Fig. 1. (B) Minimum post-stimulus call delay, m , for each of 8 males tested via playback in the 2 populations. A male's m is determined as the average of his 3 shortest delays. The population's m is the average of the individual male values. (C) Index of preference by females in the 2 populations for the leading of 2 synthetic call stimuli broadcast in close succession. For a given population, the maximum leader-follower call separation at which the index remained $\geq 50\%$ of the highest index value observed across all call separations was designated as f ; in all but one population this highest index value was significant (H_0 : index = 0.0; 1-tailed sign test, $\alpha = 0.05$; each female that preferred the leader in $>50\%$ of trials was labelled '+' and labelled '-' if $< 50\%$; the sign test was performed on the labels of all females tested for responses to a given call separation). *Index of preference > 0.0 .

strong preferences for leading calls²⁵. Thus, we could determine whether male resetting and female preference have actually coevolved or only represent a spurious association.

We reasoned that if male resetting adjustment evolved in response to female preference for leading calls, the timing of these 2 traits would be interconnected such that males seldom produce calls that most local females perceive as following ones and therefore ignore. As in other acoustic species using resetting mechanisms, male *E. diurnus* initiate few or no calls during a delay interval of minimum length m following the onset of a neighbor's call or a synthetic song stimulus²¹ (Fig. 3A,B). Females show a pronounced preference for a leading over a following call if the latter begins during an interval f after the onset of the leading call (Fig. 3C). When m is $\geq f$, males will avoid broadcasting ineffective following calls, and when $m = f$, males will maximize their call rate and improve their chance of broadcasting leading calls as well. We sampled 17 *E. diurnus* populations from southern and central France and northeastern Spain chosen to cover a broad geographic range, genetic variation as suggested by earlier phylogeographic studies²², and a diversity of male songs and chorusing patterns (Fig. 4A). In each population we determined m by testing male acoustic responses to the playback of song stimuli and f by testing female movement toward the leading of 2 song stimuli. We also developed a neighbor-joining (NJ) tree, based on microsatellite markers²⁶, to serve as our 'working phylogeny' of *E. diurnus* populations.

Results

We report that the minimum delay m in male calling following a song stimulus is strongly correlated with, and roughly matches, the maximum separation f between 2 calls for which females prefer the leading one ($\rho = 0.87$, $p < 0.001$; Fig. 4B). m was slightly shorter than f in 13 of 17 populations, possibly reflecting a conflict that males face between producing calls deemed leading by at least some females and avoiding the production of following ones and that it is resolved in favor of the former.

In further analyses we dealt with several factors that potentially generated the matching of male and female timing parameters as an artifact. First, we applied the method of independent contrasts (PIC) to our NJ tree of populations to remove any phylogenetic signal from the m - f correlation²⁷. Under most circumstances inter-population gene flow would invalidate the use of PIC to treat intra-specific relationships^{28,29}, but the negligible migration in *E. diurnus*, which are flightless, and the distinctive population genetic structure fully justify its application here. Our NJ tree (Fig. 5A) clearly reveals a branching topology that is consistent, in its details, with another tree based on COI³⁰, indicating that our working phylogeny is most probably an accurate depiction of

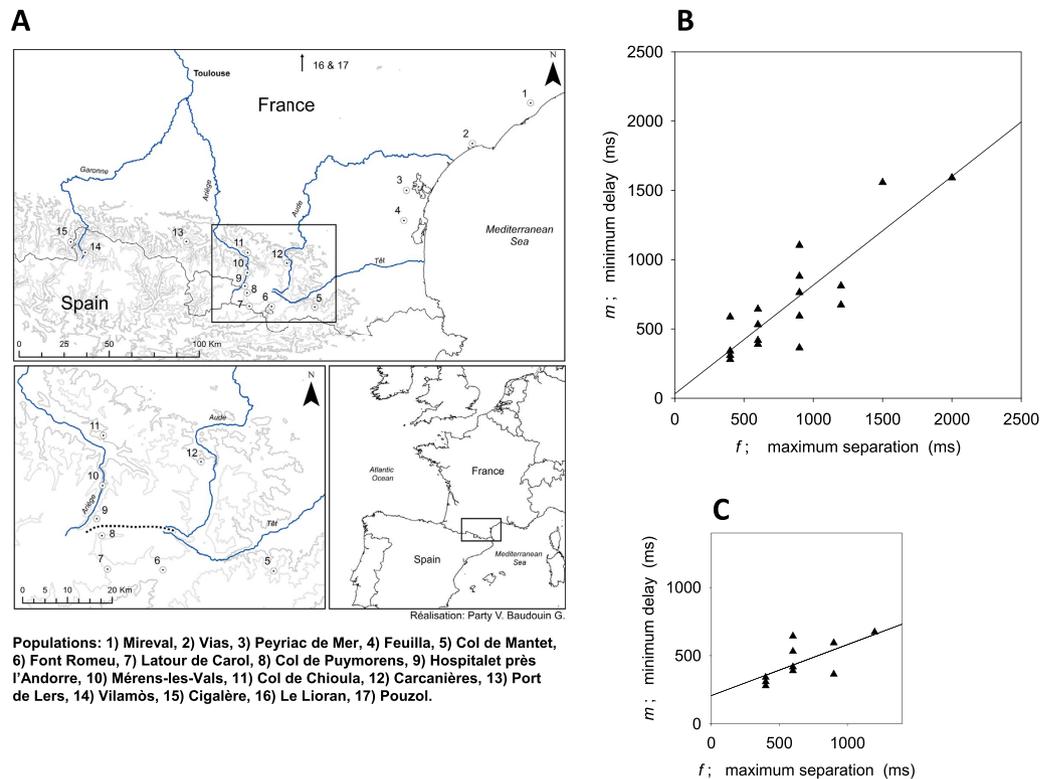


Figure 4. Correlation of male and female timing parameters across populations. (A) Map of southern France and northeastern Spain showing locations of the 17 *E. diurnus* populations sampled between 2012–2015 (see ref. 30); map was generated with ArcGIS version 10.0 for desktop, ESRI (<http://www.esri.com/software/arcgis/arcgis-for-desktop>). (B) Ordinary least-squares linear regression of m (minimum post-stimulus call delay in males; Fig. 3B) vs. f (maximum leader-follower call separation for which females prefer the leading call; Fig. 3C) for the 17 sampled populations ($m = 31 + 0.79f$; $t = 6.56$, $p < 0.001$). (C) Ordinary least-squares linear regression of m vs. f as in Fig. 4b but restricted to those 10 populations where the mean call syllable number in males was ≤ 2.1 ($m = 207 + 0.37f$). Stepwise linear regression (α to enter and α to remove = 0.15) of m on both f and mean syllable number yielded the above model that only included f .

the true one. To be conservative, we also analyzed our microsatellite data with a Bayesian clustering protocol to identify genetically distinct population clusters and assign individuals to these entities (Fig. 6). We then created a NJ tree for the 7 strongly differentiated clusters identified (Fig. 5C) and again applied the PIC correction. We found that the m - f correlation remained highly significant following PIC correction whether applied to the tree of all 17 populations sampled or to the revised tree of 7 genetically distinct clusters (Fig. 5B,D). Thus, the observed matching of male and female timing parameters is not a phylogenetic artifact of our sampled populations.

Second, we accounted for the possibility that both the male and female timing parameters simply reflect the call length (=syllable number) and free-running call rhythm in a population. In fact, the male timing parameter, m , is closely correlated with call length ($\rho = 0.94$; $p < 0.001$), a relationship that could allow the several outlier populations with long calls, and slow call rhythms, to unduly influence the m - f correlation. We therefore reanalyzed our data using only populations with relatively short calls, those with a mean of 1.0–2.1 syllables. In this reduced data set of 10 populations the m - f regression and correlation held ($t = 2.64$, $p = 0.03$; $\rho = 0.69$, $p = 0.029$; Fig. 4C), whereas the correlation between m and mean syllable number did not ($t = 1.65$, $p = 0.14$; $\rho = 0.50$, $p = 0.14$). Our analysis is the first confirmation that male rhythm adjustments have coevolved with female preferences for call timing.

Discussion

2 parallel studies of acoustic communication in *E. diurnus* reinforce our conclusion that temporally-structured chorusing can emerge from receiver psychology. A test of female preference among chorusing males whose calling differed in several acoustic parameters showed that call timing, i.e. the number of leading calls, was the most influential character on male attractiveness³¹. Males are evidently under strong selection pressure to adjust their calling with a resetting mechanism. And in playback tests of synthetic chorus stimuli imitating the alternation or synchrony that occur naturally (Fig. 2) vs. modified chorus stimuli that do not occur, females expressed no preference for the natural stimuli³⁰. This result, which is comparable to findings in an experimental study of synchronized visual signaling in fiddler crabs³², argues against the signal interference and combined intensity hypotheses¹² for the evolution of chorusing in *E. diurnus*. Similarly, the simultaneous occurrence of both alternation and synchrony in *E. diurnus* choruses (Fig. 2) is not consistent with explanations invoking enhanced defense

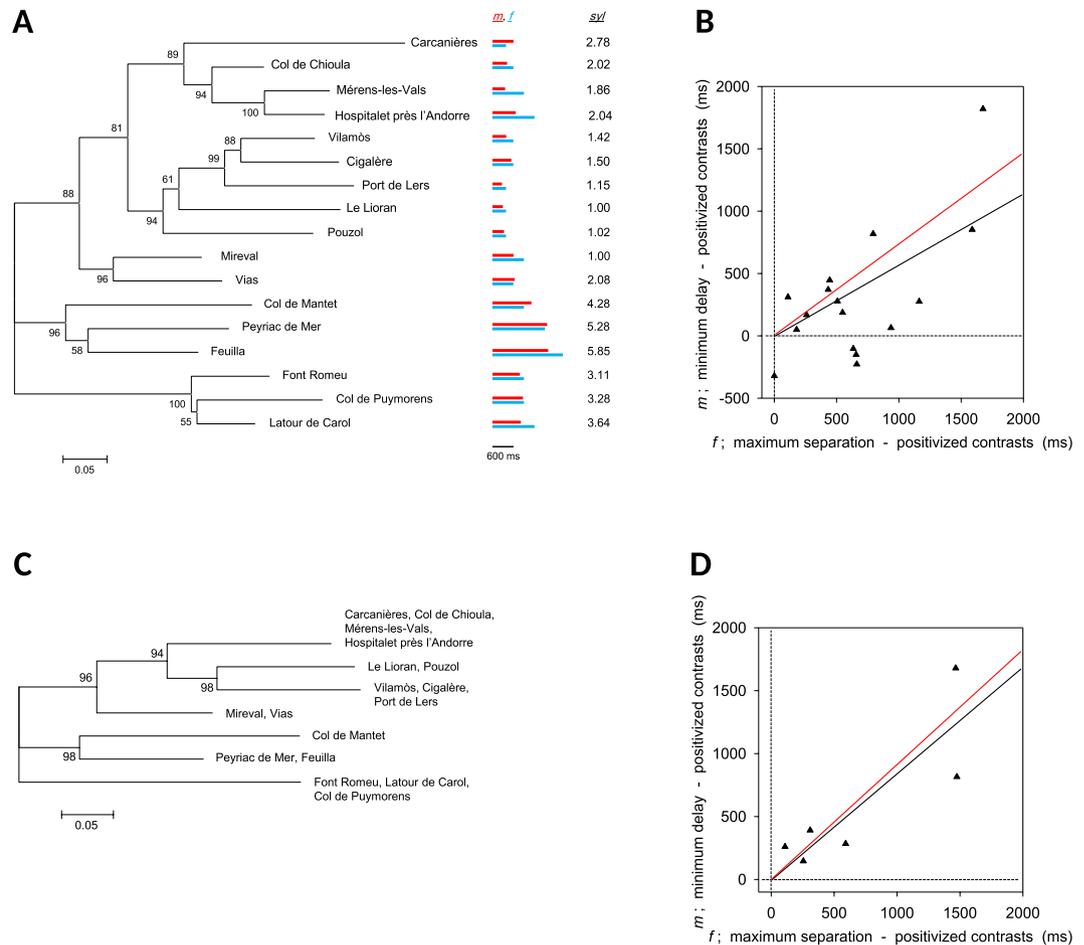


Figure 5. Comparative phylogenetic analysis of male and female timing parameters. (A) Unrooted neighbor-joining (NJ) tree of 17 *E. diurnus* populations from southern France and northeastern Spain (Fig. 4A) generated from microsatellite loci. Scale at bottom left indicates 0.05 nucleotide substitutions per site; values over branches represent posterior probabilities. Red and blue bars to the right of each population indicate the values of *m* (minimum post-stimulus call delay in males) and *f* (maximum leader-follower call separation for which females prefer the leader); see Figs 3B,C and 4B. *syl* is mean syllable number in the male call. **(B)** Correlation between *m* and *f* among the 17 populations corrected by phylogenetically independent contrasts (PIC). Black line is ordinary least-squares linear regression through the origin for the 16 standardized, positivized contrasts ($\rho = 0.76$, $p < 0.001$; df reduced by 2 to account for polytomy); red line is reduced major axis regression. **(C)** Unrooted NJ tree of 7 genetically distinct clusters determined via Bayesian clustering (Fig. 6). Each cluster comprises 1–4 of the 17 populations. **(D)** Correlation between mean *m* and *f* among the 7 clusters from Fig. 5C corrected by PIC ($\rho = 0.94$, $p < 0.01$; $n = 6$ contrasts).

against predators and parasites. These hypotheses propose that synchronous signals might reduce the cognitive ability of natural enemies to localize a prey or host individual, or that males that synchronize signals can thereby listen for the approach of natural enemies during the silent intervals between successive signals^{9,10}. However, a high incidence of alternated signals, as in *E. diurnus* choruses, would greatly reduce these potential advantages. Regularly alternated signals are also explained as a specific adaptation allowing males to broadcast signals and to assess rival neighbors in the absence of acoustic interference⁹. But again, the high incidence of synchronized signals in *E. diurnus* choruses would severely mitigate these effects.

Our findings on *E. diurnus* best support the null, or emergent property, hypothesis in which female preferences for relative call timing select for male rhythm adjustments which collectively yield patterns of synchrony and alternation. We do not claim that all or even most chorusing, in singing insects or in any other animal group, simply emerges from receiver psychology. Rather, our experimental findings show that this origin is possible and is a viable alternative to the adaptationist model. In a broader context, the emergence of chorusing from receiver psychology is an example of a self-organized system, a process that originates in local interaction within pairs and triads of individuals and lacks central control³³. Self-organization is responsible for many complex systems in biology, e.g. coordinated activity of animal groups. But unlike most of the systems under consideration, the *E. diurnus* males who generate a chorus do not benefit from their collective production *per se*, and no evidence suggests that the females who listen to choruses pay any particular attention to their overall pattern in choosing a mate^{10,17}. Emergent

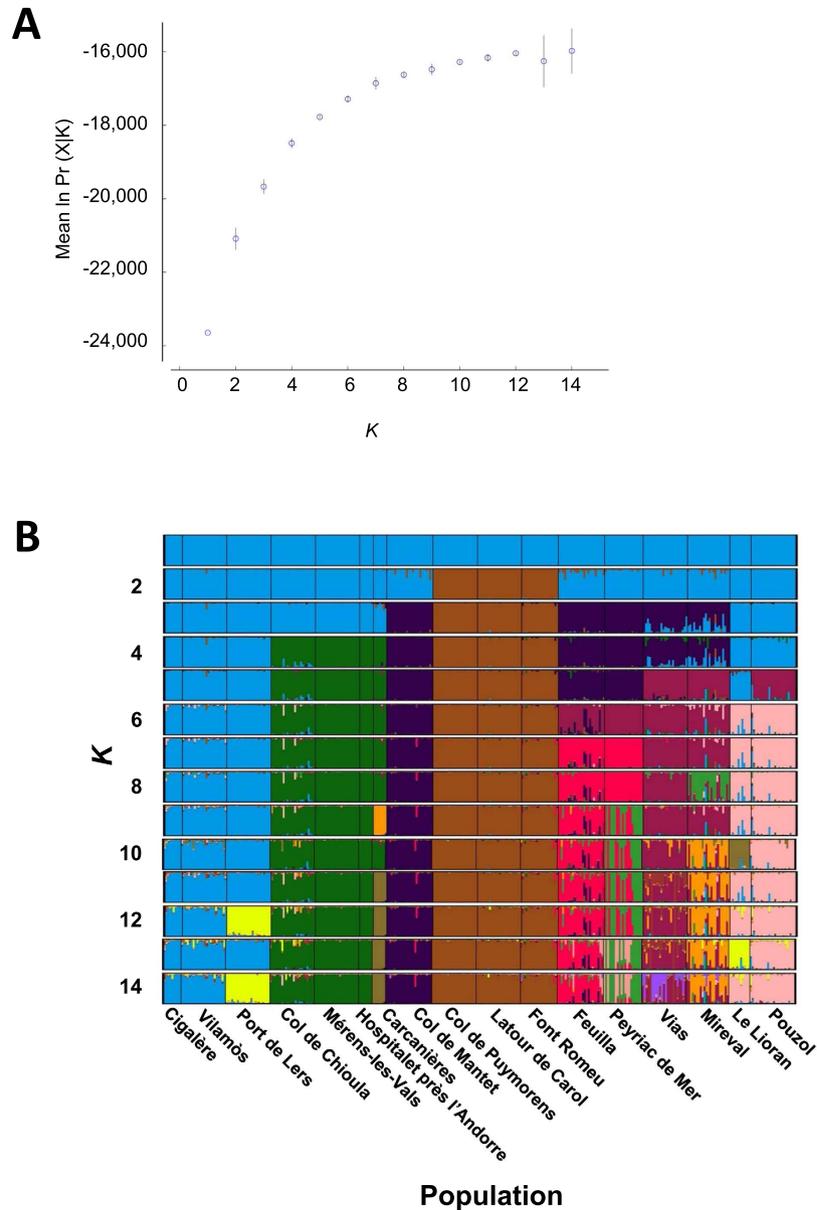


Figure 6. Clustering of genetically similar *E. diurnus* populations. (A) Mean and standard deviation of \ln (probability of data) for each inferred number of clusters ($K = 1-14$) as estimated from Bayesian STRUCTURE analysis. Values are computed over 20 runs per K . (B) Bayesian STRUCTURE plots representing distinct clusters inferred from the 17 *E. diurnus* populations sampled (Fig. 4A). Each of the 14 horizontal panels represents a STRUCTURE plot based on a given number of inferred clusters ($K = 1-14$); in each plot distinct clusters are coded by a different color. The 335 individuals sampled from all 17 populations are each represented by a narrow vertical line, and the probability ($q = 0$ to 1) of an individual's assignment to a distinct cluster is indicated by the height of the line segment bearing the color of that distinct cluster. Thus, individuals represented by lines composed of 2 or more segments of different colors may belong to the several distinct clusters whereas those represented by lines composed of a single color are likely to be pure. Fig. 5C depicts the distinct clusters inferred with $K = 7$.

phenomena such as *E. diurnus* chorusing thus play an important role in evolutionary biology by reminding us that not all complex behavior we observe represents a specialized adaptation to presumed selection pressures.

Methods

Sampling and playback experiments. In each *E. diurnus* population sampled males and females were collected and then tested at approximately the same time. In 14 of the 17 populations test insects were collected as nymphs or young adults in the field; in 3 populations they were the laboratory-reared offspring of insects collected in the field during the previous year. $n = 8-18$ males in all but 1 population (Cigalère), where $n = 3$; $n = 6-18$ females in all but the Cigalère population, where $n = 4$.

We determined the timing of the resetting mechanism in *E. diurnus* males by playback experiments conducted in the laboratory at 22–24 °C. The synthetic call stimulus used for playback with a given population was constructed from a natural call syllable having average acoustic characteristics in that population; this representative syllable was then digitally repeated to produce a call with a syllable rate and syllable number equivalent to the population mean values. Using a sound level meter (model 1982; General Radio; Concord, Massachusetts, U.S.A.) calibrated with a microphone sensitive to high frequency and a computer (see refs 31 & 34 for method), we adjusted the amplitude of the stimulus to 80 dB SPL (peak reading; 0 dB = 20 μPa) at the location of the test male; this amplitude value represented males singing approximately 1 m distant in the field. Males collected in the field were obtained as nymphs or young adults; they were at least 15 d post adult molt and had not mated for a minimum of 10 d at the time of testing.

We determined the timing of preference for leading, as opposed to following, calls in *E. diurnus* females by playback experiments conducted under the same laboratory conditions. 2 synthetic call stimuli broadcast in close succession served as the basic stimulus. For each of 6–8 different call separation intervals ranging from 30–2000 ms, a female was tested individually in 4 successive trials on a y-maze at which loudspeakers broadcasting leading and following calls separated by the given interval were placed by the left and right arms; broadcasts of leading and following calls were switched between the 2 arms in successive trials. The maze was constructed of wood and consisted of a base and two cylindrical 70-cm arms separated by 90° and angled upward at 30°. Laboratory walls surrounding the apparatus were covered with acoustic insulation foam to reduce echoes from the loudspeaker broadcasts. Females were placed at the base of the y-maze and allowed 2 min to move toward the fork and continue onto one of the arms, considered as her choice. The index of preference for a given leader-follower call separation in a population was determined as $[(p_L - 0.5)/0.5]$, where p_L is the proportion of choices for the leader summed across the 4 trials and all test females. Thus, the index reflected both the proportion of females that preferred a leading call and the strength of each individual female's preference. Because each sampled female was tested in the same number of trials, each individual female contributed equally to the population's index. Call stimuli were the same as those used to test timing of the resetting mechanism in males. The call stimulus for a given population was copied to the second channel of a stereo sound file, and the relative timing of the 2 channels was adjusted to the desired call separation; this separation interval was measured from onset of the leader to onset of the follower. The repetition rate for the stereo call stimulus was set equivalent to the mean call rate observed among interacting males in the population. Females collected in the field were obtained as nymphs or young adults; they were at least 15 d post adult molt and had neither mated nor been exposed to males or male song for a minimum of 10 d at the time of testing. See ref. 30 for additional details on protocol.

Molecular genetics and phylogeny. Genomic DNA was extracted from hind femora of 335 *E. diurnus* adults using the DNA Easy Blood and Tissue kit (Qiagen, France) following the manufacturer's instructions. DNA quality and molecular weight were confirmed in 1% agarose gel. A novel set of polymorphic microsatellite markers was developed for *E. diurnus* through pyrosequencing technology (Genoscreen, Lille France)²⁶. In total, 18 microsatellite loci, including 4 loci previously reported^{35,36}, were amplified in 7–26 specimens from 17 localities using a cost-effective M13 fluorescent protocol³⁷ and multiplexed PCR. Fragment analysis was conducted on a 3730 xl DNA analyzer (Applied Bio Systems; AB) using the GeneScan 500 LIZ (AB) as an internal size standard and 1 to 2 μl of PCR product (1:20 dilution). Genotypes were resolved in GeneMapper version 5.0 (AB). Allele frequencies were estimated in POPTREEW³⁸, and an unrooted neighbor-joining (NJ) tree of populations was constructed based on the D_A distance; this distance has been found to be an appropriate measure for obtaining the correct topology of population trees based on microsatellite markers. The robustness of nodes was estimated using 10,000 bootstrap replicates.

Phylogenetic independent contrasts (PIC) were conducted in MESQUITE version 3.04^{39,40}. The degrees of freedom used in evaluating correlation coefficients were adjusted for polytomy⁴¹.

We used a Bayesian clustering approach (STRUCTURE version 2.3.4⁴²) to identify the most likely number of distinct clusters (K) in our dataset and assign individuals to their most probable cluster. Prior to analysis, we removed individuals where >20% of the data were missing. Clustering analyses were performed with $K = 1–14$ and repeated with 20 runs to assess the consistency of results. We used the admixture model with correlated allele frequencies; no *a priori* information on populations was incorporated in the analysis. Runs were performed using a burn-in period of 10^4 followed by 10^5 MCMC iterations. Results from the 20 runs were analyzed with CLUMPAK⁴³. The estimated number of clusters (K) was taken to be that value of K with the highest probability of the data ($\Pr(X|K)$). This probability continuously increased from $K = 1–12$ (Fig. 6A), but it reaches a plateau and values of K higher than 7 increase the 'assignment noise'; i.e. variance in individual assignments across repeated runs for a given K . At $K = 7$, the percentage of 'mixed' individuals, admixture reflecting shared ancestral polymorphism or recent introgression, is low (7%) (Fig. 6B), the differentiation between clusters is high (average $F_{ST} = 0.25$, $p < 0.001$), and the overall structure as divided among clusters remains stable across repeated runs. Based on these considerations, we chose this level of structuring as representative of isolated populations used in PIC analysis.

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Author Contributions

M.D.G., R.S., Y.E.-G. and V.P. designed and performed the research and analyzed the data. M.D.G. wrote the paper.

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Title: Animal choruses emerge from receiver psychology

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Supplementary note S1

Monte Carlo Simulation of Inhibitory Resetting

When 2 males sing with similar free-running rhythms and mutually adjust with the same resetting mechanism, simulation predicts calling in synchrony or alternation^{20,21}.

Synchrony occurs where singers rebound from inhibition with promptness similar to their rhythm during solo calling; alternation occurs where the rebound from inhibition is substantially faster. Via a chain reaction, the basic interaction between 2 neighboring males can yield synchrony that includes many individuals. By definition, though, out-of-phase alternation should be restricted to a pair of individuals. Paradoxically, the simple interaction initiated by an alternating pair spreads if individuals selectively attend to 1 or 2 neighbors only^{20,44,45}. In these local groups a given singer alternates with his nearest neighbor(s) and – by default – synchronizes with his neighbor's neighbors (see Fig. 2), a feature that interlaces the small groups with each other and yields an expanding chorus.

The full model for the general inhibitory-resetting mechanism, as depicted in Fig. 1, is :

$$T = s \cdot [(d + l/v) - (r - t)] + (T + \varepsilon) + (y - x), \text{ where :}$$

- 1) T is the modified call period following interaction with a stimulus (or neighbor);
- 2) s is the duration of rhythm generator's rebound (rb_3 in Fig. 1) following inhibition at the latest possible time during the call cycle (after stimulus delay d_3 in Fig. 1) divided by the call period (T); it measures the greatest rapidity with which the generator can rebound from

26 inhibition relative to its rebound from basal level during free-running call cycles. s also
represents the slope of the phase response curve (PRC), which regresses response phase
28 $[T - T] / T$ against stimulus phase (d / T) during interactive calling;

3) d is the delay between the focal male's concurrent call and the stimulus, measured as the
30 interval from onset to onset;

4) l is the distance between the focal male and the stimulus;

32 5) v is the velocity of sound;

6) r is the interval during which the rhythm generator descends from its peak to its basal level
34 during a free-running cycle;

7) t is the effector delay between the time when the rhythm generator attains its peak level
36 and the onset of the triggered call;

8) T is the mean call period during a free running cycle;

38 9) ϵ is a stochastic element that accounts for intrinsic variation in the call period;

10) y is the length of the stimulus;

40 11) x is the length of the focal male's call.

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