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A novel statistical method for behaviour sequence analysis and its application to birdsong

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Complex vocal signals, such as birdsong, contain acoustic elements that differ in both order and duration. These elements may convey socially relevant meaning, both independently and through their interactions, yet statistical methods that combine order and duration data to extract meaning have not, to our knowledge, been fully developed. Here we design novel semi-Markov methods, Bayesian estimation and classification trees to extract order and duration information from behavioural sequences and apply these methods to songs produced by male European starlings, *Sturnus vulgaris*, in two social contexts in which the function of song differs: a spring (breeding) and autumn (nonbreeding) context. Additionally, previous data indicate that damage to the medial preoptic nucleus (POM), a brain area known to regulate male sexually motivated behaviour, affects structural aspects of starling song such that males in a sexually relevant context (i.e. spring) sing shorter songs than appropriate for this context. We further test the utility of our statistical approach by comparing attributes of song structure in POM-lesioned males to song produced by control spring and autumn males. Spring and autumn songs were statistically separable based on the duration and order of phrase types. Males produced more structurally complex aspects of song in spring than in autumn. Spring song was also longer and more stereotyped than autumn song, both attributes used by females to select mates. Songs produced by POM-lesioned males in some cases fell between measures of spring and autumn songs but differed most from songs produced by autumn males. Overall, these statistical methods can effectively extract biologically meaningful information contained in many behavioural sequences given sufficient sample sizes and replication numbers.

In many vertebrates, vocal communication is central to the coordination of social behaviour. This has been particularly well studied in songbirds, which adjust vocal sequences contained in songs to convey meaning about social status and motivational state (Catchpole & Slater, 2008). Vocal elements can differ in the duration (i.e. the length) of time that each element is produced in such a way that the durations of elements are not necessarily independent of their order. The importance of the inextricable relationship between duration and order in sequenced behaviours has not been well studied in part because statistical tools for doing so are not readily available. The goal of the present study was to develop statistical tools to fill this gap and to apply them to basic questions about vocal communication in songbirds.

Some seasonally breeding birds, including European starlings, *Sturnus vulgaris*, sing throughout the year, but the function of song and aspects of song structure change seasonally. Starlings and other songbirds show seasonal changes in broad measures of song structure such that songs produced during the breeding season are often longer (Eens, Pinxten, & Verheyen, 1991; Lampe & Espmark, 1987; Riters et al., 2000; Van Hout, Eens, Balthazart, & Pinxten, 2009), contain a larger repertoire of song element types (Eens et al., 1991) and are more stereotyped by some measures (Nottebohm, Nottebohm, & Crane, 1986; Smith, Brenowitz, Beecher, & Wingfield, 1997) compared to song produced outside of the breeding season. Structural features of male song appear vital to reproductive success. Females of several species prefer as mates males that produce relatively long songs (Bensch & Hasselquist, 1992; Eens et al., 1991; Gentner & Hulse, 2000; Nolan & Hill, 2004) with larger repertoires (Baker, Bjerke, Lampe, & Espmark, 1986; Eens et al., 1991; Searcy & Marler, 1981) and a high degree of stereotypy (Woolley & Doupe, 2008).

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Birdsong consists of species-specific arrangements of acoustic elements. The organization of these elements can vary widely across species, but in general the simplest elements of song are referred to as 'notes'. A series of notes that occurs together regularly is referred to as a 'syllable', and a repeated collection of syllables is referred to as a song 'phrase' or 'motif'. Collections of similar phrases or motifs are referred to as song 'types', which when separated by a gap of silence are referred to as 'bouts' (reviewed in Brenowitz, Margoliash, & Nordeen, 1997). In some species, song bouts can be divided into relatively distinct categories containing similar phrase types, as described in detail below for starlings (Eens, 1997; Hausberger, 1997; Mountjoy & Lemon, 1995). Birds may vary the sequential ordering of phrase categories and the amount of time spent producing distinct phrase categories. This suggests the possibility that meaningful information may be conveyed by the order and duration of phrase categories, yet this has not been extensively examined in part because pertinent statistical tools are not available to extract this information. In the present study, we address this gap in our knowledge by developing and employing novel statistical tools and using them to explore the extent to which the duration and ordering of phrase categories differ in the songs of male starlings singing to attract females in a spring (breeding) condition compared to the songs of males singing in an autumn (nonbreeding) condition.

We additionally explored the utility of these statistical methods by using them to shed light on how the brain fine-tunes and adjusts vocal behaviour seasonally. The medial preoptic nucleus (POM) is a brain area that is implicated in sexual motivation across vertebrate taxa, including birds (Balthazart & Surlemont, 1990; Balthazart, Absil, Gerard, Appeltants, & Ball, 1998; Balthazart, Surlemont, & Harada, 1992; Riters & Ball, 1999; Watson & Adkins-Regan, 1989). Lesions to the POM in male starlings during the breeding season strongly disrupt the production of sexually motivated courtship song (Alger & Riters, 2006; Alger, Maasch, & Riters, 2009; Riters & Ball, 1999) and implicate this area in song structure. Males with POM lesions sing shorter songs, and females approach POM-lesioned males less often than control males (Alger et al., 2009). These studies suggest that POM lesions make song less attractive to females, yet analyses have not been performed to examine the effects of POM lesions on the detailed structure of song. It has been proposed that lesions to the POM remove the sexual motivation to sing. Thus, POM-lesioned birds in spring condition are predicted to produce more autumn-like songs (i.e. songs that are not sexually motivated). Here we use the novel statistical techniques developed for comparison of spring and autumn song to test this prediction.

We recorded singing behaviour of male European starlings in two contexts: (1) males in photoperiodic and hormonal conditions mimicking the spring breeding season and in the presence of a female conspecific; and (2) males in conditions mimicking the autumn nonbreeding season in a social all-male flock. We also recorded singing behaviour of males in spring condition with bilateral POM lesions in the presence of a female conspecific. We examined differences in song structure among the three groups using semi-Markov methods, Bayesian estimation and classification trees.

METHODS

Animals

We captured 58 male and 48 female adult European starlings between December 2006 and February 2007 in Madison, Wisconsin, U.S.A. The birds were housed indoors in same-sex groups in stainless-steel cages on an 18:6 h light:dark cycle to induce a photorefractory state, a condition characterized by regressed gonads and a lack of sexual behaviour (Falk & Gwinner, 1988). All experiments were in accordance with the National Institutes of Health Guidelines and approved by the Institutional Animal Care and Use Committee of the University of Wisconsin (protocol code L00366).

Hormone Manipulations

Males were induced into one of two hormonal states, photostimulated (spring-like) and photorefractory (autumn-like), for song recording. We induced a spring-like state in 46 of the 58 males via direct hormonal and photoperiod manipulations. These males were gonadectomized and subsequently implanted with testosterone (T) so that POM lesion effects could be examined independent of any involvement of the POM in the regulation of T. Briefly, males were anaesthetized with isoflurane, a small incision was made just anterior to the last rib, and gonads were removed using forceps (Alger et al., 2009; Alger & Riters, 2006). The incisions were sutured and males were allowed to recover on a heating pad. These males were then placed on an LD 6:8 h photoperiod for at least 8 weeks to induce photosensitivity, a photoperiod under which birds will become fully reproductively active in response to T but will not enter a state of photorefractoriness (Falk & Gwinner, 1988). These gonadectomized subjects received two subcutaneous 14 mm Silastic implants (1.47 mm inner diameter, 1.96 mm outer diameter; Dow Corning, Midland, MI, U.S.A.) filled with 10 mm of crystalline T (Sigma, St Louis, MO, U.S.A.) at the time of lesion surgery and were then placed on an LD 11:13 h photoperiod when they were moved into observation rooms for song recording (detailed below). Past work has shown that these photoperiods and T treatment maximize the likelihood that castrated male starlings will display song in a sexually relevant context in captivity (Alger & Riters, 2006; Riters & Ball, 1999) and result in circulating T concentrations that are within the physiological range of breeding males for at least 8 weeks (Duffy, Bentley, Drazen, & Ball, 2000). All birds recovered from gonadectomy and implant surgery without complications and were active and alert within minutes postsurgery.

The other 12 males were assigned to the autumn group and were housed on an LD 16:8 h photoperiod for at least 8 weeks, a photoperiod that induces and maintains photorefractoriness, an autumn-like condition characterized by regressed gonads and a lack of sexual behaviour. These males were not gonadectomized or implanted with hormones.

As in past studies from our laboratory (e.g. Alger & Riters, 2006; Alger et al., 2009), females to be used as behavioural stimuli were housed on an LD 11:13 h photoperiod for at least 12 weeks. These females received subcutaneous oestradiol-containing implants to facilitate sexual interest in males and male song production. Each female received two 18 mm Silastic implants (1.47 mm inner diameter, 1.96 mm outer diameter, Dow Corning) filled with 13 mm of 17-beta-oestradiol (Sigma). The hormone implantation procedure was identical to that used for male T implants.

Lesion Procedure

Spring-like males were randomly assigned to the spring POM-lesioned group ($N = 30$) or the spring control group ($N = 16$). We assigned more birds to be lesioned than to receive a sham operation because we expected that, due to natural variation in brain size among wild-caught starlings, some of the lesions would miss the POM. Higher numbers of POM-lesioned birds were also included because POM-lesioned animals are less likely to produce songs given the known role of this brain structure in courtship song (Alger et al., 2009; Alger & Riters, 2006; Riters & Ball, 1999). (Indeed