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Behavioral response to song and genetic divergence in two subspecies of white-crowned sparrows (*Zonotrichia leucophrys*)

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32 **Running title:** Song as an isolating mechanism

33 **ABSTRACT**

34 Divergence in sexual signals may drive reproductive isolation between lineages, but behavioral
35 barriers can weaken in contact zones. Here, we investigate the role of song as a behavioral and
36 genetic barrier in a contact zone between two subspecies of white-crowned sparrows
37 (*Zonotrichia leucophrys*). We employed a reduced genomic dataset to assess population structure
38 and infer the history underlying divergence, gene flow and hybridization. We also measured
39 divergence in song and tested behavioral responses to song using playback experiments within
40 and outside the contact zone. We found that the subspecies form distinct genetic clusters, and
41 demographic inference supported a model of secondary contact. Song phenotype, particularly
42 length of the first note (a whistle), was a significant predictor of genetic subspecies identity and
43 genetic distance along the hybrid zone, suggesting a close link between song and genetic
44 divergence in this system. Individuals from both parental and admixed localities responded
45 significantly more strongly to their own song than to the other subspecies song, supporting song
46 as a behavioral barrier. Putative parental and admixed individuals were not significantly different
47 in their strength of discrimination between own and other songs; however, individuals from
48 admixed localities tended to discriminate less strongly, and this difference in discrimination
49 strength was explained by song dissimilarity as well as genetic distance. Therefore, we find that
50 song acts as a reproductive isolating mechanism that is potentially weakening in a contact zone
51 between the subspecies. Our findings also support the hypothesis that intra-specific song
52 variation can reduce gene flow between populations.

53

54 **INTRODUCTION**

55 Discrimination between diverged sexual signals can contribute to reproductive isolation by
56 reducing gene flow between populations (Dobzhansky 1940; Mayr 1963; West-Eberhard 1983;
57 Coyne & Orr 2004). Behavioral responses to a sexual signal provide a measure of the salience of
58 that signal in mate choice (Searcy 1992) and in territorial interactions (Kroodsma 1986; Nowicki

59 *et al.* 1998). Numerous empirical studies suggest that mating signals used to attract mates and
60 repel competitors can also promote behavioral isolation among closely related populations
61 (reviewed in West-Eberhard 1983; Andersson 1994; Price 1998; Panhuis *et al.* 2001).
62 Concordance in geographical patterns of sexual signal and genetic variation occurs in a number
63 of taxa (e.g. crickets, *Shaw et al.* 2007; mice, *Campbell et al.* 2010; gibbons, *Thinh et al.* 2011;
64 and frogs, *Warwick et al.* 2015), supporting the hypothesis that divergence in mating signals
65 between populations can act as a behavioral reproductive isolating mechanism.

66 Much emphasis has been placed on the importance of song, and in particular learned
67 song, in facilitating speciation in birds (Marler & Tamura 1964; Nottebohm 1969; Baker &
68 Cunningham 1985; Grant & Grant 1996; Martens 1996; Price 1998, 2008; Slabbekoorn & Smith
69 2002; Podos & Warren 2007). As a long-distance signal, song is often the first aspect of the
70 phenotype that can be assessed by would-be mates or competitors (Catchpole & Slater 2008),
71 and so has potential as a behavioral barrier to gene flow (Coyne & Orr 2004). Many birds
72 produce distinct songs, and birds typically respond strongest to the song of their own species
73 (reviewed in Andersson 1994; Martens 1996), supporting a role for song as an isolating
74 mechanism. Within a species, there is also substantial geographic variation in song (reviewed in
75 Podos & Warren 2007). Both male and female receivers often discriminate between songs of
76 different cultural populations (Searcy *et al.* 1997; Derryberry 2007; Seddon & Tobias 2007),
77 which should reduce gene flow between populations with diverged signals. However, there is
78 little evidence for song acting as an intra-specific barrier to gene flow between cultural
79 populations (reviewed in Slabbekoorn & Smith 2002). The majority of empirical studies that
80 focus within a species do not find genetic substructuring based on song divergence for songbirds
81 (oscines) (e.g. Payne & Westneat 1988; Lougheed & Handford 1992; Soha *et al.* 2004; Ruegg *et*
82 *al.* 2006; Leader *et al.* 2008; Ortiz-Ramírez *et al.* 2016), nor for non-oscines in which vocal
83 learning evolved independently (e.g. Wright & Wilkinson 2001; Saranathan *et al.* 2007;
84 Gonzalez & Ornelas 2014). Thus, although there is abundant evidence that song acts as an
85 isolating mechanism between bird species, when and how intra-specific song variation facilitates
86 reproductive divergence is less clear (Slabbekoorn & Smith 2002; Lachlan & Servedio 2004).

87 Interactions between hybridizing lineages present an opportunity to investigate how intra-
88 specific song divergence affects the process of mate selection and resource acquisition. Hybrid

89 zones are natural laboratories for studying the speciation process because they facilitate the
90 testing of behavioral and genetic barriers between differentiated lineages (Endler 1977; Barton &
91 Hewitt 1985; Hewitt 1988; Harrison 1993). Although studies of hybridizing lineages typically
92 find song divergence in allopatry (e.g. Halfwerk *et al.* 2016), songs are often more convergent in
93 sympatry, due to local acoustic adaptation or interspecific learning (Secondi *et al.* 2003; Haavie
94 *et al.* 2004; Qvarnström *et al.* 2006; Kenyon *et al.* 2011), or hybridization itself (de Kort *et al.*
95 2002). Likewise, in many cases individuals in hybrid zones discriminate between diverged songs
96 (Patten *et al.* 2004; Turčoková *et al.* 2011; Greig & Webster 2013), while in other cases,
97 individuals do not discriminate between non-local and local songs (Matessi *et al.* 2000; Gee
98 2005; den Hartog *et al.* 2008), or there is asymmetric song recognition (Kershner & Bollinger
99 1999; Dingle *et al.* 2010; Ruegg *et al.* 2012; McEntee 2014; Pegan *et al.* 2015). Concordance
100 between 1) genetic and 2) song divergence, along with 3) behavioral discrimination between
101 lineage-specific songs suggests that songs have the potential to maintain, if not drive
102 reproductive isolation. However, few studies test for an association among all three components,
103 and fewer studies place these patterns in the context of evolutionary history. This context can
104 help us understand how behavioral isolating barriers function in the transition from populations
105 to species (Coyne & Orr 2004; Edwards *et al.* 2005).

106 Although recently diverged lineages offer a window into the speciation process (Hewitt
107 1988), their evolutionary history can be problematic to determine (Durrett *et al.* 2000; Pettengill
108 & Moeller 2012). Historical demographic inference based on the coalescent can be used to
109 estimate the relative time since divergence between lineages, which may indicate the role of
110 historic ecological or biogeographic processes (e.g. Hickerson *et al.* 2006), as well as to calculate
111 the degree of historical gene flow between current lineages (e.g. Carling *et al.* 2010; Field *et al.*
112 2011). Model-based approaches employing coalescent-based analyses of multilocus sequence
113 data can also test alternative hypotheses of evolutionary histories (Rosenberg & Nordborg 2002;
114 Gutenkunst *et al.* 2009; Excoffier *et al.* 2013), which can provide a framework for interpreting
115 reproductive barriers. Examples of evolutionary histories for recently diverged lineages include
116 primary divergence with either strict isolation or ongoing symmetrical or asymmetrical
117 migration, migration after a period of allopatric divergence (e.g., secondary contact), and
118 panmixia. Differentiating between primary divergence and secondary contact can provide

119 information on whether behavioral divergence could have occurred in allopatry. If evidence of
120 asymmetric gene flow between taxa coincides with a pattern of asymmetric recognition of songs,
121 then this pattern would provide support for song as an incomplete behavioral barrier (e.g.
122 Halfwerk *et al.* 2016). In comparison, concordance between symmetric song discrimination and
123 secondary contact with reduced gene flow would suggest song acting as a behavioral barrier. A
124 supported model of panmixia, on the other hand, would indicate a limited role for reproductive
125 isolation between lineages (e.g. Oomen *et al.* 2011).

126 Here, we investigate whether song is a reproductive isolating mechanism in an oscine
127 species widely studied for song evolution: the white-crowned sparrow (*Zonotrichia leucophrys*).
128 The question of whether song is a behavioral barrier to gene flow has been asked in the white-
129 crowned sparrow for decades (Baker 1975; Baker *et al.* 1984; Baker & Cunningham 1985;
130 MacDougall-Shackleton & MacDougall-Shackleton 2001; Soha *et al.* 2004), because of a strong
131 pattern of discrete song types, i.e. dialects, across small geographic scales (Marler & Tamura
132 1964) with male (Nelson & Soha 2004) and female (Petrinovich & Patterson 1981)
133 discrimination between dialects. Empirical data for genetic differentiation between cultural
134 dialect populations within subspecies of white-crowned sparrows is mixed (Baker *et al.* 1982 as
135 revisited by Soha *et al.* 2004, MacDougall-Shackleton & MacDougall-Shackleton 2001).
136 However, behavioral studies suggest that examining this question in the context of hybridization
137 between subspecies may lend insight into this question. Experiments with the white-crowned
138 sparrow demonstrate that males have a genetic predisposition to learn the song of their own
139 subspecies, and females have a genetic predisposition to pay closer attention to the song of their
140 own subspecies (Whaling *et al.* 1997; Nelson 2000), suggesting an innate behavioral barrier to
141 gene flow between subspecies despite learned song.

142 We focus on two subspecies distributed along the western coast of North America: the
143 Puget Sound subspecies (hereafter *Z. l. pugetensis*), and the Nuttall's subspecies (hereafter *Z. l.*
144 *nuttalli*). *Z. l. pugetensis* is migratory and breeds from northern California to southern British
145 Columbia, whereas *Z. l. nuttalli* is a year-round resident that breeds in coastal central and
146 northern California (Grinnell 1928; Blanchard 1941; Banks 1964). The subspecies are
147 hypothesized to have diverged in glacial refugia during the Pleistocene (Banks 1964; Baker *et al.*
148 1984), and a putative contact zone exists in northern California (Banks 1964; Mewaldt *et al.*

149 1968; Corbin & Wilkie 1988). Previous studies on *Z. l. nuttalli* and *Z. l. pugetensis* documented
150 cultural differences (Baker 1987) as well as behavioral discrimination between subspecific songs
151 (Lampe & Baker 1994), but found limited genetic divergence based on allozymes (Corbin 1981;
152 Corbin & Wilkie 1988) and mitochondrial haplotypes (Weckstein & Zink 2001). If the two
153 subspecies are distinct and hybridizing, we expect to find (1) two genetic clusters with admixture
154 between them, as well as (2) support for a historic demographic model of secondary contact. If
155 song functions as an isolating mechanism between the subspecies, we expect to find (3) song
156 divergence between the subspecies, (4) differential male response to subspecific songs, and (5)
157 an association between song divergence, genetic divergence and the strength of discrimination
158 between songs.

159

160 **MATERIALS AND METHODS**

161 **Genetic sampling and sequencing**

162 We sampled 190 individuals from 17 localities along a coastal transect spanning the
163 ranges of *Z. l. nuttalli* and *Z. l. pugetensis* (Fig. 1; Table 1). We collected blood samples (20 uL)
164 by brachial venipuncture from 132 mist-netted males in 2004 and 2005 and released birds after
165 metal banding. We transferred blood to EDTA-saturated filter paper, and stored in airtight
166 containers on DriErite at room temperature. Our sample also included tissues from 51 vouchered
167 specimens collected in 2010. Voucher specimens are deposited in the Museum of Natural
168 Science at Louisiana State University. We also collected vocal data for these 183 males (see
169 Song recording and analysis). Seven additional samples, including four females, were provided
170 as tissue loans from the Museum of Vertebrate Zoology and the Burke Museum. We extracted
171 total genomic DNA using a DNeasy blood and tissue extraction kit following the manufacturer's
172 recommended instructions (Qiagen, Valencia, CA).

173 We sent DNA extracts to the Institute of Genomic Diversity at Cornell University in
174 Ithaca, NY, USA, for genotyping-by sequencing (GBS). GBS reduced-representation libraries
175 were prepared and analyzed according to the methods of Elshire et al. (2011) using the restriction
176 enzyme PstI (CTGCAG) for digestion and creating a library with 95 unique barcodes, one for
177 each individual for each plate. Samples were sequenced on two lanes of the Illumina HiSeq
178 platform, generating 551,083,045 reads.

179 Single-nucleotide polymorphisms (SNPs) were called by processing the raw 100bp
180 single-end sequence reads using the UNEAK pipeline (Lu *et al.* 2013), an extension of the Java
181 program of TASSEL 4.0 (Bradbury *et al.* 2007). Reverse complement tag-pairs were collapsed,
182 and loci with greater than 20% missing data, as well as minor allele frequency less than 1%,
183 were excluded. Samples were defined as failed if the number of sequences produced for that
184 sample was less than 10% of the mean number of sequences for all samples sequenced in that
185 flow cell lane – four failed samples were excluded from further analysis.

186 The UNEAK pipeline identified a total of 79,130 biallelic SNPs. After filtering in the
187 pipeline, the final data matrix for all 186 individuals contained 1583 SNPs. This reduction in loci
188 was due in part to a handful of individuals with very few reads overall. To address this, a
189 restricted dataset was created by removing individual samples that produced fewer than 500,000
190 reads. The restricted dataset resulted in 6419 SNPs for 169 individuals, with total missing data
191 (number of missing genotypes per locus per sample) equal to 16.9%. All subsequent analyses
192 were conducted with this restricted dataset.

193

194 **Population Structure Analysis**

195 To characterize patterns in genetic structure and assign individuals to populations, we used the
196 program STRUCTURE v. 2.3.4 (Pritchard *et al.* 2000). Structure analyses were performed using
197 a burn-in length of 200,000, and 500,000 MCMC repetitions to test clusters ranging from $K=1$ to
198 $K=20$ to allow for sub-structuring within sampling sites, with 10 independent replicates, after
199 which all parameters converged. Parameter settings also included an admixture model of
200 ancestry and correlated allele frequencies. An individual was classified as parental *Z. l. nuttalli* if
201 $Q \leq 0.1$, parental *Z. l. pugetensis* if $Q \geq 0.9$, and admixed if $0.1 < Q < 0.9$. The optimal number
202 of clusters (K value) was calculated using ΔK likelihood evaluations (Evanno *et al.* 2005) in
203 Structure Harvester (Earl & vonHoldt 2012). We used CLUMPP (Jakobsson & Rosenberg 2007)
204 to identify potential multi-modality and account for label switching among replicates and
205 Distruct (Rosenberg 2004) to visual admixture proportions. We recognize that STRUCTURE is
206 merely an exploratory starting point for downstream population-based analyses, and it can be
207 hazardous to read its results as actual inference (Falush *et al.* 2016). Therefore, we also assessed
208 population structure with a model-free method based on multidimensional statistics: principal

209 component analysis (PCA) implemented in the R (R-Core-Team 2015) package adegenet v.2.0
210 (Jombart & Ahmed 2011). We used the function scaleGEN to scale allele frequencies and
211 replace missing genotype data with the mean allele frequencies. We performed the PCA with the
212 function dudi.pca. For visualization each individual was labeled according to sampling location,
213 allowing us to examine relationships among individuals without *a priori* assumption about
214 subspecies assignment. Alternative methods of analysis (e.g. fastStructure (Raj *et al.* 2014) and
215 DAPC (Jombart *et al.* 2010)) showed qualitatively similar findings and are not presented here.

216

217 **Inference of historical demographic parameters**

218 To distinguish the demographic history of these populations and to estimate key parameters of
219 interest, such as divergence time and effective population size, we used the composite-likelihood
220 simulation-based approach of fastsimcoal2 (Excoffier *et al.* 2013). We generated the observed
221 joint, folded site frequency spectrum (SFS) using custom python scripts and $\partial a \partial i$ (Gutenkunst *et al.*
222 *al.* 2009). $\partial a \partial i$ provides a facility for projecting an SFS from a larger sample size to a smaller
223 sample size. For RAD-like datasets, which often contain significant missing data, projecting
224 down can increase the number of usable SNPS by averaging over resamplings of the larger
225 dataset. We explored multiple possible values for projection, and selected two values for full
226 downstream analysis: one larger (75 x 75) and one smaller (20 x 20), as measured in number of
227 individuals per population. The larger projection was selected to maximize the number of
228 segregating sites. The smaller projection was selected to minimize total size of the SFS without
229 sacrificing demographically important signal in the data, in an effort to explore the tradeoff
230 between computation time and accuracy of inference. We tested five different historical
231 demographic models for goodness of fit to the observed data: (1) primary divergence with strict
232 isolation, (2) divergence with ongoing symmetrical and (3) asymmetrical migration, (4)
233 migration after a period of allopatric divergence (e.g. secondary contact), and (5) panmixia. For
234 all models we explored two partitions of the data: (1) admixed individuals assigned to subspecies
235 based on admixture proportions from the aggregated STRUCTURE runs for $K = 2$ and (2) only
236 parental individuals by excluding those with admixture proportions between 0.1 and 0.9. We
237 selected wide, uninformative, uniform search ranges for all estimated parameters. We performed
238 50 independent runs per model, per data partition to obtain likelihood values for the observed

239 data under each given model. For each run we performed 100,000 simulations and 40
240 expectation-conditional maximization (ECM) cycles. We evaluated model fit with both
241 information theoretic (AIC) and likelihood (LRT) based methods. We generated 95% confidence
242 intervals for demographic parameters of interest using 100 parametric bootstrap replicates. For
243 each bootstrap replicate we simulated a new SFS with the same number of SNPs as our observed
244 data using the maximum likelihood parameters and the best fitting model from the model
245 selection step. We re-estimated parameters across replicates for the simulated SFS and
246 aggregated maximum likelihood parameters. We generated bootstrap confidence intervals using
247 the python package Scikits-Bootstrap (<https://github.com/cgevens/scikits-bootstrap>).

248

249 **Song recording and analysis**

250 Within these two subspecies, males produce one stereotyped song type (e.g. dialect). Most males
251 in each location produce the same dialect, and males in different locations produce different
252 dialects. We recorded 208 males defending territories during the breeding season in 16 different
253 localities (we did not have songs for site 8), each with their own unique song dialect, in 2004,
254 2005, and 2010, with an average of 12 individuals (range = 5–34) per site (Table 1). Song
255 dialects in these localities have been stable over 30 years (Derryberry 2009; Luther & Derryberry
256 2012), so have not likely changed across this six-year spread in sampling. Recordings were made
257 using a Sony TCM-5000EV cassette recorder, a PRO-302 Unidirectional Dynamic microphone,
258 and a Sony PBR330 parabolic reflector. All songs were digitized with 16-bit precision at a 25
259 kHz sampling rate using Syrinx 2.2b (Burt 2001) and an Echo Digital Audio sound card. All
260 songs were high pass filtered to eliminate noise below 1500 Hz. We measured 8 acoustic
261 parameters shared by all song types: song maximum and minimum frequencies (Hz); the
262 dominant (peak) frequency of the whistle; the duration of the whistle, the introduction, and the
263 average syllable duration; and the rate of trill note delivery and frequency bandwidth of the trill
264 (Table S1). All measurements were taken using Signal version 3.1 or 5 (Beeman 1999). We took
265 minimum and maximum frequency measurements at –36 dB relative to the peak amplitude
266 frequency in the song from digital spectrograms (256 pt transform, frequency resolution = 97.7
267 Hz). We calculated frequency bandwidth as the difference between the maximum and minimum
268 frequencies. We measured dominant frequency as the frequency at which the most sound energy

269 was transmitted during production of the relevant song section from a smoothed power spectrum
270 (smoothing resolution 100 points). Temporal variables were measured from oscillograms (time
271 waveforms). Trill rate was calculated as the number of notes produced per second. Following
272 Podos (2001), we calculated a ninth acoustic parameter, vocal performance, as the orthogonal
273 distance between each song and an upper-bound regression for the plot of trill frequency
274 bandwidth as a function of trill rate for 375 white-crowned sparrow songs from 15 different
275 dialects that has been shown to be robust to different methods for estimating the performance
276 trade-off between bandwidth and trill rate (Derryberry 2009; Wilson *et al.* 2014). Songs closer to
277 the limit are higher performance. All raw song data were transformed to a scale with a mean of 0
278 and a standard deviation of 1 (a z-score) to allow for scale free comparisons.

279 We assessed if song acoustic parameters could be used to distinguish the two subspecies
280 using two approaches. First, we ran a discriminant function analysis (DFA) of the individual
281 song variables using JMP v.12 (Sall 2015). Songs from genetically admixed individuals were
282 classified to subspecies based on their admixture proportions from STRUCTURE. Next, we
283 summarized 7 of the acoustic parameters using a PCA in JMP. We excluded trill rate and
284 frequency bandwidth from the PCA, as these were used to calculate vocal performance. This
285 yielded four independent factors with an eigenvalue greater than 1, explaining a total of 75% of
286 the original song variation (Table 2). To assess whether songs have diverged between
287 subspecies, we used a linear mixed model approach with locality (n=16) as a random effect and
288 subspecies as the predictive factor.

289

290 **Territorial playback experiment**

291 To test subspecies-specific discrimination among songs, we measured the response of free-
292 living, territorial adult males using territorial playbacks, a standard experimental design that
293 quantifies male response to simulated intrusion on their territories (McGregor *et al.* 1992). We
294 conducted playback experiments in two parental localities (sites 2 and 12) and two admixed
295 localities (sites 6 and 7), hereafter referred to as playback localities. Playbacks were conducted
296 between May and June in 2010 (sites 2, 7, 12) and in 2013 (site 6), when males were actively
297 defending breeding territories. Focal males were not genotyped.

298 Stimuli were presented in a paired, balanced design. In each parental locality we assessed
299 male response to songs from their own location ('own') and to songs from a parental locality of
300 the other subspecies ('other'), and in each admixed location we assessed male response to 'own'
301 local song versus 'other' for each of the two subspecies. We used 10 exemplars for each song
302 category and tested 10 males for each comparison. Each male heard a different pair of exemplars
303 to avoid pseudoreplication (Kroodsma 1990; Kroodsma *et al.* 2001). Order of presentation and
304 selection of exemplars were randomized across males. Stimuli from site 2 were selected from
305 recordings made in 2004, sites 7 and 12 from 2005, and site 6 from 2010. Adult males in the wild
306 live an average of 16 months (Cortopassi & Mewaldt 1965). Thus, it is unlikely that males tested
307 were familiar with the males that produced the stimulus songs.

308 We separated treatments by 48 hours to minimize habituation and did not test neighbors
309 on the same day. Songs were amplitude normalized and broadcast at a constant level (80 – 82 dB
310 SPL 1m) and at a natural rate of six songs per minute from a speaker (Altec Lansing IMT320
311 inMotion) near the center of the focal male's territory as determined by behavioral observations.
312 We measured four response variables: mean distance from the speaker during the (1) 3-min
313 playback period and the (2) 3-min post-playback period, (3) number of flights over the speaker
314 during the playback period and (4) song rate (songs/min) during the playback period. To
315 facilitate accurate measures of distance, we placed markers at 4 meters and 8 m on either side of
316 the speaker before the trial began. Distances and observed behaviors were recorded at 10-second
317 intervals. Males were considered to have a stronger response to the stimulus when they
318 approached the speaker more closely (Searcy *et al.* 2006), flew over the speaker more often, and
319 produced songs at a higher rate. One observer narrated observations while another recorded the
320 observations onto datasheets. Experiments were not blind to stimulus type.

321 For each experiment, we reduced the four behavioral response measures using PCA and
322 used the PC scores in statistical testing in R (R-Core-Team 2015). The original behavioral
323 variables were not statistically independent (Rice 1989) and separate tests would not reflect the
324 multivariate nature of the males' responses (McGregor *et al.* 1992). Playback and post-playback
325 distance were highly correlated, so we calculated an average distance to include in PCAs. We
326 retained PCs with an eigenvalue greater than 1 for analyses, or the minimum number of PCs
327 required to explain 50% or more of the variance in the original variables (Table 3). To test

328 whether males discriminated between 'own' and 'other' within each locality, we analyzed paired
329 comparisons separately using Wilcoxon signed-rank tests on the respective PCs. Repeated
330 measures MANOVAs were then used to test for differences in male response due to (1) locality
331 and (2) genetic status (putative parental vs. putative admixed). Joint analyses standardized the
332 post-playback data to the first 3-min. For combined analyses using MANOVAs, male response
333 was normally distributed (all Shapiro-Wilk $P > 0.25$) and equal in variance (all Leven's $P >$
334 0.18). Effect size (Cohen's d) and power to reject a false null hypothesis were also determined
335 (Cohen 1988). We calculated the power for our given N , effect sizes (estimated from means and
336 standard deviations), and alpha level of 0.05 using the G* Power 3.1 (Faul *et al.* 2009) for
337 Wilcoxon signed-rank tests (matched pairs).

338

339 **Song dissimilarity, behavioral response, and F_{st}**

340 We asked whether variation in male response to 'own' versus 'other' song was explained by
341 acoustic dissimilarity, as well as pairwise F_{st} between the parental (2 and 12) and admixed (6 and
342 7) playback locations. We calculated a dissimilarity score between all songs used for playback
343 analyses using the dynamic time warping function in Luscinia v.2.02.10.15 (Lachlan 2007). This
344 function searches for the optimal alignment of two signals and then calculates a dissimilarity
345 score based on temporal and spectral characters. For each playback experiment, we averaged
346 song dissimilarity scores and the response difference to 'own' vs. 'other'. We calculated genetic
347 distance among the playback localities using a pairwise F_{st} matrix in Arlequin v3.5 (Excoffier &
348 Lischer 2010) based on the 6419 SNP dataset (Table S2). We then used linear regression to
349 compare male response to song dissimilarity and male response to genetic distance in R (R-Core-
350 Team 2015).

351

352 **Multiple Matrix Regression with Randomization**

353 To quantify the independent contributions of song and geographic distance on genetic
354 differentiation, we used a multiple matrix regression with randomization (MMRR) (Wang 2013).
355 Unlike a partial Mantel, MMRR uses a randomized permutation procedure to account for non-
356 independence between variables (Wang 2013). We quantified isolation by song (IBS) and
357 isolation by distance (IBD) with the “MMRR” function in R for 10,000 permutations, using

358 pairwise genetic distance as the response variable and geographic and song distances as the
359 explanatory variables. We calculated genetic distance among locations using a pairwise F_{st}
360 matrix in Arlequin v3.5, based on the 6419 SNP dataset (Excoffier & Lischer 2010). We
361 calculated the geographic distance matrix from GPS coordinates using the “earthdist” function in
362 the R package fossil (Vavrek 2011). We calculated the song distance matrices for each of the 9
363 song parameters as well as song PC1 using the “write.matrix” function in the R package MASS
364 (Venables & Ripley 2002). We excluded Sinkyone (site 8; Table 1) from analyses because we
365 did not have song data.

366

367 **RESULTS**

368 **Population structure**

369 The optimal number of populations in the Bayesian assignment probability analysis using
370 STRUCTURE for all replicates was $K=2$, based on the Evanno method, with $\Delta K = 922.63$ (Fig.
371 2). Increasing K did not provide a better explanation of the data as measured by log likelihood.
372 Based on admixture proportions (Q values), individuals from localities 1 – 5 were confidently
373 assigned to the southern subspecies (*Z. l. nuttalli*), localities 6 – 8 were admixed, and localities 9
374 – 17 were assigned to the northern subspecies (*Z. l. pugetensis*). For the PCA, we found two
375 separate clusters corresponding to the two subspecies (Fig. S1). The admixed MacKerricher (site
376 7) and Sinkyone (site 8) samples were distributed between the two clusters, whereas Manchester
377 (site 6) samples did not cluster with the rest of the *Z. l. nuttalli* samples.

378

379 **Historical demographic inference of secondary contact**

380 Including admixed individuals in the dataset consistently increased the fit of all models;
381 therefore, we report only the results including admixed individuals in the data matrix. Likewise,
382 the reduced projection dataset provided a poor fit for all the models, so we report results only of
383 the larger projection. All model comparison criteria decisively supported the secondary contact
384 model over models of continuous symmetric or asymmetric migration, indicating that some
385 period of isolation was important in establishing the divergence between these subspecies (Fig.
386 S2, see Table S3 for a comparison of all models). However, we caution here that we could not
387 possibly evaluate all historical scenarios, and therefore cannot fully reject a model of primary

388 differentiation. Our parameter estimates indicate incomplete isolation beginning during the last
389 glacial cycle (~45kya), followed by a short period of complete isolation after the Last Glacial
390 Maximum (LGM) (~9kya), and secondary contact only relatively recently (~2kya). Recent
391 migration rates (~10 individuals per generation) were on the order of 5x higher than the
392 migration rate between the time of initial divergence and isolation (~2 individuals per
393 generation), indicating a period of drastically reduced connectivity (Table 4).

394

395 **Song variation between subspecies**

396 Songs have diverged between subspecies, although not in all parameters (see measurements of
397 the 9 acoustic parameters in Table S1 and factor loadings for the first four PCs derived from
398 song parameters in Table 2). In general, *Z. l. nuttalli* produce songs with longer whistles and
399 shorter other notes. Using all acoustic parameters, all but two songs (1%) were correctly
400 classified to subspecies by a DFA. A forward, stepwise DFA revealed that the best variable to
401 distinguish between the subspecies was whistle length. Whistle length had a jack-knifed
402 classification accuracy of 80%; adding song minimum frequency and note length increased
403 accuracy to 90%. Using DFA, songs from admixed individuals in Manchester (site 6) and
404 MacKerricher (site 7) were classified as *Z. l. nuttalli*. A linear mixed model revealed that genetic
405 subspecies identity was a significant predictor of song structure for PC1 (F=22, DF=1,
406 $P < 2.29 \times 10^{-6}$) but not for PC2-4 (PC2: F=0.1, DF=1, P=0.8; PC3: F=0.6, DF=1, P=0.44; PC4:
407 F=0.4, DF=1, P=0.5). Plotting song PC1 against geographic distance illustrates that the
408 subspecies have diverged in song, and the songs of admixed individuals group with *Z. l. nuttalli*
409 (Fig. 3).

410

411 **Behavioral response to playbacks**

412 Coding of playback populations as parental in Bolinas (site 2) and Bandon (site 12), and as
413 admixed in Manchester (site 6) and MacKerricher (site 7), was corroborated by genetic
414 clustering analyses (see *Results* Population structure, Table 1). Note that admixture proportions
415 in Manchester (site 6) are 71% *Z. l. nuttalli* and in MacKerricher (site 7) are 51%. Within the two
416 parental playback localities (sites 2 and 12), males responded more strongly to their local song
417 than to the song of the other subspecies (site 2: PC1: $S = -25.5$, $P < 0.0059$, effect size Cohen's

418 $d=1.5$; site 12: PC1: $S = -23.5$, $P < 0.0137$, $d = 0.92$; Table S4, Table 5). In the two admixed
419 playback localities (sites 6 and 7), males gave equal responses to local and non-local songs of
420 their more genetically similar subspecies, *Z. l. nuttalli* (site 6: PC1: $S = 3.5$, $P < 0.78$, $d = 0.27$, PC2:
421 $S = 1.5$, $P < 0.92$, $d = 0.22$; site 7: PC1: $S = -12.5$, $P < 0.23$, $d = 0.22$; Table S4, Table 5), but
422 responded more strongly to local song than to *Z. l. pugetensis* song (site 6: PC1: $S = -21.5$,
423 $P < 0.0273$, $d = 0.86$, PC2: $S = 1.5$, $P < 0.92$, $d = 0.09$; site 7: PC1: $S = -21.5$, $P < 0.0273$, $d = 0.99$; Table
424 S4, Table 5).

425 In testing whether male response to own versus other song varied among playback
426 localities, the locality main effect was significant ($F_{3,56} = 4.16$, $P = 0.0099$), indicating that males
427 from the four localities (sites 2, 6, 7 and 12) varied in their overall level of response to simulated
428 intruders. The song main effect was also significant ($F_{1,56} = 40.1$, $P = 0.0001$), indicating that
429 males responded more strongly to simulated territorial intruders with their own song than
430 intruders with songs from other locations. The interaction term for this test was not significant
431 ($F_{1,56} = 0.42$, $P = 0.42$), indicating that males in all locations gave a stronger response to 'own'
432 than to 'other' songs.

433 In testing whether male response to 'own' vs. 'other' varied according to whether the
434 subject males are from parental or admixed populations, the genetic status main effect was
435 significant ($F_{1,58} = 10.35$, $P = 0.0021$), indicating that putative admixed males responded more
436 strongly to territorial intruders than did putative parental males (Fig. 4). Consistent with the
437 previous model, the song main effect was also significant ($F_{1,58} = 41.3$, $P = 0.0001$), indicating
438 that overall males responded more strongly to their 'own' than to the 'other' song phenotype. The
439 interaction term for this test was not significant ($F_{1,58} = 2.69$, $P = 0.11$), indicating that both
440 putative parental and putative admixed males showed similar levels of discrimination between
441 'own' and 'other' songs, although there was a trend towards weaker discrimination among
442 putative admixed males (Fig. 4).

443

444 **Song dissimilarity and F_{st} predict strength of discrimination**

445 Dissimilarity between own and other stimuli predicted the strength of behavioral discrimination.
446 Individuals were more different in response to own versus other song when there was greater
447 dissimilarity between own and other song, such that they responded significantly less to songs

448 more dissimilar to their own ($R^2=0.77$, $F_{1,4} = 13.41$, $P<0.02$; Fig. S3). Likewise, pairwise genetic
449 distance predicted the strength of behavioral discrimination. Individuals were more different in
450 response to own versus other song when there was a larger F_{st} between the focal playback
451 locality and stimulus locality, such that individuals responded less to songs from populations
452 more genetically distant from their own ($R^2=0.88$, $F_{1,4} = 29.23$, $P<0.006$; Fig. S4).

453

454 **Isolation by Song**

455 Whistle length and song PC1, which included whistle length and average note length as
456 significant loadings, were both stronger predictors of genetic distance than geography across
457 localities. The regression coefficient for song PC1 distance ($\beta_S = 0.51$, $3 p = 0.0001$) was over
458 twice as large as the regression coefficient for geographic distance ($\beta_D = 0.2$, $p = 0.12$), and the
459 regression coefficient for whistle length distance ($\beta_S = 0.53$, $p = 0.0002$) was over seven times as
460 large as the regression coefficient for geographic distance ($\beta_D = 0.075$, $p = 0.63$), suggesting that
461 isolation by song explained genetic distance more strongly than isolation by distance for these
462 parameters (Fig. S5)

463

464 **DISCUSSION**

465 Overall we found acoustic, behavioral, and genetic evidence that *Z. l. nuttalli* and *Z. l.*
466 *pugetensis* are distinct evolutionary units and that song is acting as a barrier to gene flow
467 between them. Historical demographic inference suggests that the subspecies diverged relatively
468 recently, and subspecific differences in song have been maintained in the face of hybridization.
469 Putative parental individuals discriminate between the two subspecies based on song, as do
470 putative admixed individuals. We found strong evidence that as songs become more dissimilar,
471 males respond less to these songs in an important functional context: territory defense. There was
472 some evidence that song is a weaker barrier in the hybrid zone, as putative admixed individuals
473 tended to discriminate less strongly between songs of the two subspecies than did putative
474 parental individuals, though the effect was not statistically significant. On further examination of
475 song divergence, we found that whistle length – an important species recognition cue in song
476 learning in this species – may also function in subspecies recognition.

477 Our genetic clustering analyses found that *Z. l. nuttalli* and *Z. l. pugetensis* are two

478 distinct genetic populations. This contrasts with previous attempts that could not distinguish the
479 subspecies using allozyme or mitochondrial loci (Corbin 1981; Weckstein & Zink 2001). In both
480 our study and others, pairwise genetic distances among localities both within and between the
481 subspecies were low (Corbin 1981; Zink & Barrowclough 1984). While these two subspecies are
482 genetically distinct in allopatry, population assignment tests revealed genetically admixed
483 individuals at three localities in the hybrid zone. Our simulation-based demographic analyses
484 suggest that this admixture could be explained by secondary contact, with glacial refugia during
485 the last glacial maximum a plausible mechanism for allopatric differentiation. However, further
486 testing of more complex models with selection is warranted in future studies (Roux *et al.* 2016).
487 While many studies of individuals with intermediate admixture proportions assume that taxa are
488 exchanging genes in secondary contact, few explicitly test this model against other evolutionary
489 scenarios (Payseur & Rieseberg 2016, but see Nadachowska-Brzyska *et al.* 2013). Coalescent
490 simulations are a powerful tool for investigating the history of populations, but there are several
491 drawbacks. For one, it can be computationally demanding for genome-scale data, especially as
492 sample size and model complexity increase. Additionally, the stochastic nature of the
493 coalescence process introduces some uncertainty into the estimated demographic parameters
494 (Terhorst & Song 2015). Finally, we interpret these analyses with the caveat that although our
495 results supported secondary contact, it is impossible to evaluate all possible historical scenarios
496 of differentiation.

497 Songs were divergent between *Z. l. nuttalli* and *Z. l. pugetensis*. The best parameters to
498 distinguish between the subspecies were whistle length and average note duration, and *Z. l.*
499 *nuttalli* had longer whistles and shorter durations of other notes. Putative admixed individuals
500 from Manchester (6) and MacKerricher (7) had songs more similar to *Z. l. nuttalli*, and
501 individuals from Manchester (6) were also more genetically similar to this subspecies. The
502 pattern of song divergence may provide insight into the features of song that individuals use to
503 discriminate between subspecies. We found that whistle length was the best song parameter to
504 distinguish between the subspecies. The whistle is a likely candidate as a conspecific marker for
505 recognition, as it is universally present across song dialects for both subspecies and is the
506 introductory component of their song. Although song is culturally inherited, song learning is
507 directed by a genetic template (Nelson *et al.* 1995; Soha & Marler 2001a; b). Several song

508 learning experiments with white-crowned sparrow nestlings indicate that the whistle is innate
509 rather than learned (Whaling *et al.* 1997), important for acoustic imprinting (Margoliash 1983),
510 and may reflect an innate template in conspecific song memorization, production and recognition
511 (Whaling *et al.* 1997; Soha & Marler 2000). Thus, the whistle's importance in song learning and
512 recognition for nestlings may also influence adult recognition of potential competitors and mates.
513 Innate recognition of conspecifics may be especially important given that in the non-breeding
514 season, southern localities contain a mixture of overwintering *Z. l. pugetensis* and resident *Z. l.*
515 *nuttalli* (Blanchard 1941). A rich area of future study lies in exploring regions of the genome
516 responsible for divergence in song between the subspecies, potentially related to innate aspects
517 of song learning.

518 Males responded less strongly to heterotypic song in parental populations. Signals that
519 elicit a stronger territorial response from males are typically interpreted as signals more effective
520 at maintaining a territory and competing for mates (Searcy & Nowicki 2005), (but see Baker &
521 Mewaldt 1978; Baker *et al.* 1981 for the argument that a stronger response to heterotypic song
522 can facilitate reproductive isolation). Therefore, we interpret a lower response to heterotypic
523 song as evidence of a behavioral barrier between *Z. l. nuttalli* and *Z. l. pugetensis*, such that
524 individuals of one subspecies would not be as effective in territory defense and mate acquisition
525 in a population of the other subspecies. These results are consistent with previous studies that
526 found males in parental populations are more responsive to their own subspecies song, both in
527 white-crowned sparrows (Lampe & Baker 1994) and in other taxa (Turčoková *et al.* 2011; Greig
528 & Webster 2013). Discrimination was not explained solely by familiarity with the local song
529 type, as putative admixed individuals did not discriminate between their own songs and non-
530 local songs *Z. l. nuttalli*, which more closely matched their genotype.

531 Male territorial responses to playbacks of bird songs are less logistically challenging than
532 measuring female response and are therefore more typically used to test signal discrimination as
533 an indirect measure of reproductive isolation (e.g. Irwin *et al.* 2001; Dingle *et al.* 2010; Podos
534 2010; Derryberry 2011; Turčoková *et al.* 2011), although theory also suggests that male-male
535 competition can contribute directly to isolation (Ellers & Slabbekoorn 2003). A useful follow-up
536 experiment could involve testing admixed and parental female preferences for admixed and
537 parental songs, as testing females would provide more direct evidence of whether song is acting

538 as a behavioral barrier between the subspecies. A study in the *Z. l. oriantha* subspecies indicated
539 that females prefer their natal-dialect song over a foreign-dialect or heterospecific song
540 (MacDougall-Shackleton *et al.* 2001), so we predict that females will discriminate between
541 subspecies songs in the parental populations.

542 We not only found that individuals discriminated between homotypic and heterotypic
543 song, but also that the strength of discrimination between songs was predicted by similarity of
544 the stimulus song to the receiver's song. Finding this association supports the hypothesis that
545 divergence in the signal itself is driving behavioral discrimination between the signals. Co-
546 variation between signal and response has been found in some playback studies (e.g. Sosa-López
547 *et al.* 2016), but not others (e.g. den Hartog *et al.* 2008). However, these studies focus on the
548 strength of behavioral response to a stimulus (e.g. height of behavioral response PC1) rather than
549 the strength of discrimination between stimuli (e.g. slope of behavioral response PC1). Our
550 pairwise design enabled us to relate the difference in song stimuli directly to the difference in
551 behavioral responses to those stimuli. The strength of discrimination between stimulus songs was
552 also positively correlated with pairwise genetic distances for those playback localities. These
553 associations among genetic distance, song divergence, and behavioral response support the role
554 of song as a behavioral isolating mechanism in the hybrid zone.

555 We also attempted to infer what processes are driving patterns of genetic, acoustic, and
556 behavioral divergence between subspecies. Multiple forces of selection may act on song, causing
557 divergence among populations. Acoustic signals may diverge and converge via sexual and social
558 selection (Fisher 1930; West-Eberhard 1983), acoustic adaptation to environmental conditions
559 affecting sound transmission (Morton 1975; Wiley & Richards 1982; Derryberry 2009),
560 morphological divergence in shape and size that constrains signal production (Podos 1996;
561 Podos *et al.* 2004), genetic or cultural drift (Lemon 1975; Lynch 1996; Irwin *et al.* 2008) or a
562 combination of these social, ecological, and stochastic factors (Mundinger 1982; Price 1998;
563 Wilkins *et al.* 2013). Divergent migratory behavior and/or allopatric temporal isolation between
564 the subspecies could also play a role in genetic differentiation (e.g. Ruegg *et al.* 2012; Delmore
565 & Irwin 2014), given that *Z. l. pugetensis* is migratory and *Z. l. nuttalli* is a year-round resident.
566 However, migratory behavior as an isolating mechanism is not mutually exclusive with our
567 hypothesis that song is a behavioral barrier between the subspecies. After controlling for

568 geographic distance, we still found a significant association between whistle length and genetic
569 differentiation, which suggests that this song feature could be driving genetic divergence
570 between the subspecies. Many studies have looked for an association between song distance and
571 genetic variation in white-crowned sparrows (Baker 1975; Baker *et al.* 1982; MacDougall-
572 Shackleton & MacDougall-Shackleton 2001; Soha *et al.* 2004) and other taxa (Wright &
573 Wilkinson 2001; Nicholls *et al.* 2006; Alstrom *et al.* 2007; Irwin *et al.* 2008; Kenyon *et al.* 2011;
574 Sosa-López *et al.* 2016), but few other than our study have found this association independent of
575 geographic variation (but see MacDougall-Shackleton & MacDougall-Shackleton 2001; Rendell
576 *et al.* 2012).

577

578 Conclusions

579 Our aim in this study was to evaluate when and how song acts as an isolating mechanism
580 in order to gain insight into the evolution and maintenance of behavioral barriers. Our findings
581 provide strong support to the hypothesis that song is a behavioral barrier to gene flow between *Z.*
582 *l. nuttalli* and *Z.l. pugetensis*, although clearly other barriers to mating are incomplete as
583 introgression is ongoing. We investigated both the signal – bird song – and receiver response to
584 that signal, and found that both songs and behavioral responses to song are divergent between the
585 subspecies, although these differences may be weakening in the contact zone and facilitating
586 hybridization. Our finding that song and genetic distance predict the strength of behavioral
587 discrimination between songs gives insight into the processes driving the evolution of this
588 behavioral barrier.

589

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608

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939 **DATA ACCESSIBILITY**

940 Sampling information and SNP calls for each sequenced individual is available at the Dryad
941 Digital Repository (<http://dx.doi.org/10.5061/dryad.5jc0t>). Scripts for the historical demographic
942 inference are available on Github (<https://github.com/isaacovercast/WCS>). Songs for each
943 recorded individual were archived at the Borror Laboratory of Bioacoustics.

944

945 **AUTHOR CONTRIBUTIONS**

946 EPD and RTB conceived of the study. EPD conducted the behavioral experiments and song
947 analyses. IO conducted historic demographic modeling in fastsimcoal. SEL conducted all other
948 analyses and wrote the manuscript. All authors provided edits and comments to the manuscript.

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961 **TABLES AND FIGURES**

962

963 **Table 1.** Sampling information for each locality. Q values from STRUCTURE refer to mean admixture proportions for
 964 individuals in each locality.

Site	Locality	County	State	Latitude	Longitude	Year	Q Value	Song N	Genetic N	Subspecies
1	San Francisco	San Francisco	CA	37.803	-122.478	2005	0.061	12	3	nuttalli
2	Bolinas	Marin	CA	37.908	-122.722	2005	0.024	34	15	nuttalli
3	Schooner Bay	Marin	CA	38.083	-122.914	2004	0.041	9	14	nuttalli
4	Abbotts Lagoon	Marin	CA	38.122	-122.953	2010	0.023	9	5	nuttalli
5	Sonoma	Sonoma	CA	38.419	-123.105	2010	0.098	11	8	nuttalli
6	Manchester	Mendocino	CA	38.981	-123.702	2010	0.292	5	10	admixed
7	MacKerricher	Mendocino	CA	39.489	-123.8	2005	0.487	15	9	admixed
8	Sinkyone	Mendocino	CA	39.832	-123.85	2010	0.487	0	3	admixed
9	Ferndale	Humboldt	CA	40.544	-124.358	2010	0.896	10	9	pugetensis
10	Eureka	Humboldt	CA	40.742	-124.239	2005	0.96	12	8	pugetensis
11	Trinidad	Humboldt	CA	41.258	-124.099	2010	0.957	10	9	pugetensis
12	Bandon	Coos	OR	43.071	-124.435	2005	0.989	12	14	pugetensis
13	Bullards Beach	Coos	OR	43.127	-124.416	2005	0.986	6	5	pugetensis
14	Nehalem	Tillamook	OR	45.684	-123.938	2005	0.99	16	19	pugetensis
	Frances	Pacific	WA	46.557	-123.4		0.995	0	2	pugetensis

15	Ocean Shores	Grays Harbor	WA	46.928	-124.17	2005	0.975	7	15	pugetensis
	Enumclaw	King	WA	47.248	-122.013		0.996	0	1	pugetensis
16	Dosewallips	Jefferson	WA	47.692	-122.895	2005	0.987	6	9	pugetensis
17	San Juan Island	San Juan	WA	48.461	-123.014	2004	0.98	34	11	pugetensis

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965 **Table 2.** Factor loadings for the first five principal components (eigenvalue > 1) derived from
 966 song variables.

Song Parameter	PC1	PC2	PC3	PC4
Eigenvalue	1.27	1.21	1.07	1.03
Percent variation	23	21	16	15
Whistle length (ms)	0.59			
Avg. note length (ms)	-0.59	0.24	0.15	
Song maximum frequency (Hz)	0.18	0.66	-0.17	-0.16
Song minimum frequency (Hz)	0.2	-0.45	-0.52	-0.39
Whistle dominant frequency (Hz)	-0.41		-0.21	-0.69
Introduction length (ms)	-0.26	-0.28	-0.46	0.56
Vocal performance		-0.46	0.65	-0.17

967
 968 **Table 3.** Factor loadings for the principal components (eigenvalue > 1) derived from behavioral
 969 responses to individual playback experiments and all experiments standardized and combined.

Experiments (Own vs. Other)	Response variables	PC1	PC2
Bolinas (2) vs.	Average distance	-0.76	
Bandon (12)	Song rate	0.87	
	Fly overs	0.65	
	Eigenvalues	1.75	
	Cum. Percent Variance	58.5	
Manchester (6) vs.	Average distance	0.02	0.99
Bolinas (12)	Song rate	-0.76	0.05
	Fly overs	0.76	0.01
	Eigenvalues	1.17	1
	Cum. Percent Variance	39	72.4
Manchester (6) vs.	Average distance	-0.78	0.47

Bandon (12)	Song rate	0.89	0.1
	Fly overs	0.3	0.92
	Eigenvalues	1.5	1.08
	Cum. Percent Variance	50	86
MacKerricher (7) vs. Bolinas (2)	Average distance	-0.83	
	Song rate	0.63	
	Fly overs	0.73	
	Eigenvalues	1.62	
	Cum. Percent Variance	54	
MacKerricher (7) vs. Bandon (12)	Average distance	-0.83	
	Song rate	0.8	
	Fly overs	0.71	
	Eigenvalues	1.8	
	Cum. Percent Variance	61	
Combined Own vs. Other	Average distance	-0.8	
	Song rate	0.79	
	Fly overs	0.63	
	Eigenvalues	1.7	
	Cum. Percent Variance	55	

970

971 **Table 4.** Maximum likelihood parameter point estimates and 95% bootstrap confidence intervals

972 for the secondary contact model.

Parameter	Max	Bootstrap Values		
	Likelihood Point Estimate	Median	Lower 95%	Upper 95%
Ancestral N_e	74422	54872	49553	67125
<i>Pugetensis</i> N_e	7478	35096	15114	63980
<i>Nutalli</i> N_e	8802	17785	7779	31860

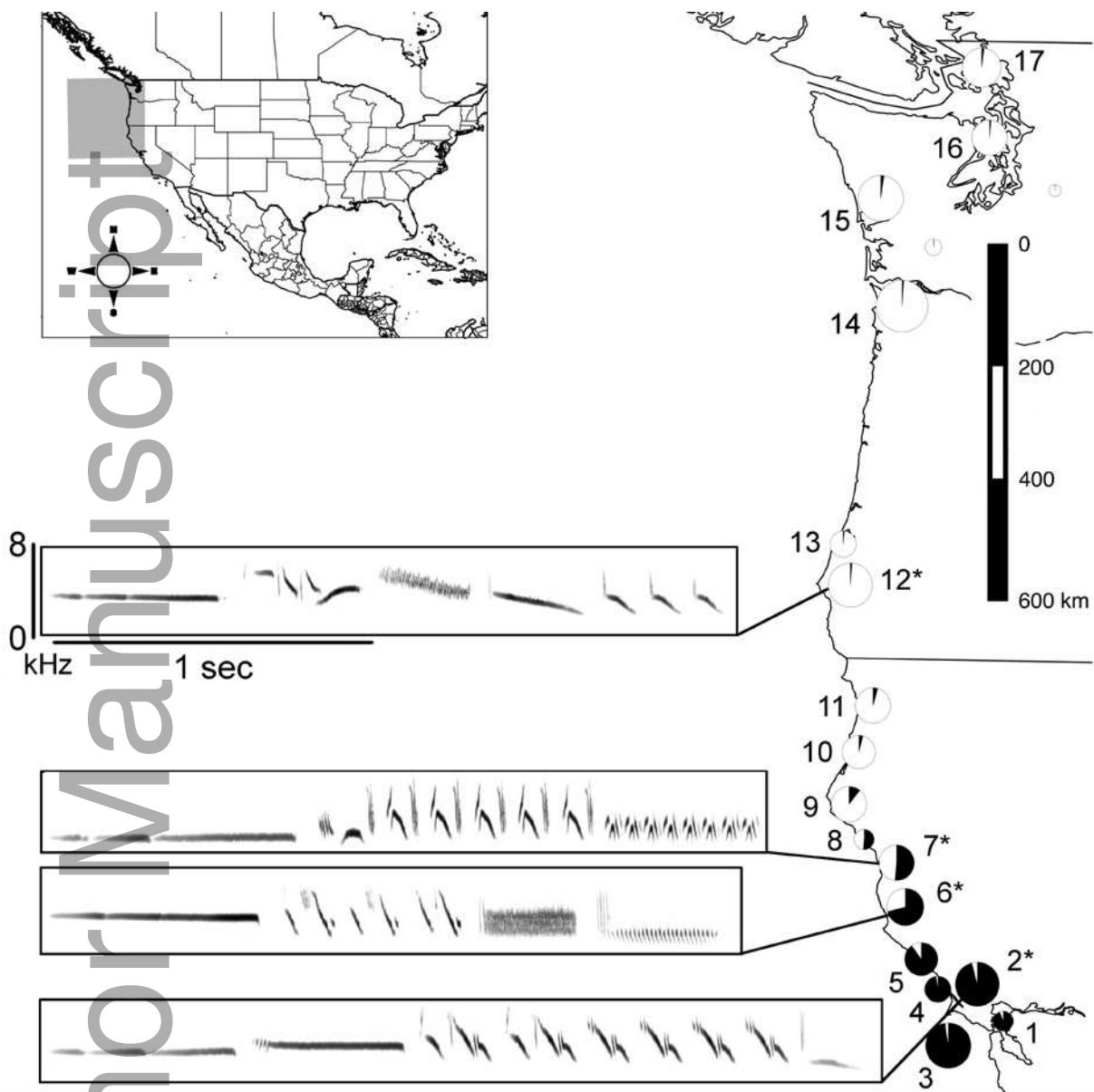
Ancient migration rate	0.00013	0.00006	0.00003	0.00013
Recent migration rate	0.00078	0.00374	0.00289	0.00506
Time of initial isolation with migration	45372	45324	40585	63789
Time of full isolation	9142	6225	8447	37283
Time of secondary contact	2281	6139	8431	36695

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974 **Table 5.** Results of statistical comparisons of response to 'own' vs. 'other' for each pairwise
 975 comparison using Wilcoxon sign-ranked tests. Significant *P* values indicated by a (*), and *d* is
 976 the post hoc calculated effect size.

Experiments (Own vs. Other)	Response variable	N	<i>S</i>	<i>P</i>	<i>d</i>
Bolinas (2) vs. Bandon (12)	PC1	10	-25.5	0.0059*	1.5
Manchester (6) vs. Bolinas (12)	PC1	10	3.5	0.78	0.27
	PC2	10	1.5	0.92	0.22
Manchester (6) vs. Bandon (12)	PC1	10	-21.5	0.0273*	0.86
	PC2	10	1.5	0.92	0.09
MacKerricher (7) vs. Bolinas (2)	PC1	10	-12.5	0.23	0.22
MacKerricher (7) vs. Bandon (12)	PC1	10	-21.5	0.0273*	0.99
Bandon (2) vs. Bolinas (12)	PC1	10	-23.5	0.0137*	0.92

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978

979 **Fig. 1.** Sampling map of numbered localities along a transect from the southern subspecies *Z. l.*

980 *nuttalli* to the northern subspecies *Z. l. pugetensis*. Song spectrograms correspond to the sites of

981 behavioral playback experiments. Pie charts represent admixture proportions from STRUCTURE

982 for each locality and circle size corresponds to the number of individuals selected for sequencing

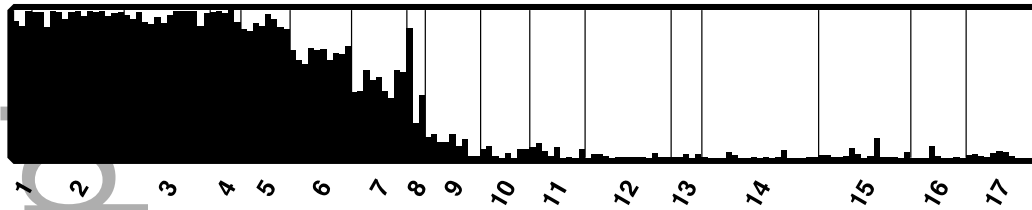
983 at each site. Gray pie charts indicate two samples collected outside of main localities. Asterisks

984 indicate localities for territorial playback experiments.

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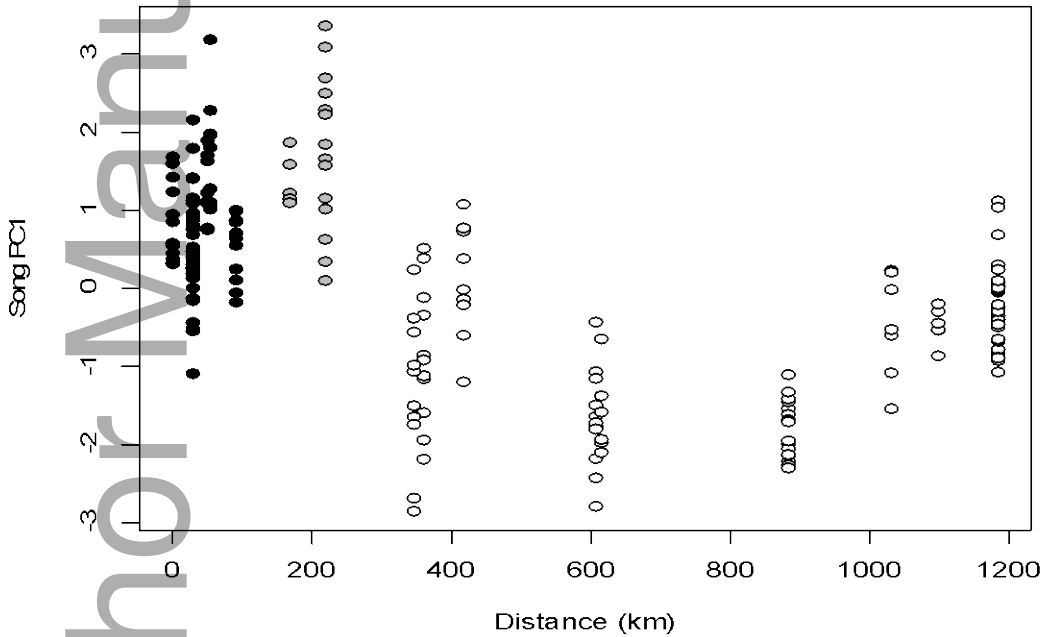
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988

989 **Fig. 2.** Probability of assignment to *Z. l. nuttalli* (black) and *Z. l. pugetensis* (white) as
990 determined from a STRUCTURE analysis using 6419 SNPs for $K = 2$ across 17 localities.

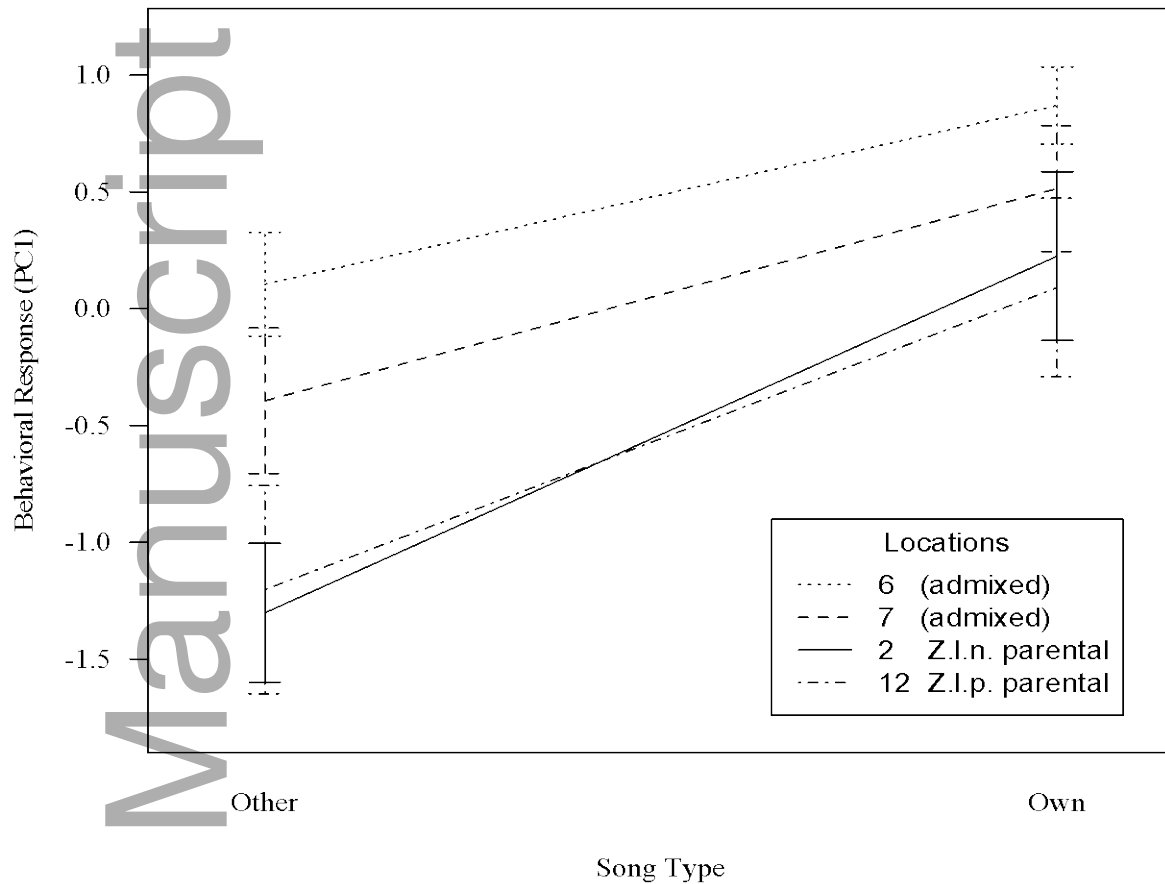
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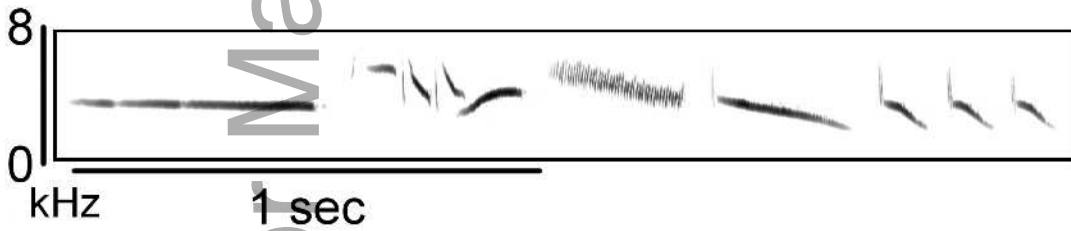
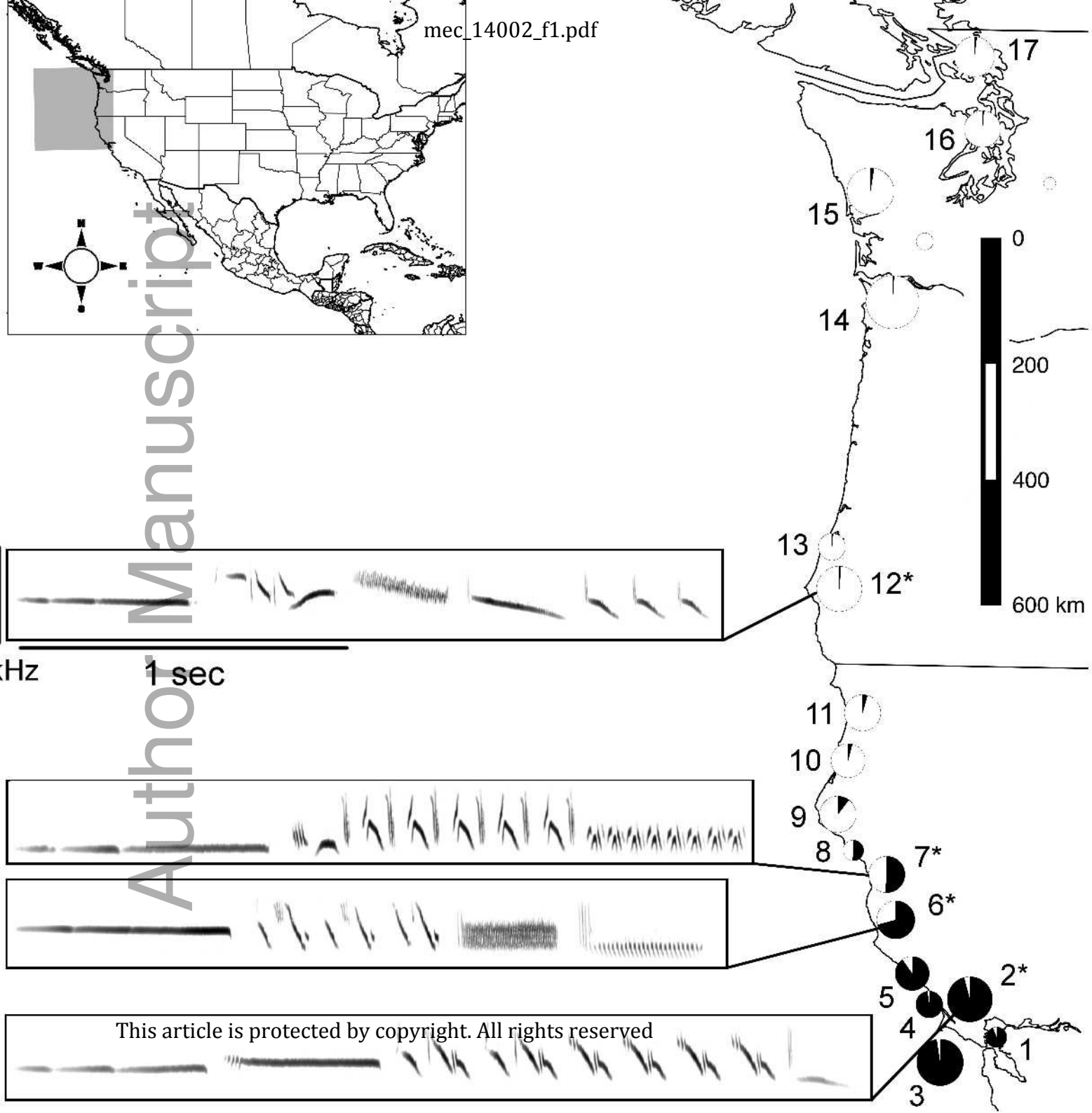
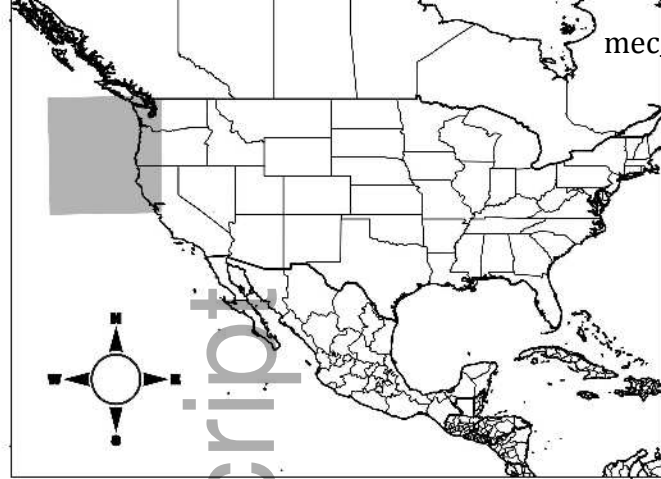
992

993 **Fig. 3.** Plot of song PC1 for *Z. l. nuttalli* (black), admixed individuals (grey), and *Z. l. pugetensis*
994 (white) across hybrid zone transect.

995



996
 997 **Fig. 4.** Results of territorial playback experiments comparing male responses to their ‘own’
 998 songs and the songs of the ‘other’ subspecies in four localities along a hybrid zone transect.
 999 Localities (from south to north): 2. Bolinas (solid line), 6. Manchester (dotted lines), 7.
 1000 MacKerricher (dashed lines), and 12. Bandon (dash dot lines). Larger values of PC1 indicate a
 1001 stronger behavioral response to simulated territorial intrusion. A steeper slope indicates a
 1002 stronger discrimination between local and foreign stimuli. Bars represent standard error.



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