What determines lek size? Cognitive constraints and per capita attraction of females limit male aggregation in an acoustic moth

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It has been proposed that leks arise because of increased mating benefits in aggregations of displaying males, and some evidence supports this hypothesis. But observations also indicate that lekking aggregations include only a small percentage of the males in a population, implying that certain factors limit lek size. Potential factors include increasing travel costs to find and form large but distant aggregations, greater attraction of predators and higher levels of aggression. Any one of these constraints may cause the number of females arriving at larger leks to decelerate such that per capita male attractiveness, and hence mating success, declines above an optimum lek size. None the less, relatively little experimental work has examined what determines lek size. In particular the possibility that cognitive aspects might constrain lek size has rarely been considered. We studied this question in Achromia grisea, an acoustic moth in which singing males form small aggregations that attract females. We created artificial leks in the laboratory and tested their relative attractiveness to females; we also tested male preferences to form and join such aggregations. Females preferred male aggregations over solitary singers, but the marginal per capita attractiveness of an aggregation of n = 2 males versus n males waned for n ≥ 5. Similarly we found that males were attracted to other males singing in the vicinity, but this effect disappeared for n ≥ 4. We infer that lek size is limited because the marginal per capita attractiveness of larger leks only occurs for small groups. This constraint probably arises because females distinguish leks by overall song rate but are neuroethologically incapable of discriminating rates above a threshold value corresponding to groups of four to six males. These findings emphasize the critical role that neural constraints may play in determining fundamental parameters of complex behaviours such as lekking.

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group of displaying males and therefore find it difficult to localize any one male, and because any one male’s exposure to predation is ‘diluted’ by virtue of his many signalling neighbours (Hoogland & Sherman, 1976; Lack, 1968; Oring, 1982; Trail, 1987; Wiley, 1991). However, the female may also play a driving role here, as she may be afforded these same benefits when she enters a lek and engages in mate choice.

If leks arise because they offer males higher per capita mating success, a question that logically follows is what determines lek size? Because we generally do not observe a single, massive lek at which all males in a population have aggregated, lek size does appear to be limited in most animal species (Högland & Alatalo, 1995). Are these limits set by costs that males might incur were they to form and join massive aggregations? Such limits could arise if aggression, disease transmission or general ‘stress’ rise faster than the group’s attractiveness to females as male number increases (Alatalo, Högland, Lundberg, & Sutherland, 1992; Aspi & Hoffmann, 1998; Gosling & Petrie, 1990). Additionally, the group’s attractiveness to predators and other natural enemies might rise appreciably as male number increases (Boyko, Gibson, & Lucas, 2004; Magnhagen, 1991). In these situations a male’s fitness might peak at an intermediate group size and then decline in larger aggregations (Isvaran & St Mary, 2003; Jiguet & Bretagnolle, 2006). The time and energy that males would expend on travel to form large aggregated may represent another factor limiting group size (Högland & Alatalo, 1995).

Similar constraints may be expected to act on females and ultimately limit the size of male leks. Whereas females may be preferentially attracted to groups of males to the extent that the ratio of arriving females to displaying males is higher in groups than for lone males, the preferential arrival of females at leks may decelerate at large groups such that this ratio begins to decline (Isvaran & St Mary, 2003). As a result, per capita male mating success may exhibit a parallel decline at larger groups. Deceleration of the number of arriving females may occur because it is not economical for females to invest in the travel needed to locate a large, but distant, lek (Gibson, Taylor, & Jefferson, 1990). Females might also suffer from male harassment at very large leks, as well as from the disease transmission and general stress already noted as potentially disturbing males at these aggregations (reviewed in Clutton-Brock, Deutsch, & Nefdt, 1993; Högland & Alatalo, 1995). And, females may find it difficult to evaluate and identify a ‘superior’ male in a large lek and actually end up pairing with males ‘inferior’ to those they would choose in smaller leks even though these include a restricted number of potential mates representing a reduced range in ‘quality’ (Hutchinson, 2005).

Perceptual constraints, including psychophysical and neuroethological factors, in conjunction with the economic factors described above, may also explain the shape of the sexual preference function in females. For instance, in tungara frogs, Physalaemus pustulosus, psychophysical laws explain the decline of the preference of females for more elaborate male calling songs (Akre, Farris, Lea, Page, & Ryan, 2011). Similarly these constraints may contribute to the decelerating number of female arrivals at larger leks. This possibility has not been widely considered in behavioural ecology, but a full understanding of lek formation requires that we identify the perceptual mechanisms by which females discriminate lek size (Högland & Alatalo, 1995; Shuttleworth, 2010). For example, female preference for larger leks may be constrained by properties of their sensory receptors and nervous system: the coding of an overall signal of a lek in electrical impulses, i.e. action potentials, may be limited by the maximum rate of impulses that the nervous system can generate. Thus, even where the magnitude of an overall signal increases with lek size, at some point the overall lek signal would saturate female receptors and females’ preference for a lek of n + 1 or n + 2 males over an n-male lek would disappear. At this point the number of female arrivals would begin to plateau, and males may no longer benefit from leaving one group of n individuals to join another such group and increase its size to n + 1. At best, the same number of arriving females would be expected, and this number would have to be shared with an additional male. Thus, perceptual constraints in female ability to discriminate lek size would ultimately lead to selection on males to limit their lek size. Such constraints may be particularly likely in species in which individuals cannot directly evaluate the number of group members owing to perceptual and cognitive limitation and instead rely on indirect means to assess group size (Brannon & Roitman, 2003; Gómez-Laplaza & Gerfal, 2011; McComb, Packer, & Pusey, 1994). These constraints are also likely where discrimination of group size is made at a distance because economic factors would prohibit the travel needed to visit the several leks in a population and inspect them at close range.

In view of the possibility that females and males may each drive the evolution of lek size, we then ask which influences do observed lek size reflect? Do the influences of males and females coincide such that the typical number of males that assemble in leks matches the aggregation size for which the ratio of arriving females to displaying males is maximum? If not, does the mismatch between the two values imply the occurrence of sexual conflicts? For example, an elevated predation pressure on large aggregations of displaying males might influence males to form smaller groups than those at which the female: male ratio would be highest. The perceptual mechanisms by which lek size is assessed also need to be considered. If these mechanisms constrain males while sensing the size of their own lek differently than they constrain females that are assessing the size of distant leks, mismatch may occur.

Despite the great interest in leks in sexual selection research, there has been relatively little empirical study of the factors limiting lek size. We have relatively little information on the relationship between the actual lek sizes that males form and the lek sizes at which per capita male attractiveness and mating success are maximum (Alem, Koselj, Siemers, & Greenfield, 2011; Isvaran & St Mary, 2003; Jiguet & Bretagnolle, 2006). Moreover, there is little understanding of the neuroethological and psychophysical processes involved in assessment of leks and how these mechanisms may ultimately limit lek size (Högland & Alatalo, 1995; Shuttleworth, 2010).

We addressed these questions in an acoustic pyralid moth, Achroia grisella (lesser wax moth), in which males gather in small aggregations and broadcast an ultrasonic advertisement song that attracts receptive females in the vicinity (Greenfield & Coffelt, 1983; Spangler, Greenfield, & Takessian, 1984). This species is easily manipulated and studied experimentally in the laboratory: Because females respond to synthetic song stimuli as readily as to live males (Spangler et al., 1984), an experimenter can establish various numbers of singing males in small groups, and the orientation and movement of females as well as males to these experimental groups can be observed (Alem & Greenfield, 2010). We relied on these special features to implement a series of experiments designed to measure per capita male attractiveness as a function of lek size, the tendency of males to aggregate in leks, and the mechanisms by which females distinguish leks and potentially assess their size.

METHODS

Study Species

Achroia grisella is a cosmopolitan symbiont of the Western honeybee, Apis mellifera, and is now found in most parts of the
world where apiculture is practised. The moths usually infest honeybee colonies with low worker populations wherein the moth larvae feed on organic detritus (Künke, 1930). Adults tend to remain in the vicinity of the colony after eclosion, and mating takes place there. Males sing for 6–10 h each night from eclosion until senescence, and small groups of singing males, typically 2–10 individuals, are observed to assemble in the field and in the laboratory (Brandt, 2003; Greenfield & Coffelt, 1983). The song is a rhythmic train of short pulses (100–120 μs) of high-frequency sound (70–130 kHz) produced while the male remains stationary on the substrate and fans his wings more or less continuously. Fanning causes a pair of tymbals at the forewing bases to buckle during each wing upstroke and downstroke and emit a pair of pulses, one from each tymbal, separated by a short silent gap (an ‘asynchrony interval’ of 100–1000 μs). The acoustic energy broadcast by a singing male is somewhat omnidirectional, with amplitude recorded 1 cm in front of a male averaging 95 dB peSPL (see Jang & Greenfield, 1996; Snedden, Tosh, & Ritchie, 1994; Spangler et al., 1984). Females may be attracted by male song from distances up to 1 m, and video-tracking analyses of laboratory trials showed that females move among the several singing males in a group before arriving at a particular individual (Alem & Greenfield, 2010). Tests using live males or loudspeaker broadcasts of synthetic song stimuli demonstrate that female choice is largely based on acoustic characters of male song: mean and peak amplitude, pulse rhythm and the mean asynchrony interval separating the two pulses in a pulse pair. Studies of different _A. grisella_ populations indicate that females generally prefer males whose songs are delivered at a faster pulse rhythm, at higher amplitude and with a longer asynchrony interval (Jang, Collins, & Greenfield, 1997; Jang & Greenfield, 1996).

A previous study of _A. grisella_ provided some evidence that females are more strongly attracted to larger than to smaller groups of singing males, but only when the difference in male number was two and not one (Alem et al., 2011). That study also suggested that males singing in groups might benefit from reduced per capita predation by insectivorous bats, an effect arising due to enhanced vigilance or to the dilution of exposure to predators afforded by group display. However, the range of group sizes tested in that study (two to four males) was too limited to allow any determination of the limits on lek size, and the specific tendency of males to form leks was not investigated.

**Population Studied and General Protocol**

We used _A. grisella_ from a population collected near Tours, Dept Indre et Loire, France (47°19′N, 0°46′E) in 2007 for all testing of lek attractiveness to females and the tendency of males to form leks (experiments 1, 2 and 5). Tests of the mechanisms by which females distinguish leks from solitary males (experiments 3, 4) were done with a population collected in Kansas, U.S.A. (38°57′N, 95°15′W). We recognize the potential for differences between _A. grisella_ populations in the shape of the preference function and in the threshold of response to pulse pair rates (Rodriguez & Greenfield, 2003; Zhou, Kelly, & Greenfield, 2010; Zhou et al., 2008) to influence optimal lek size (see Discussion). However, females in the two populations show similar preferences for more extravagant signals (Alem, 2012), and we therefore assumed that the general mechanism of lek discrimination was conserved. To confirm this assumption, we also sampled several Indre et Loire insects in tests distinguishing leks from solitary males and compared these supplementary observations with the findings from Kansas insects (see Appendix).

All insects were kept in a laboratory at the IRBI in Tours, France where they were maintained in outbred populations in adherence with the ASAB/ABS Guidelines for the Use of Animals in Research. Moths were reared on a standard diet of flours, honey, beeswax, glycerol, nutritional yeast and water (modified from Dutky, Thompson, & Cantwell, 1962) and were held under a 12:12 h L:D photoperiod at 25 ± 1 °C. Insects used in all experiments were individually isolated in 30 ml plastic cups immediately following pupation to ensure that they would be unmated. This criterion was mandatory for females, which normally mate only once and become refractory thereafter. We also applied it to males so that all individuals in our tests would have a standard mating history. In addition to being unmated, all males were between 1 and 4 days old and females were between 0 and 3 days old when tested to ensure that they had not yet begun to senesce. Adults neither feed nor drink and males seldom survive more than 10 days. Female survival is markedly shorter (Brandt, Ludvar, & Greenfield, 2005; Greenfield & Coffelt, 1983). Previous tests showed that the acoustic features of male song and female responsiveness to male song begin to decline after 5 days (Brandt & Greenfield, 2004; Jang et al., 1997). These declines were observed in the Indre et Loire population tested here, but we also confirmed that there were no significant changes in male and female behaviour during the initial 4 days following adult moult.

We conducted all tests in a room whose walls and ceiling were covered with acoustic insulation foam that greatly reduced echoes from male song and song stimuli broadcast from loudspeakers. Temperature was held at 25 ± 1 °C as in rearing, and all trials were run during the first 6 h of the photoperiodic night, the daily interval of maximum activity in _A. grisella_. A 25 W red incandescent bulb situated overhead provided illumination for our observations without unduly disturbing the insects. All experiments were conducted within an 80 cm diameter screen arena placed in the centre of the acoustically insulated room. Except in the experiment testing lek formation, singing males were always held in cylindrical screen cages (3 cm height × 3 cm diameter) that we positioned at designated coordinates within the arena. Males sang readily in these cages, and previous tests had confirmed that the screen of the arena and cages did not interfere with transmission of song (Jang et al., 1997). Manipulations and experimental set-up were not harmful to the moths and individuals were transferred back to their original population after tests.

**Experiment 1: Female Preference for Leks of Singing Males**

Our first experiment was designed to determine the differential between female visits to groups of singing males versus lone singers. We conducted trials in which the test female was released in the centre of the arena while a lone male was situated in a cylindrical cage 24 cm distant on one side and a group of singing males were situated in individual cages 24 cm distant on her opposite side. The cages holding each of the grouped males were separated by 2 cm and arranged along a line perpendicular to the axis connecting the lone male and the group. An appropriate number of empty screen cages were placed adjacent to the lone singer’s cage so that the visual stimulus of the cages, should that stimulus be a factor, would be equal on both sides of the females (Fig. 1a). We verified that all males were singing during a trial by observing their wing fanning and monitoring their song with a bat detector (Ultrasound Detector D230; Pettersson Elektronik AB; Uppsala, Sweden). A female was given 180 s to leave the release point, arrive within 2 cm of either the lone male or the group, and remain there for a minimum of 10 s. Females that did not meet these criteria (approximately 15%) were deemed nonresponsive. Otherwise the female was considered to have chosen either the lone male or the group.

We tested female preferences for groups of two, three, four and five males versus a lone singer. Twenty females were used in each
of these four tests, and individual females were never reused in more than one test. We used a pool of 160 males for the experiment, and for each successive test, we effected a random selection, with replacement of the individuals that would assume the role of lone singer and that of group member. Thus, the role of lone singer was not habitually occupied by a particularly inferior or superior male. We also regularly switched the lone singer and the group between the two sides of the arena to minimize the potential for a position bias affecting female movement.

We determined the percentage of responding females that chose the lek, and then divided the number of females that chose the lek by the number of males in the lek to determine per capita attractiveness. We divided the per capita attractiveness of the lek by the number of females that chose the solitary male to specify relative per capita attractiveness of the lek.

Experiment 2: Marginal Female Preference for Larger Leks

Our objective in experiment 2 was describing the pattern of female preference for larger leks and evaluating the potential role of neuroethological constraints in limiting lek size. We used a protocol very similar to that employed in experiment 1 to determine the differential between female visits to groups of slightly dissimilar numbers of males. Here, we tested the marginal female preferences for four versus two, five versus three, six versus four, seven versus five, eight versus six and nine versus seven males. We chose to test the discrimination of groups of $n$ versus $n + 2$ males because females tested with $n$ versus $n + 1$ males did not show any significant preference (Alem et al. 2011; see also results of experiment 1 below). Additionally, the choice of $n$ versus $n + 2$ males represents the situation that arises when a male decides to leave his lek for an equal sized one in the vicinity. We used 20 females in each of these six tests, never reusing individual females in more than one test. We effected a random selection, with replacement of the individual males ($N = 220$) that would comprise the groups such that the smaller, or larger, group was not necessarily represented by inferior or superior males.

As in experiment 1, we determined the percentage of responding females that chose the larger lek, and then divided the number of females that chose this lek by the number of males in the lek to determine per capita attractiveness. We determined the per capita attractiveness of the smaller lek in the same fashion, and then divided the per capita attractiveness of the larger lek by the per capita attractiveness of the smaller lek to specify the relative (=marginal) per capita attractiveness of the larger lek. According to neuroethological constraints, we predicted that female preference for larger leks would progressively decrease and eventually plateau with lek size increments. Consequently, the marginal per capita attractiveness of the larger lek should eventually dip below 1.

Experiment 3: The Distinguishing Acoustic Features of Leks

To understand the pattern of preference for aggregated males we then studied how females detect and distinguish leks from solitary males. We recorded the songs of individual males while they were in isolation from other males and when they were assembled in a lek of three males to determine whether individuals modified their singing in a lek. Males were held individually in screen cages, and we formed leks by grouping three cages holding singing males in a triangular formation with 3 cm separating the cages. We used 33 individual males, and organized them randomly to form 11 leks. We recorded 50% of these males first as isolated singers and second as participants in a lek. The other 50% were recorded in the reversed sequence. We allowed the males approximately 30 min to become accustomed to the cage before recording. An interval of 1 h typically separated a male’s two recordings.

We recorded a male with a condenser microphone (model CM16/CMPA; Avisoft Bioacoustics, Berlin, Germany; frequency response: ±3 dB, 20–150 kHz) situated a standard 2.5 cm above and oriented directly towards his cage. Recordings were digitized with an analogue to digital converter (model UltraSoundGate 416–200; Avisoft Bioacoustics) set at 16 bits and 500 000 samples/s. We sampled brief 10 s segments of these recordings and analysed the samples with Avisoft SASLab Pro to determine each male’s pulse pair rhythm and mean peak amplitude (see Jang & Greenfield, 1996). To record the three-male leks, we used three microphones situated as above and mutually isolated with acoustic foam. Thus, we greatly reduced the ‘crosstalk’ from neighbours in a male’s recording. We monitored the three recordings in real time on a computer screen to ensure that all males in the lek were singing, and we saved the three recordings to a three-channel digital file.
We also recorded the three-male leks, as well as isolated individuals, with a single microphone situated at 20 cm in order to compare pulse rhythm and mean peak amplitude of groups with those of solitary males via the same one-channel recording format.

The songs of individual males in a lek are not evenly spaced in time, making it difficult to define the overall pulse pair rhythm of a lek. To be objective, we only considered pulse pairs that began at least 2 ms after the onset of the last pulse pair in the lek as separate and contributing to the overall rhythm. We used 2 ms because it is twice the length of most asynchrony intervals within pulse pairs. Following presumed neuroethological constraints, we also used a longer, 5 ms criterion, the approximate length of the refractory period following an action potential in A. grisella (Jang & Greenfield, 1996; cf. Sulykke, Larsen, & Michelsen, 1988), to designate separate pulse pairs in leks. These criteria, particularly the 5 ms one, were chosen so that the overall rhythm we reported for a lek might reflect the rhythm perceived by a female listening to it.

Experiment 4: How do Females Discriminate Leks from Solitary Males?

We conducted a series of three playback experiments to determine: (1) whether females can distinguish leks by acoustic features alone, (2) whether females use the overall rhythm broadcast by leks to distinguish them, and (3) whether females attend to additional features of the overall song broadcast by leks when distinguishing them.

(1) In experiment 4a, we tested females with a choice of two-song stimuli, the recording of a solitary male and of a lek of three males, each recording made on a single channel in experiment 3. We released the test female in the centre of the arena and pre-males, each recording made on a single channel in experiment 3. Song stimuli, the recording of a solitary male and of a lek of three of them. Following presumed neuroethological constraints, we also used a longer, 5 ms criterion, the approximate length of the refractory period following an action potential in A. grisella (Jang & Greenfield, 1996; cf. Sulykke, Larsen, & Michelsen, 1988), to designate separate pulse pairs in leks. These criteria, particularly the 5 ms one, were chosen so that the overall rhythm we reported for a lek might reflect the rhythm perceived by a female listening to it.

We tested each female in five trials. Each successive trial of a given female was delayed by a minimum 30 min to minimize habituation. In each of a female’s five trials we used different solitary and lek song stimuli taken from the recordings made in experiment 3 to avoid potential pseudoreplication. The sequence in which these stimuli were presented was re-randomized for each of the test females. We regularly switched the solitary and the lek song stimuli between the two sides of the arena. For analysis, we only retained females that responded in all five trials, a criterion that eliminated two of the seven females tested.

(2) Recordings in experiment 3 showed that the overall songs of leks differed from the songs of solitary males in their faster overall pulse pair rhythms, whereas the mean song amplitudes broadcast by leks were comparable to those of solitary males (see Results). In experiment 4b, we therefore made synthetic pulse train stimuli that were delivered at the rhythms representative of the songs of solitary individuals and of three-male leks, and we presented these in binary choice trials to determine whether overall song rhythm alone allows females to distinguish leks from a distance. The faster rhythm of the overall song broadcast by leks was not regular, however, and we sought to replicate its temporal features in our playbacks. We tabulated the intervals between successive pulse pairs in the leks recorded in experiment 3, and we arranged these intervals in random order to construct a synthetic lek song stimulus. Holding the total number of pulse pairs constant at 130 pulses/s (see Results), we repeated the procedure to generate five different synthetic lek song stimuli, and we used each of these five stimuli in our playback experiments to avoid pseudoreplication.

In constructing our synthetic song stimuli, we began with a pulse taken from a recording made in experiment 3 of a solitary male whose song characters were average for the population. We copied this standard pulse after an asynchrony interval of 550 μs to create a standard pulse pair, and we then copied this standard pulse pair at the intervals specified by the random order for lek song stimuli. To construct a synthetic song stimulus representing an isolated male, we used the same standard pulse pair and copied it repeatedly at 12.5 ms intervals to create a regular rhythm of 80 pulse pairs/s, an average value (at 25 °C) for the population.

As in experiment 4a, we tested each female in five successive trials. Each of a female’s five trials used a different lek song stimulus paired with the solitary song stimulus. The order in which the five lek song stimuli were presented was re-randomized for each female. For analysis, we only retained females who responded in all five trials, a criterion that eliminated one of the six females tested.

(3) As noted in experiment 4b, the overall song broadcast by leks was distinguished by an irregular rhythm as well as by an elevated number of pulse pairs/s. In experiment 4c, we therefore asked whether females identified leks by certain features of this irregular rhythm not found in the song broadcast by lone males. In particular, we tested whether females favoured a synthetic song stimulus that featured occasional accelerations in rhythm over a stimulus having the same overall number of pulse pairs/s but delivered at a constant rhythm. Both song stimuli, constant rhythm and modified with accelerations, were taken from the synthetic song stimulus representing a solitary male in experiment 4b. That stimulus was left unmodified (80 pulse pairs/s; pulse pair period = 12.5 ms) for the constant rhythm stimulus. The modified stimulus included brief accelerations of two consecutive pulse pair periods of 4.0 ms that were inserted in a slowed, regular rhythm (pulse pair period = 14.0 ms) at random intervals subject to several constraints: a minimum of two regular periods separated consecutive accelerations, 10 accelerations were inserted each second, and the overall number of 80 pulse pairs/s was maintained. We predicted that females might respond preferentially to song stimuli that include accelerations of rhythm because an earlier study (Limousin & Greenfield, 2009) found female preferences for song that included occasional increases in amplitude.

We tested 33 females individually in five successive trials. For analysis, we only retained the 22 females that responded in each trial.

Experiment 5: Do Singing Males Join Other Male Singers?

We used a modification of the protocol employed in experiments 1 and 2 to determine the tendency of singing males to move towards and sing next to other singing males versus moving towards a location more distant from singing neighbours and singing there alone. That is, what is the tendency of males to form leks? Here, we repeated experiment 1 except that we released a male rather than a female at the arena centre. We placed either one, two, three, four or five individually caged, singing males in a cluster situated 30 cm distant from the arena centre on one side of the test male and the appropriate number of empty cages in a cluster situated 30 cm from the centre on his other side (Fig. 1b). The test male was given 5 min to settle for a minimum of 5 s at least 1 cm away from his release point and sing there. Males that did not leave
Experiment 1: Female Preference for Leks of Singing Males

We found that a significantly higher percentage (binomial test: two-tailed $P < 0.0001$) of females were attracted to male aggregations than to lone males for three of the four aggregation sizes (three, four and five males) tested. For aggregations that included two males, 70% of the tested females were attracted to the lek (binomial test: two-tailed $P = 0.115$; Fig. 2a). Moreover, for all aggregation sizes, the differential attraction of females was sufficiently great that the per capita attractiveness of aggregated males exceeded that of a solitary male (Fig. 2a). Relative per capita attractiveness values of the lek were significant (Monte Carlo resampling, one-tailed $P \leq 0.034$) for lek sizes from two to five. There was no indication of a decline in differential attraction of females as lek size increased from two to five.

Experiment 2: Marginal Female Preference for Larger Leks

We found that a significantly higher percentage (binomial test: two-tailed $P \leq 0.001$) of females were attracted to the larger male aggregation in tests of leks composed of three versus one male, four versus two males and six versus four males (Fig. 2b). In these three choice tests, as well as in tests of leks composed of seven versus five males, a male in the larger lek enjoyed a higher per capita attractiveness. However, in tests of leks composed of eight versus six males and of nine versus seven males an equal number of females chose the larger and smaller lek, and males in the smaller lek therefore enjoyed a higher per capita attractiveness. Marginal per capita attractiveness values of the larger lek were significant (Monte Carlo resampling protocol, one-tailed $P \leq 0.002$) for tests of three versus one male, four versus two males and six versus four males (Fig. 2b). As predicted by neuroethological constraints, the marginal attractiveness of the larger lek in tests of $n$ versus $n + 2$ males generally declined, and it disappeared beyond $n = 5$. On the other hand, we observed no particular reluctance by females to move towards large leks, for example during a choice of nine versus seven males. The latency of arriving at a chosen lek following a female’s release in the arena centre did not differ between the six choice tests (Kruskal–Wallis test: $H_5 = 1.344, P = 0.930$).

Experiment 3: The Distinguishing Acoustic Features of Leks

Recordings of males singing while solitary and while in a lek of three singing males showed that a given male increased his pulse pair rhythm approximately 10% (paired $t$ test: $t_{33} = -6.87, P < 0.001$) when in a group. This increase corresponds to $8.01 \pm 6.80$ (mean $\pm$ SD) pulse pairs/s. We observed no significant change in mean peak amplitude (paired $t$ test: $t_{33} = -0.11, P = 0.92$) between solitary and lek singing.
Recordings of solitary males and three-male leks, each made on a single channel, showed that the overall pulse pair rhythm of a lek significantly exceeded the pulse pair rhythm of solitary males whether we used the 2 ms criterion or the 5 ms one for identifying consecutive pulse pairs likely to be perceived by females (two-sample t test: $t_{33} = -6.67, P < 0.001$; Fig. 3). However, we observed no difference in mean peak amplitude between solitary males and leks ($t_{33} = -0.105, P = 0.91$). We note that the overall pulse pair rhythm of a lek did not increase proportionally with the number of males: With the 2 ms criterion, the overall pulse pair rhythm varied from 90–120 pulse pairs/s while solitary, not 240–260 pulse pairs/s but rather ranged from 130 to 140 pulse pairs/s. With the 5 ms criterion, the overall pulse pair rhythm was further reduced to 110–130 pulse pairs/s. These reductions in overall pulse pair rate of a lek reflect the overlap or near overlap of pulse pairs broadcast by two or more males.

Recordings sampled from several Indre et Loire insects did not reveal any differences with the Kansas insects in terms of acoustic parameters of males singing while solitary and while in a lek (see Appendix).

**Experiment 4: How do Females Discriminate Leks from Solitary Males?**

Three of the five females that responded in all five of their playback trials testing a choice between the recording of a lek and of a solitary male chose the lek in every trial. The other two females chose the lek in four of five trials (Fisher’s combined probability test: $X^2_{10} = 27.49, P < 0.01$). Thus, females have the ability to distinguish leks on the basis of their song alone.

In tests using synthetic song stimuli we found that three of the five females that responded in all five of their playback trials chose the synthetic lek stimulus in every trial. The remaining two females chose the lek in three of five trials ($X^2_{10} = 23.57, P = 0.01$). These findings indicate that females may distinguish leks from solitary males on the basis of the faster overall pulse pair rhythm broadcast by leks.

In our final tests using synthetic song delivered with accelerations of rhythm as occur naturally and without these accelerations, we found no preference for either song stimulus among the 22 females that responded in all five of their playback trials ($X^2_{44} = 20.75, P = 0.99$; $X^2_{44} = 28.52, P = 0.97$). Even though the overall song of a lek is distinguished by accelerations of rhythm that are not found in the song of solitary males, there was no evidence that females evaluated this feature.

**Experiment 5: Do Singing Males Join Other Male Singers?**

Overall, more males moved into the zone of attraction than remained in the surrounding area in every test, but males’ tendency to join and form leks did not increase linearly with lek size: Test males exhibited significant movement towards a solitary singing male and a group of three singing males (binomial tests: two-tailed $P = 0.02$ and $P = 0.008$) but not towards groups of two, four and five males (Fig. 4). Males that moved into the zone of attraction around the lek in the five choice tests settled 11.2 ± 0.91 cm (mean ± SD, $N = 77$) from the centre of the group of cages holding the singing males. We observed no influence of lek size on the distance at which the test males settled (ANOVA: $F_{4,107} = 0.679, P = 0.609$).

**DISCUSSION**

We confirmed that females preferred male leks over solitary males, and they did so to the extent that the per capita attractiveness of leks increased with lek size. However, we found that the marginal per capita attractiveness of slightly larger aggregations to females clearly ceased at leks of intermediate (four to six males) size. Acoustic measurements showed that the song broadcast by leks did not differ from that of solitary males in terms of amplitude but rather in terms of a markedly faster overall pulse pair rate. We found that females preferred signals that were distinguished only by the faster rate characteristic of leks, but they showed no preference for rhythm accelerations, which were also

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**Figure 3.** Oscillograms of males singing while (a) solitary and (b) in a lek of three. Waveforms represent 500 ms segments of one-channel recordings. (a) Pulse pair rhythm = 76 pulses/s; mean peak amplitude = 0.452. (b) Overall pulse pair rhythm = 140/110 pulses/s (2/5 ms criterion); mean peak amplitude = 0.329.

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**Figure 4.** Male tendency to join and form lekking aggregations in experiment 3. Black and grey segments of each vertical bar represent the proportions of tested males that moved towards and remained within the 30 cm radius ‘zone of attraction’ (see Fig. 1) around a lek composed of one to five males and those that remained outside this zone, respectively. Horizontal solid line shows the 0.5 expectation for male distribution, which is a conservative value given that the zone of attraction is much smaller than the part of the arena outside the zone. In total 19, 23, 18, 26 and 23 males responded in tests with groups of one, two, three, four and five lekking males, respectively. * $P < 0.05$; ** $P < 0.01$; two-tailed binomial test.
characteristic of overall lek signals. In parallel with our tests of female preference, we found that males were also attracted to singing males in the vicinity. However, males did not exhibit any significant trend to join aggregations of a specific size, and we found no evidence suggesting that larger male groups repelled additional males from arriving and joining them.

On the Role of Neuroethology

Does neuroethology play a role in shaping female preferences and in setting a limit to lek size? Results from experiments 2, 3 and 4, considered alongside an earlier study (Jang & Greenfield, 1996), suggest that basic neural constraints operate in setting the maximal aggregation size of six to seven males at which the marginal per capita attractiveness to females exceeds 1.0. We have no evidence that A. grisella females directly count the males in an aggregation to assess differences in lek size (cf. Shettleworth, 2010 on animal numeracy), and we infer that females assess leks via some characteristic of their overall signals. Both acoustic measurements of overall lek signals and female preference tests indicated that this characteristic is the overall pulse pair rate. The primacy of pulse pair rate is consistent with acoustic principles and specific features of A. grisella song.

Whereas larger aggregations are decidedly louder in certain acoustic species (e.g. Williams & Smith, 1991), our amplitude measurements of overall lek signals and solitary males indicate that sound intensity cannot serve as the proxy with which female A. grisella discriminate lek size. This finding is not surprising, given the way in which the combined sound amplitude of two or more simultaneous sound sources is summed (summation of independent, incoherent sound sources by the standard formula from linear acoustics; Crocker, 1998) and the very low duty cycle (proportion of one period in which the acoustic signal is present; <0.02) of A. grisella song. The summation of simultaneous, incoherent sounds shows that the combined amplitude of two equal sound sources is only 3.0 dB higher than a single source, a differential slightly greater than the limit that A. grisella females may detect (Jang & Greenfield, 1996; Limousin & Greenfield, 2009), and this increment only increases to 4.8 dB for three sources. But even these small increments are unlikely to occur in A. grisella leks: The low duty cycle of the song, taken in conjunction with the absence of any synchrony between the rhythms of neighbouring males, would prevent exactly simultaneous sounds from occurring but rarely. Additionally, individual males did not increase their basic amplitude when singing in a lek relative to their solitary song. This latter finding is consistent with observations that in the presence of neighbouring signallers males increase their pulse pair rhythm but not their basic amplitude (Jia, Greenfield, & Collins, 2001).

In A. grisella, pulse pair rhythm is the likely proxy that females use to distinguish leks from solitary males and larger leks from smaller ones. Females clearly distinguish a 15% difference in pulse pair rhythm (Jang & Greenfield, 1996), and acoustic measurements of overall signals of three-male leks (pulse pair rhythm from 130 to 140 pulses/s) indicate that they are at least 30% faster than the signals of solitary males (ca. 80 pulses/s), even when the 5 ms criterion for measuring overall rhythm is applied (pulse pair rhythm from 110 to 130 pulses/s; see also the Appendix). But would the evaluation of rhythm and neuroethological constraints lead to a maximum lek size of four to six males?

The earlier study (Jang & Greenfield, 1996) examining female response to pulse pair rhythm in A. grisella reported that the preference for a fast rhythm versus an average one (100 pulse pairs/s in the population studied) rises steadily as the fast rhythm increases until attaining 140 pulse pairs/s, at which value the preference plateaus. Moreover, the preference wanes at values above 160 pulse pairs/s. This plateau probably occurs at a pulse pair rate that elicits the maximum possible action potential rate in the receptor (tympanal nerves). Song stimuli delivered at 140 pulse pairs/s have a ‘short interpulse pair interval’ of 5 ms, which may approximate the refractory period following an action potential in A. grisella (Jang & Greenfield, 1996; cf. Suryikke et al., 1988). Thus, slightly faster stimuli would not elicit a higher action potential rate because the receptor is saturated, and stimuli that are considerably faster may actually elicit a lower action potential rate because some pulse pairs do not invoke an action potential: The interval between successive pulse pairs is shorter than the refractory period, the minimum delay between action potentials in the receptor. Because relative action potential rates in the left and right receptors (ears) probably determine behavioural preference for stimuli in a binary choice test (Pollack, 1998), females prefer 140 pulse pairs/s over 100 pulse pairs/s, but the preference for 160 pulse pairs/s over 100 pulse pairs/s is not any stronger. Thus, it may be inferred that there would be no marginal preference for 160 pulse pairs/s over 140 pulse pairs/s, values that would approximate the overall rhythm broadcast by a lek of five to seven males. Consequently, as seen in our current study, female preference for leks of n + 2 males over leks of n males wanes for n ≥ 5 because an overall pulse pair rate of 140 pulses/s has been attained and the receptors are saturated.

Assuming that pulse pair rhythm allows females to discriminate leks, why do A. grisella females apparently ignore the rhythm accelerations that also reveal leks with some degree of reliability? Possibly, females only perceive mean rhythm evaluated over an extended time interval, although in evaluating song amplitude females may perceive both mean and peak values (Limousin & Greenfield, 2009). In vertebrates, a varying signal character such as occasional rhythm acceleration might serve to release receiving individuals from habituation to ‘monotonous’ signals (Hartshorne, 1956; Seary, 1992), but an inhibitory effect of a constant rhythm has never been observed in A. grisella. Rhythm accelerations might also reflect acoustic signal competition or rapid adjustments in signal timing that minimize call overlap between neighbouring males (Brumm & Slater, 2007; Greenfield, 1994; Tobias & Seddon, 2009). On the other hand, such accelerations could represent a reliable signal of group cooperation between lekking individuals (Wiley & Wiley, 1977). However, none of these are satisfactory explanations in A. grisella, as accelerations in overall lek rhythm do not arise from a given male responding to his neighbours. Rather, the accelerations simply emerge from intermale differences in rhythm and an absence of fine coordination between neighbouring singers, and female receivers may simply lack the ability to perceive these brief fluctuations in signal timing.

On the Match Between Lek Size and Female Preference

Does marginal per capita attractiveness of a male aggregation to females, a value probably set by female perceptual constraints, determine lek size in A. grisella? A comparison of female and male responses suggests that marginal female preferences and male lek formation are roughly matched, and there is no evidence of intersexual conflict. The lek size at which the marginal per capita attractiveness is the largest, three males, is equivalent to the largest lek size, three males, that attracts significantly more males than the opportunity to behave as an isolated singer does. Thus, males do not seem to form groups beyond a size at which a slightly smaller one nearby would offer its males a higher per capita attractiveness (cf. Isvaran & St. Mary, 2003). These findings are consistent with observations in the field, where stable leks were typically fewer than five males (Brandt, 2003; Greenfield & Coffelt, 1983).
We did not observe any evidence suggesting that larger male groups repelled additional males from arriving and joining them. However, were all males free to move in the arena and interact directly with each other, it is likely that aggression, which is commonly observed among A. grisella males (Cremers & Greenfield, 1998; Greenfield & Coffelt, 1983), would have occurred and prevented large (≥5), stable groups from persisting. We also note that males attracted to singing males do not settle and sing very close to them but rather remain a minimum distance (approximately 11 cm) away. Thus, lek formation may involve two opposing forces in male behaviour, mutual attraction and repulsion due to aggression, suggesting that the match may be mediated, in part, by the aggression between males in leks.

Several authors indicate that multiple factors may jointly favour lek formation (Höglund & Alatalo, 1995; Isvaran & St. Mary, 2003), and a common influence in addition to female preference is predation (e.g. Alonso, Alvarez-Martinez, & Palacin, 2012; Boyko et al., 2004). In A. grisella both sexes exhibit specialized defensive behaviour in response to the echolocation signals of insectivorous bats (Greenfield & Baker, 2003; Greig & Greenfield, 2004; Rodriguez & Greenfield, 2004). A recent study showed that female attraction and predation pressure by greater horseshoe bats appear to act in parallel in favouring male aggregation in leks (Alem et al., 2011). However, the range of tests conducted in this study was too small to specify an upper limit that bat predation per se might impose on lek size. And should the limit imposed by bat predation differ from the limit set by female attraction, a lek size intermediate between the two limits may be favoured. Moreover, should the magnitude or incidence of predation pressure vary in different populations, a variable limit may be set.

Are both male and female A. grisella subject to the same perceptual constraints, and would this putative similarity ultimately influence the aggregation of males in leks and the marginal per capita attractiveness of leks to females? Possibly, as we have no reason to expect any sexual dimorphism in basic neural function of the acoustic receptor (Hoy & Robert, 1996). If so, the approximate match observed between lek size and limits to marginal per capita attractiveness may simply emerge as a by-product of common neural constraints. That is, the tendency of individual males to move towards and join larger leks may decelerate as lek size increases because of the same perceptual constraints that limit the marginal attraction of larger leks to females. However, male—male aggression must also play some role in limiting lek size, and intensity of this aggression may have evolved in response to the way in which females are attracted to leks. Moreover, economic aspects of female mate searching, as well as of male searching for male aggregations to join, need to be considered. At present, we have little information on the cost of searching in either sex (Alem, 2012; Kokko & Wong, 2007), and we cannot evaluate whether the lek sizes favoured by economics coincide with the sizes dictated by neural constraints. In parallel, predator attraction may also impose selection on mating signals and display (Akre et al., 2011), and it would be important to know the extent to which perceptual constraints explain the attraction of bats to leks of singing males and influence lek size in A. grisella. These gaps in knowledge notwithstanding, our study highlights the critical role that basic neural and cognitive constraints may play in complex behaviour and, in particular case, in determining fundamental parameters of lekking aggregations.

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APPENDIX. SPECIFIC ACOUSTIC SIGNALS OF LEKS IN THE INDRE ET LOIRE POPULATION

As in experiment 4, and with the same protocol and acoustic equipment, we recorded the songs of solitary males and three-male leks of the Indre et Loire population on a single channel. We sampled 18 unmated 1–4-day-old males and then analysed the recordings with Avisoft SASLab Pro. We determined the pulse pair rhythms and the mean peak amplitudes for solitary males as well as for the leks. We found that the average pulse pair rhythm of a solitary male was 83 ± 5 pulses/s and the overall pulse pair rhythm of leks was 170 ± 37/118 ± 14 pulses/s (2 ms criterion; 5 ms criterion; mean ± SD). Mean peak amplitude (sound pressure level calculated on an arbitrary linear scale; only measured in nine males and the three corresponding leks) of songs broadcast by solitary males and leks were 0.28 ± 0.1 and 0.3 ± 0.03, respectively.

Similarly to what we found for the Kansas population in experiment 4, recordings of solitary males and three-male leks showed that the overall pulse pair rhythm of a lek significantly exceeded the pulse pair rhythm of solitary males (Mann–Whitney U test: U = 0.00, N₁ = 18, N₂ = 33, P < 0.001), while no difference was observed in the mean peak amplitude (two-sample t test: t₂₀ = −0.084, P = 0.93). Moreover, as for the Kansas population, when using the 2 ms criterion, the overall pulse pair rhythm of a lek of three males in the Indre et Loire population, each singing at 83 ± 5 pulse pairs/s (mean ± SD) while solitary, was not 240–250 pulse pairs/s but 170 ± 37 pulse pairs/s (mean ± SD). With the 5 ms criterion, the overall pulse pair rhythms ranged from 100 to 140 pulse pairs/s. These ranges are not different from the ranges of overall rhythms observed in the Kansas population (two-sample t test for 2 ms criterion: t₂₀ = 0.615, P = 0.556; for 5 ms criterion: t₂₀ = −0.041, P = 0.97). Additionally, pulse pair rhythms of Indre et Loire and Kansas solitary males were not statistically different (t₁₉ = 1.97, P = 0.064).

These results indicate that the acoustic features of solitary males and lek signals were similar in the Indre et Loire and the Kansas populations: only the pulse rhythm increased significantly in a lek, and not proportionally to the number of males. Therefore females of these two populations have access to the same acoustic information for lek discrimination. Accordingly Indre et Loire females might show a decelerating preference for larger leks comparable to the preference observed in Kansas females (see Results).