Introductory whistle is sufficient for early song recognition by golden-crowned sparrow nestlings

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Many songbird species have a predisposition to learn conspecific songs, suggesting song learning may be guided by an innate auditory template. Evidence for such a template includes preferential response to conspecific song in early life, even before song learning begins. A prime example of an innate cue for selective song learning is the introductory whistle of white-crowned sparrows, *Zonotrichia leucophrys*. The songs of its sister species, the golden-crowned sparrow, *Zonotrichia atricapilla*, also contain an introductory whistle, which differs in structure from that of white-crowned sparrows. Here we tested the ability of nestling golden-crowned sparrows in a sympatric population to discriminate between conspecific and heterospecific songs based on introductory whistles alone, prior to the onset of song learning. Golden-crowned sparrow nestlings responded with more chirps to playbacks of conspecific whistles than to heterospecific (white-crowned sparrow) whistles, and they responded similarly to full conspecific songs and conspecific whistles alone. We suggest that the introductory whistle alone is sufficient for song recognition in the golden-crowned sparrow. We discuss similarities and differences in the role of the introductory whistle between these sister taxa, and how this divergent song phrase may share a role in species recognition in both sister species. Identifying the cues underlying song recognition prior to song learning could be key to understanding the evolution of behavioural isolation between closely related songbird species.

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Organisms across many taxa must learn species-appropriate behaviours based on experience with conspecifics. Learning is involved in the development of mate preferences in a wide array of organisms, including spiders, damselflies, guppies and many bird species (reviewed in Verzijden et al., 2012). When behaviour relating to mate attraction is learned, however, organisms may mistakenly learn to produce or prefer heterospecific signals in lieu of conspecific signals (Baptista & Morton, 1981; Slagsvold, Hansen, Johannessen, & Lifjeld, 2002). Thus, selection should favour learning strategies that minimize heterospecific learning when there are fitness costs to learning the wrong species' signals. For example, selection to minimize learning errors may explain the time constraints (Nottebohm, 1969) and innate conspecific preferences (Marler & Peters, 1977; Slagsvold et al., 2002) that often characterize song learning in oscine songbirds. This suggests that there are heritable mechanisms that direct cognitive processes at the earliest stages of song learning.

The auditory template model (Marler, 1990) posits that an innate, crude template exists in newly hatched songbirds, against which learners compare songs of potential tutors. Such a template should constrain the learning process and increase the probability that only conspecific song elements are learned, even when juveniles are exposed to both conspecific and heterospecific tutors. Prior studies on several songbird species have demonstrated species discrimination abilities in fledglings based on their behavioural and physiological responses to song playbacks (Dooling & Searcy, 1980; Nelson & Marler, 1993), and even in nestlings prior to the onset of song learning (McFarlane, Söderberg, Wheatcroft, & Qvarnström, 2016; Shizuka, 2014). These studies suggest that songbirds are already able to recognize conspecific song before learning begins, as the auditory template hypothesis predicts.

What song elements might young songbirds use to recognize conspecific songs prior to song learning and how might the use of particular song elements evolve? In general, an ideal auditory cue for species recognition might consist of a simple acoustic element that is invariant within species and absent in other sympatric species (Nelson, 1989). Existing evidence suggests that specific notes or phrases, or the relative frequency between song phrases that fit these criteria are used for species recognition (Becker, 1982;

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Colombelli-Négrel et al., 2012; Hauber, Russo, & Sherman, 2001; Hurly, Ratcliffe, & Weisman, 1990). In the case of a song learning cue, an additional criterion is that the cue must be discriminable to young birds before the song learning process begins. During the initial stages of population divergence, the cues involved in species recognition are likely to be shared in recently diverged taxa. If selection against hybridization favours species recognition, then evolution may proceed in two ways. First, one species may shift to using a new feature for species recognition (e.g. the feature that is most divergent between the taxa). Alternatively, the divergent taxa may use the same feature for recognition, but the feature itself may diverge. Testing these hypotheses requires a system in which the specific features involved in song recognition are already known and there is evidence of reproductive isolation between recently diverged taxa.

The New World sparrow genus *Zonotrichia* is exceptionally well studied and presents an ideal opportunity to explore the evolution of song recognition. The white-crowned sparrow, *Zonotrichia leucophrys*, has been the subject of particularly intensive research and many details of its song learning and recognition programme have been resolved (reviewed in Soha, 2017). In this species, the introductory whistle of its song has been implicated to play a critical role in song learning. The flat introductory whistle is species-universal: white-crowned sparrows show geographical variation in song, but all subspecies and regional dialects begin with a pure-tone whistle (Soha & Marler, 2000). This whistle is an innately encoded component of song: young male white-crowned sparrows raised in isolation produce simple songs consisting primarily of whistles (Marler, 1970). Moreover, the introductory whistle serves as a cue to memorize syllables that follow it; Soha and Marler (2000) showed that young male white-crowned sparrows preferentially memorized heterospecific syllables following a conspecific whistle, rather than conspecific syllables without an introductory whistle. Finally, recent genetic and behavioural evidence from two subspecies suggests that there is fine-scale variation in the duration of this introductory whistle between subspecies, and that individuals respond more strongly to songs of their own subspecies (Lipshutz, Overcast, Hickerson, Brumfield, & Derryberry, 2016).

The white-crowned sparrow shares its range with its sister species, the golden-crowned sparrow, *Zonotrichia atricapilla*, and the two species are commonly found in the same breeding habitat near tree-line in parts of Alaska and northwestern Canada. The two species are reciprocally monophyletic in all existing phylogenies (Weckstein, Zink, Blackwell-Rago, & Nelson, 2001; Zink, 1982). The scant record of hybrid individuals (Miller, 1940; Morton & Mewaldt, 1960) and presence of fixed nuclear genetic differences (Weckstein et al., 2001) suggest that there is strong reproductive isolation between these two species. Shared mitochondrial haplotypes between at least some populations of golden-crowned and white-crowned sparrows indicates that they may have experienced gene flow in the past, but the subspecies of white-crowned sparrow, *Z. l. gambelii,* that is sympatric with golden-crowned sparrows in the breeding season does not show this pattern (Weckstein et al., 2001). Thus, the existing evidence suggests that these are sister species, with little to no ongoing hybridization in areas of sympatry.

Like white-crowned sparrows, golden-crowned sparrows show considerable geographical variation in song across their ranges, but all begin their song with a species-characteristic introductory whistle (Shizuka, Lein, & Chilton, 2016). However, whereas white-crowned sparrow introductory whistles have no frequency modulation, the golden-crowned sparrow introductory whistle always contains descending frequency modulation in all dialects (Fig. 1; Shizuka et al., 2016). Moreover, golden-crowned sparrows can distinguish between conspecific song and the songs of sympatric white-crowned sparrows at 6−8 days of age (Shizuka, 2014). Thus,

both white-crowned and golden-crowned sparrows seem to share two features: a species-universal introductory whistle and the ability to recognize conspecific songs at the onset of song learning. This raises the question: is the introductory whistle sufficient for song recognition at the earliest stages of life? We addressed this question by conducting a series of playback experiments of conspecific and heterospecific songs and whistles to golden-crowned sparrow nestlings.

METHODS

We conducted this study at Hatcher Pass Management Area, Alaska in June and July, 2015. This golden-crowned sparrow population is sympatric with white-crowned sparrows, and nestlings are exposed to both species' songs in the nest. We found golden-crowned sparrow nests by following females during nest building, incubation or feeding of nestlings.

Whistle stimuli were prepared using Raven Pro 1.4 (Cornell Lab of Ornithology, 2011) by extracting a single whistle from the full songs of each of five unique male white-crowned sparrows (heterospecific treatment) and five golden-crowned sparrows (conspecific treatment), as shown in Fig. 1. All golden-crowned sparrow songs were recorded in another population within the local dialect region, but more than 100 km away and thus the recorded individuals were unfamiliar to the subjects. White-crowned sparrow songs were from the local subspecies, *Z. l. gambelii*, and likewise recorded away from the study site. The extracted whistles were standardized for root mean squared amplitude. All stimuli files were sampled at 48 kHz and 16 bits per sample. Whistles were repeated every 10 s for 2 min, and 1 min of white noise was added before and after the 2 min of whistles.

Based on results of a prior study (Shizuka, 2014), we conducted experiments when approximately 6 mm of the longest primary feather was exposed in all nestlings, which corresponded to 7−8 days after hatching. All chicks from a nest were removed at once and held in an insulated cloth lunchbox when not being tested or measured. Nestlings were placed individually in a portable pet carrier ($26 \times 27 \times 48$ cm) and randomly assigned to either a heterospecific whistle treatment ($N = 7$), conspecific whistle treatment ($N = 7$), heterospecific full song treatment ($N = 13$), or conspecific full song treatment ($N = 16$). Songs were played back from iPod Nano mp3 players (Apple) using iHome model IM60 and IM70 (SDI Technologies, Inc., Rahway, NJ, U.S.A.) speakers placed outside of the pet carrier. Playback volume was standardized to 60 dB SPL at 1 m from the speaker. If a chick was chirping during trial set-up, we waited until it stopped chirping to begin the trial; if the chick then resumed chirping during the pre-track period of white noise at the start of the trial, this was recorded as the pre-track response.

We measured the behavioural response as the number of chirps during the 2 min whistle presentation period, and measured the pre-track response as the number of chirps during the 1 min pre-playback period of white noise. Chicks assigned to either the conspecific or heterospecific whistle treatment or the full heterospecific song treatment that showed no response to their treatment playback subsequently received a full local conspecific song treatment as a positive control. Chicks that showed no response to the positive control ($N = 8$) were excluded from the analysis (raw data provided as Supplementary Material).

Data were analysed with a linear mixed model using the function lmer in the package lme4 (Bates, Maechler, Bolker, & Walker et al., 2015) implemented in R v.3.2.4 (R Core Team, 2016). We first generated a global model including chirp response as the response variable, with pre-track response, exposed primary feather length (a proxy for developmental stage) and two levels of