Migratory orientation in a narrow avian hybrid zone

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Background. Zones of contact between closely related taxa with divergent migratory routes, termed migratory divides, have been suggested as areas where hybrid offspring may have intermediate and inferior migratory routes, resulting in low fitness of hybrids and thereby promoting speciation. In the Rocky Mountains of Canada there is a narrow hybrid zone between Audubon's and myrtle warblers that is likely maintained by selection against hybrids. Band recoveries and isotopic studies indicate that this hybrid zone broadly corresponds to the location of a possible migratory divide, with Audubon's warblers migrating south-southwest and myrtle warblers migrating southeast. We tested a key prediction of the migratory divide hypothesis, that genetic background would be predictive of migratory orientation among warblers in the center of the hybrid zone.

Methods. We recorded fall migratory orientation of wild-caught migrating warblers in the center of the hybrid zone as measured by video-based monitoring of migratory restlessness in circular orientation chambers. We then tested whether there was a relationship between migratory orientation and genetic background, as measured using a set of species-specific diagnostic genetic markers.

Results. We did not detect a significant association between orientation and genetic background. There was large variation among individuals in orientation direction. Mean orientation was towards the NE, surprising for birds on fall migration, but aligned with the mountain valley in which the study took place.

Conclusions. Only one other study has directly analyzed migratory orientation among naturally-produced hybrids in a migratory divide. While the other study showed an association between genetic background and orientation, we did not observe such an association in yellow-rumped warblers. We discuss possible reasons, including the possibility of a lack of a strong migratory divide in this hybrid zone and/or methodological limitations that may have prevented accurate measurements of long-distance migratory orientation.

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Abstract:

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49 genetic background and orientation, we did not observe such an association in yellow-rumped
50 warblers. We discuss possible reasons, including the possibility of a lack of a strong migratory
51 divide in this hybrid zone and/or methodological limitations that may have prevented accurate
52 measurements of long-distance migratory orientation.

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55 **Introduction:**

56 Breeding and wintering ranges of many species are separated by thousands of kilometers.
57 Long-distance seasonal migration exhibited by many taxa moving between these disjunct areas is
58 a complex and energetically demanding task that has been studied for decades (Berthold 1996).
59 However, the physiological mechanisms, controls and senses involved in navigation of many
60 taxa are still unclear, as are the contributions of various cues to migratory behavior (Berthold and
61 Terrill 1991; Alerstam 2006). Much of our understanding of migratory directionality and
62 navigation comes from studies of blackcap warblers (*Sylvia atricapilla*) in Europe. In an
63 influential series of studies, Helbig and colleagues (Helbig 1991; Berthold et al. 1992; Helbig
64 1994; Helbig 1996) reared blackcaps from populations that exhibited different migratory routes.
65 They found that, even in captivity in a common environment, populations recapitulated their
66 natural routes, as assayed by Emlen funnels (Emlen 1970). These studies also found that lab-
67 crossed hybrids between the parental types tended to show intermediate migratory orientations.
68 Few studies have collected similar data in wild populations, primarily due to logistical
69 challenges. Such observations are particularly pertinent to studies of migratory divides, where
70 populations that differ in migratory directionality come into contact and interbreed (e.g., Helbig
71 1991; Bensch et al. 1999, 2009; Irwin and Irwin 2005; Ruegg 2007; Rohwer and Irwin 2011;
72 Ilieva et al. 2012). For example, if hybrids between divergent populations have a mixture of
73 alleles responsible for migration and exhibit an intermediate migratory orientation, as has been
74 observed in lab-raised blackcap warblers (Helbig 1991; Helbig 1994; Helbig 1996), such a novel
75 phenotype may be inferior and represent an important fitness detriment. This is because, in a
76 number of systems, intermediate routes have been suggested to take hybrid individuals over
77 regions that are provide greater challenges in finding food (e.g., deserts) or navigating (e.g.,

78 mountains). If so, inferior routes of hybrids can cause lower survival and/or lower fecundity (if
79 arriving on the breeding grounds in poor condition hampers reproduction), thereby contributing
80 to postmating reproductive isolation and promoting further differentiation and speciation of the
81 two populations (Helbig 1991; Irwin and Irwin 2005).

82 To date, the only study to directly assay the migratory behaviour of wild individuals
83 differing in their genetic constitution across a migratory divide is in a study of Swainson's
84 thrushes (*Catharus ustulatus*; Delmore et al. 2012; Delmore and Irwin 2014; Delmore et al.
85 2016). In a hybrid zone between coastal and inland forms of Swainson's thrushes in western
86 Canada, migration was studied using both light-level geolocators, to directly track migration
87 routes and wintering locations (in Central and South America), and orientation chambers to
88 measure migratory direction of birds beginning fall migration. Hybrids exhibited greater
89 variability in their routes than parentals did, with some taking routes that were intermediate to
90 parental forms and took them over the arid regions of the southwestern USA. Moreover, among
91 Swainson's thrushes within the hybrid zone, genetic background was predictive of migratory
92 route as well as orientation direction, and one particular genomic region showed a particularly
93 strong association with longitude (i.e., west to east) of migratory route and wintering location
94 (Delmore et al. 2016).

95 Given the prominence of the idea that differences in migratory behavior may cause
96 reproductive isolation within hybrid zones (e.g., Helbig 1991; Bensch et al. 1999, 2009; Bearhop
97 et al. 2005; Irwin and Irwin 2005; Ruegg 2007; Rolshausen et al. 2009, 2013; Liedvogel et al.
98 2011; Rohwer and Irwin 2011; Ilieva et al. 2012), it is important to test whether there is
99 association between genetic background and migratory orientation within a variety of hybrid
100 zones. Here, we provide another direct examination of this question with a large-scale study of

101 birds sampled on fall migration within a hybrid zone between Audubon's / myrtle warblers
102 (*Setaphaga auduboni* and *S. coronata*) in western North America. Within this hybrid zone there
103 are a full range of hybrid genotypes, indicating that multiple generations of hybrids and
104 backcrosses are present and that reproductive isolation is far from complete. However, the
105 narrowness of this hybrid zone and sizeable amounts of linkage disequilibrium in the center of
106 the zone suggest that some form of moderately strong selection maintains it (Brelsford and Irwin
107 2009). Assortative mating and other pre-mating reproductive barriers are unlikely to be strong
108 between these taxa (Brelsford and Irwin 2009; Brelsford et al. 2011; Toews et al. 2014a),
109 implying a potentially sizeable role for post-mating selection against hybrids, possibly based on
110 inferior migratory behaviour. Band recovery and isotopic data from birds outside the hybrid zone
111 suggests the two parental taxa differ in their migratory movements (Figure 1; Toews et al.
112 2014a,b). If migratory traits are genetically controlled and inherited additively—as the studies of
113 blackcaps and Swainson's thrushes suggest—we expected a correlation of orientation and
114 ancestry in the hybrid zone. Specifically, we predicted that individuals more genetically
115 Audubon's-like would orient SSW, individuals more myrtle-like would orient SE, and hybrids
116 would orient intermediate between these two (i.e. south).
117 We used a video-based orientation cage method, initially developed by Fitzgerald and
118 Taylor (2008). These orientation cages are similar in many respects to traditional Emlen funnels
119 (Emlen 1970), but in this case the behaviour of birds was scored using video cameras. As
120 compared to the scratch marks quantified with Emlen funnel experiments, this method has a
121 number of benefits, including: (1) scoring of the videos can be automated, ensuring objectivity,
122 (2) a longer period of observation can be obtained for each individual, (3) specific time periods
123 can be analyzed in isolation and (4) any behavioural changes over the course of the trial can be

quantified. We designed our study to assay the orientation of individuals in the evening of the day they were captured, which previous research suggests is predictive of later orientation (Ilieva et al. 2012). We then genotyped each individual with species diagnostic genetic markers and tested whether there was a correlation between orientation and genetic background.

Methods

Study site and orientation trials

Between August 15th and September 12th of 2011 we captured migratory yellow-rumped warblers (n = 181) near Kananaskis, Alberta, a site at the center of the Audubon's / myrtle warbler hybrid zone (Brelsford and Irwin 2009). Our sampling was concentrated in two areas: along the southern edge of Barrier Lake (51.0235°N, 115.0608°W) and within the hamlet of Lac Des Arc (51.0536°N, 115.1589°W; Figure 1C). We set up mist-nets before dawn and used passive netting along with song playback (using a tape of a variety of yellow-rumped warblers as well as begging calls from nestlings; previous experience showed that similar recordings could be used to attract all phenotypes in the hybrid zone) to increase our likelihood of catching individuals of our target species. These sites, timing, and capture methodology were chosen to capture birds that were beginning their migratory movements, but it is possible that some of the individuals sampled were close to their breeding territories or natal areas. Immediately after capturing each individual we took morphometric measurements (bill, tarsus, wing and tail length; according to Pyle 1997), photographs, a blood sample (10-40μL), and applied a unique aluminum USFWS leg band. Birds were then transported approximately three kilometers from the capture site to the location where the orientation trials were performed (51.0286°N, 115.0242°W). This site is a large, recently clear-cut field (~400m^2; <10 years old) near the Kananaskis Biogeoscience Institute and has clear views in all directions.

147 Each individual was placed individually into one of 12 outdoor holding / orientation
148 cages (Figure 2) and given water and small mealworms throughout the day. The cages were a
149 modified design based on Fitzgerald and Taylor (2008), who used similar cages to study
150 orientation in yellow-rumped warblers (Figure 2). The cages were leveled, oriented with a
151 compass, and spaced approximately 3-5 meters apart. The cage frames were made out of pine
152 boards, with the top and bottom of the cages made from composite plywood. The perch was
153 made with a 9" plastic embroidery hoop, held up by four 7/16" dowels, and placed horizontally
154 such that birds could perch on the hoop (Figure 1C). Since research suggests that birds may use
155 magnetic cues for navigation, the wood pieces were joined with non-magnetic brass screws and
156 stainless steel staples were used to affix screen mesh to the top and sides of the cage (Alerstam
157 1993; Muheim et al. 2006). During the day the sides of the cages were covered with a blue,
158 opaque tarp for sun shelter, with part of the tarp rolled up to provide sufficient airflow (Figure
159 2A). Using the same cages to both hold the individuals during the day and run the orientation
160 trials in the evening has a number advantages over traditional use of Emlen funnels (Emlen
161 1970). Most importantly, this likely reduces the stress inherent in holding individuals indoors in
162 artificial environments and moving individuals between holding and assay cages shortly before
163 the trials, as is necessary with funnels (Emlen 1970). We were careful in assessing the welfare of
164 the birds: if individuals did not immediately fly to the perch, showed any signs of stress (e.g.
165 panting), or were not eating properly they were released during the day (less than 15% of birds
166 captured).
167 Before we started each orientation trial, we removed the food and water dishes and closed
168 each tarp around the sides of each cages, allowing each individual to see only out of the top of
169 the cage, which had a full view of the sky. Through a hole in the bottom of each cage we

attached a D-Link Wireless Network Camera (DCS-932L) pointing directly up, with the top of the camera oriented northwards. Unlike cameras that point down (e.g. Muheim et al. 2014), these upward-facing cameras do not obstruct a bird's view of the sky. The cameras we used have an infrared LED light for illumination during low light conditions. This light was applied consistently across all of the trials, and we are aware of no evidence that birds can see infrared light. The cameras were set such that the video image was recorded with the right side of the video representing the west side of the cage and the left side of the video representing the east side of the cage. We recorded 320p x 240p 30 frame-per-second video from each cage simultaneously using the D-ViewCam software on a PC laptop via a D-Link router. We began recording approximately one hour before sunset and ran the trials until approximately 30 minutes past sunset. Each evening we ran the trials until the last individual stopped moving, after which we released all of the individuals. We tested each bird once and recorded the behaviour of 1 to 10 individuals each evening, averaging 6 birds per evening, with a total of 124 individuals for which we collected complete orientation data. All animal care and experimentation was conducted according to the University of British Columbia protocol Nos. A11-0054 (Project title: Orientation in migratory songbirds) and A09-0131 (Project title: Geographic variation in birds of western Canada). Field permits were provided by the Canadian Wildlife Service, Prairie and Northern Region office (AB Scientific Permit 11-AB-SC023) and Alberta Parks (11-107).

Video analysis

From the D-ViewCam software we exported each video in ".asf" format, noting the start and end time of each trial. We analyzed the video data with radR, an open source platform developed for acquiring and analyzing radar data (Taylor et al. 2010) that was more recently adapted to analyze video files. In brief, radR uses contrast to score individual pixels, and then

uses movement, area and intensity to define objects. We sampled the videos at three frames per second. For each detected object we extracted the X and Y coordinates of its centroid, as weighted by the area of the object. Given that the light conditions change over the evening, we used three groups of parameters to accurately and consistently identify the bird as the primary object relative to the background (see supplementary material for parameters used). We defined the center of the circular perch as the center of our analytical coordinate system and created an exclusion zone within the circular perch, such that the program would identify the bird only in the region from the perch outwards (Figure 3). This makes our results more comparable to previous funnel studies, which only recorded jumps where the individual contacts the side of the funnel (Fitzgerald and Taylor 2008). From this data radR generates a list of time-stamped X and Y coordinates for points where it identified an object (see supplementary material for an example of data extracted for a single individual).

Using R 3.0.3 (R-core Development Team 2013) we applied a number of additional filters, primarily to remove noise (i.e. objects that were not the bird) and remove times when the bird was not moving (i.e. sitting on the perch). To remove noise, we first removed all data where there were three or more objects identified at a single time-stamp, as three or more objects were invariably an artifact of background noise. Sometimes radR identified the bird as two separate objects, primarily when the individual was above one of the four perch dowels, such that parts of the bird stuck out on either side of the dowel. For time-stamps with two objects, we averaged the XY values that were less than 500 pixels apart to include only those times that an individual was above the dowels (i.e. the dowels are slightly less than 500 pixels wide in the video image) and visually inspected the data to confirm this was accurate. To ignore points where the bird was sitting motionless on the perch we removed consecutive time points that had a lower XY

distance than a predetermined threshold, which we calculated by studying videos of individuals sitting quietly on the perch (50 pixels for the current analysis). Each of the points that passed these filters were then transformed into an angle relative to North (N=0^o, E=90^o, S=180^o and W=270^o) and given a timestamp (in seconds) relative to the time of sunset for that evening.

From these data we estimated three behavioural traits for each individual: mean orientation, rho and activity, defined as follows. We calculated mean orientation using the "circular statistics" package in R (Agostinelli and Lund 2007). We used the mean of angles observed over the entire observational period (restricting the time range to only those times when the birds were more active did not meaningfully change the results). We used the same R package to estimate rho, an estimate of angular variance that varies between 0 and 1 (i.e. a measure of the concentration of points, with a value of 1 being perfectly concentrated). Finally, we used the total number of time points at which an object (i.e., a moving bird) was detected by radR over the trial as the total activity for each individual.

To assess robustness of estimating the mean orientation for each individual using the data generated from radR, we chose five orientation videos at random and scored them by eye, blind to the output from radR. For this we visually estimated the angle of the bird (if present in the frame) every 30 seconds, over the entire video, and also recorded whether the bird was on the perch or in mid-flight. We then calculated the difference in angle between this estimate and that obtained from radR for the full dataset and also from only those points where the bird was observed to be in flight. Our data filtering appeared to be effective as our data from a random selection of videos analyzed by eye (for 5 birds) was consistent with the output from radR. When we compared all of the points observed by eye, including those where the individual was sitting on the perch, the resultant mean angles were within +/- 22^o relative to the output from radR after

239 filtering. If we included only those points where the individual was in flight (by excluding those
240 times we observed by eye for which the individual was on the perch), which our filters within
241 radR were designed to remove, the resultant mean angles were within +/-11^{o} of the radR output.
242

243 *Molecular analysis*

244 Blood samples were taken using a small needle and capillary tube from the brachial vein,
245 stored in Queen's lysis buffer (Seutin et al. 1991), and left at ambient temperature until returned
246 to the laboratory. DNA was extracted using a standard phenol-chloroform procedure and
247 resuspended with 50 – 200 µL of buffer (depending on the size of the pellet) containing 10 mM
248 Tris-HCl and 1 mM EDTA, at pH 8.0, and stored at 4^{o} C. We sexed individuals molecularly
249 using the procedure described in Fridolfsson and Ellegren (1999). We genotyped individuals at
250 three molecular markers. The full PCR and genotyping protocol for two of the three nuclear
251 markers (*CHD1Z* and *numt-Dco1*) was presented in a previous publication (Brelsford and Irwin
252 2009). For the third nuclear marker, an 850 base pair fragment of *RIOK2*, we used the forward
253 primer 5'-ATGGGTGTTGGCAAAGAATC-3', the reverse primer 5'-
254 GCTCCTCTTCRTTWGCAACA-3', and a PCR annealing temperature of 60°C. The enzyme
255 *XmnI* cuts an allele common in Audubon's warblers, but leaves intact an allele common in
256 myrtle warblers. To generate a genetic hybrid index we scored a zero for each Audubon's allele
257 and a one for each myrtle allele for the three markers and divided this by the total number of
258 alleles (6 for males, 4 for females) resulting in an index that ranges between 0 (all Audubon's
259 alleles) to 1 (all myrtle alleles).

Combining genotype and migratory behaviour

To test whether orientation is associated with genetic background, we used two statistical approaches. First, we used a circular linear model to test whether orientation varied linearly with hybrid index (Agostinelli and Lund 2007). Second, we used a circular ANOVA to test whether mean orientation angle differed in any way (not necessarily linearly) among these five genetic groups: those with all Audubon's alleles (h-index = 0; Group A), those with mostly Audubon's alleles (0 < h-index < 0.5; Group AH), those with mixed genotypes (h-index = 0.5; Group H), those with mostly myrtle alleles (0.5 < h-index < 1; Group MH), and those with all myrtle alleles (h-index = 1; Group M). While the linear model would assume that each hybrid class as an intermediate migratory orientation compared to the two classes on either side of it, the ANOVA does not make that assumption, allowing situations in which hybrid classes might have more extreme orientations than either parental group.

To test whether there was a significant mean orientation of all of individuals considered together, regardless of their genetic background, we used a Rayleigh test. This is a procedure to test the null hypothesis that the orientation angles are distributed randomly, with the alternative being that the distribution is clumped in certain direction(s). The test statistic is *r*, or rho, and is the magnitude of the mean vector (Fitzgerald and Taylor 2008).

Results

Molecular data

We obtained genotypes from 166 of the 181 yellow-rumped warblers captured at the beginning of fall migration through the Kananaskis area, including 123 individuals with orientation data. Based on the three genetic markers, our data set consisted of individuals with a broad range of genetic backgrounds, spanning from Audubon's warblers (h-index = 0) through a

broad array of hybrid genotypes to myrtle warblers (h-index = 1), consistent with the expectation based on prior research of a broad mixture of hybrid classes (F1's, F2's, backcrosses, etc.) and pure-type individuals (Brelsford and Irwin 2009). The daily composite allele frequencies varied relatively little over the study period: the daily proportion of myrtle alleles was usually between 0.3 to 0.6, with an average over the study period of 0.4 (Figure 4A). Our molecular sexing of individuals indicated that we captured an excess of male birds with 73% of all of the individuals identified as males (Figure 4B). This was likely due to our use of song playback during mist netting.

Orientation trials

In the orientation trials, initial observations of yellow-rumped warblers in the orientation chambers indicated that their activity begins to increase approximately one hour before sunset. During this time of increased activity their behavior also changed qualitatively, from primarily sitting on the perch and/or flying occasionally to and from the bottom of the cage, to performing more short flights from the perch to the top of the cage, consistent with *zugunruhe* (i.e. migratory restlessness; Emlen 1970). The activity of the birds increased to a peak at around 20-30 minutes before sunset (Figure 5), and then gradually declined. Following sunset their activity sharply declined, such that we recorded virtually no movements after 40 minutes post-sunset. At the end of most evenings individuals usually stopped moving within 5-10 minutes of each other with remarkable consistency (the steep decline to the right of Figure 5).

Of the 123 individuals where we had genotype and orientation data, we found that 96 birds showed strong evidence of orientation behavior (i.e., within-individual $r > 0.1$; total activity > 500). We used all 123 individuals in analyses; analyses using only those individuals with strong orientation did not qualitatively change results. Of the five genetic groups, our

306 sample contained $n = 6$ for Group A (i.e., genetically Audubon's), $n = 45$ for Group AH, $n = 41$
307 for Group H, $n = 22$ for Group MH and $n = 9$ for Group M (i.e., genetically Myrtle). We did not
308 detect a significant difference in mean orientation between the genetic groups ($F = 1.23$, $P =$
309 0.09) and there was no significant linear relationship between mean orientation and genetic
310 hybrid index ($t = 0.779$, $P = 0.22$). Hence we could not reject the null hypothesis of no
311 association between genetic background and orientation.
312 While there was considerable variability in orientation among individuals, there was a
313 significant mean orientation towards 25º or NNE (Figure 6A; $n = 123$, among-individual $r =$
314 0.320, $P < 0.01$), with a 95% confidence interval between 5º and 46º. Separating the birds by
315 their genetic hybrid index, each group had mean orientations similar to N or NE, although only
316 Groups M (h-index = 1) and H (h-index = 0.5) showed evidence of significant mean orientations
317 compared to the null of an even distribution of orientation angles: Group A, 38º ($n = 6$, $r =$
318 0.634, $P = 0.09$); Group AH, 3º ($n = 45$, $r = 0.230$, $P = 0.09$); Group H, 54º ($n = 41$, $r = 0.373$, P
319 < 0.01); Group MH, 359º ($n = 22$, $r = 0.321$, $P = 0.10$); and Group M, 15º ($n = 9$, $r = 0.733$, $P <$
320 0.01).

321 **Discussion**

322 Here we have provided the second study of potential association of genetic ancestry and
323 migratory orientation in an avian hybrid zone. Our use of video-based orientation trials and
324 automated video analysis enabled us to sample a large number of individuals during fall
325 migration while also gathering high resolution and orientation chamber movement data for each
326 bird, a benefit over previous Emlen funnel methods. Despite these methodological advantages
327 and prior evidence for a possible migratory divide across the Audubon's / myrtle warbler hybrid
328 zone (Toews et al. 2014a), we did not find an association between genetic background and

329 orientation. We discuss below the possibilities that this result was due to (1) lack of any true
330 association between genetic background and migratory route within this hybrid zone, (2) short-
331 term orientation during the time of the study not being indicative of long-distance migration
332 orientation; or (3) methodological limitations that hindered the detection a true association.
333 First, the prior evidence for a migratory divide across the yellow-rumped warbler hybrid
334 zone is mixed, raising the possibility that our results are due to no actual association of genetic
335 ancestry and migratory differences in the center of the zone. Banding data and wintering range
336 maps of Audubon's and myrtle warblers indicate that Audubon's warblers largely winter in the
337 southwestern USA, whereas myrtle warblers mostly winter in the southeastern USA (Toews et
338 al. 2014a). However, sizeable numbers of myrtle warblers winter in the southwest (Hunt and
339 Flaspohler 1998). It has been postulated that most of these belong to a distinct form of myrtle
340 warbler (the subspecies *hooveri*) breeding in Alaska, the Yukon, and northern British Columbia
341 (McGregor 1899; Toews 2017). However, it is also possible that some myrtle warblers from
342 Alberta also migrate to the southwestern USA, and that the current hybrid zone between
343 Audubon's and myrtle warblers in western Alberta does not correspond to a migratory divide.
344 Isotopic data (Toews et al. 2014a) is mostly supportive of the hybrid zone coinciding with a
345 migratory divide, because sites just outside of the hybrid zone differed in their hydrogen isotopic
346 signatures in a way consistent with the expected difference between southwestern and
347 southeastern USA, and birds within the hybrid zone showed a broader mixture of signatures.
348 Taking this evidence together, it is likely that there is at least some sort of transition in average
349 migratory orientation between Audubon warblers in central British Columbia and myrtle
350 warblers in central Alberta, but it is possible that that the transition zone is very broad, with birds

in the center of the hybrid zone having no or only a weakly detectable relationship between ancestry and migratory orientation.

Second, it is possible that the behavior that we observed in the chambers is indicative only of short-term and short-distance orientation rather than longer-term and longer-distance migratory orientation. Among individuals we found a lot of scatter in directional tendencies, with a mean migratory orientation that was surprisingly towards the NNE (26^{o}), odd for birds on fall migration, which is expected to be generally southward. A possible explanation is that individuals may be moving northeast, out of the valleys in the Rocky Mountains, to later turn south. The orientation of the valley near the capture location and orientation experiment area is approximately 23^{o} (NNE; estimated using Google Earth™), very close to the observed orientation of the birds when grouped together (26^{o} NNE). Our sample likely included many hatch-year birds, raising the possibility that the northward orientation of some birds may be indicative of regional-scale post-fledging movements (such as those made by blackpoll warblers; Brown and Taylor 2015), the incidence and function of which are still unknown. While previous orientation studies have tested the effect of ecological barriers on migratory behaviour (i.e. water bodies; Sandberg and Moore 1996; Ilieva et al. 2012), this is one of the first studies to assay orientation in and around mountainous areas. Using high-resolution radar technology, Williams et al. (2001) found evidence that nocturnal migrants responded to local topological features by changing their orientation during fall migration, especially those birds migrating below 300m, as is assumed with yellow-rumped warblers. Given the type of data these methods collect, however, it is challenging to assign these types of observations to specific species or even species groups (Williams et al. 2001). For the warblers in our study, individuals might have a memory of the axis of the valley at the time of capture, and then orientation in that direction. We recommend

that future orientation studies around mountainous areas should consider including additional orientation localities in valleys of varying orientations. It would also be useful to assay individuals each evening over a longer period (i.e. 1-2 weeks) to test whether this orientation is maintained or dissipates with time. This could provide a robust test of the role of topological features in influencing migratory movements.

Finally, it is possible that the observed chamber orientations are not representative of movements the birds would make if they were outside of the chambers. All experiments using orientation chambers with captive birds run the risk that migratory movements in specific systems may not be well represented by cage movements, although it is remarkable how often there is a strong association (e.g., Helbig 1991, 1996; Alerstam 1993; Van Doren et al. 2017). The movements and pattern of activity of the warblers in our study were qualitatively similar to previous descriptions of *zugunruhe*, suggesting that the yellow-rumps in our sample were expressing behaviours consistent with migration. But given the unexpected mean orientation toward the northeast, the lack of any relationship with genetic background, and the reasonably short duration of the movement behavior each evening, we acknowledge that it is plausible that orientation cage behaviors in our study are not indicative of orientation in free-moving birds. Given this concern, we suggest that tracking studies using direct tracking technology such as radio towers and miniaturized geolocator tags may be more useful for studying large-scale migratory orientation (Taylor et al. 2011; Delmore et al. 2012; Veen 2013; Delmore and Irwin 2014).

In conclusion, we used molecular genetic methods and a video-based orientation assay that provided objective, high resolution temporal data for many individuals in a semi-natural setting. Our results show no significant association between genetic ancestry and chamber

orientation of migrating yellow-rumped warblers within a hybrid zone that was postulated to correspond to a migratory divide. These results provide an interesting contrast to Swainson's thrushes, in which genetic ancestry within a hybrid zone is predictive of migratory orientation as measured both by geolocators and orientation chambers (Delmore et al. 2016). We suggest that, with future modifications, these methods could provide a powerful tool for understanding migratory orientation in many species. In particular, when replicated in multiple locations, one could use these orientation assays to examine the effect of local topographical features. Or, if assayed orientations were verified by geolocators, one could identify potentially maladaptive migratory tendencies in hybrid individuals. We hope these methods will contribute to the understanding of migratory divides, and of migration more generally.

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Literature Cited:

Agostinelli C, Lund U. 2013. R package 'circular': Circular Statistics (version 0.4-7). https://r-forge.r-project.org/projects/circular/

Alerstam T. 1993. Bird Migration. Cambridge University Press.

Alerstam T. 2006. Conflicting evidence about long-distance animal navigation. *Science* **313**:791–794.

Bearhop S, Fiedler W, Furness RW, Votier SC, Waldron S, Newton J, Bowen GJ, Berthold P, Farnsworth K. 2005. Assortative mating as a mechanism for rapid evolution of a migratory divide. *Science* **310**:502–504.

Bensch S, Andersson T, Åkesson S. 1999. Morphological and molecular variation across a migratory divide in Willow Warblers, *Phylloscopus trochilus*. *Evolution* **53**:1925–1935.

Bensch S, Gran M, Müller N, Gay L, Åkesson S. 2009. Genetic, morphological, and feather isotope variation of migratory willow warblers show gradual divergence in a ring. *Molecular Ecology* **18**:3087–3096.

Berthold P. 1996. *Control of migration*. Chapman & Hall, London.

Berthold P, Helbig A J, Mohr G, Querner U. 1992. Rapid microevolution of migratory behaviour in a wild bird species. *Nature* **360**:668–670.

Berthold P, Terrill SB. 1991. Recent advances in studies of bird migration. *Annual Review of Ecology and Systematics* **22**:357–378.

Brelsford A, Irwin DE. 2009. Incipient speciation despite little assortative mating: the yellow-rumped warbler hybrid zone. *Evolution* **63**:3050–3060.

438 **Brelsford A, Milá B, Irwin DE. 2011.** Hybrid origin of Audubon's warbler. *Molecular Ecology*
439 **20**:2380–2389.

440 **Brown JM, Taylor PD. 2015.** Adult and hatch-year blackpoll warblers exhibit radically
441 different regional-scale movements during post-fledging dispersal. *Biology Letters*
442 **11**:20150593.

443 **Delmore KE, Fox JW, Irwin DE. 2012.** Dramatic intraspecific differences in migratory routes,
444 stopover sites and wintering areas, revealed using light-level geolocators. *Proceedings of*
445 *the Royal Society B: Biological Sciences* **279**:4582–4589.

446 **Delmore KE, Irwin DE. 2014.** Hybrid songbirds employ intermediate routes in a migratory
447 divide. *Ecology Letters* **17**:1211–1218.

448 **Delmore KE, Toews DPL, Germain RR, Owens GL, Irwin DE. 2016.** The genetics of
449 seasonal migration and plumage color. *Current Biology* **26**: 2167–2173.

450 **Emlen ST. 1970.** Celestial rotation: its importance in the development of migratory orientation.
451 *Science* **170**:1198–1201.

452 **Fitzgerald TM, Taylor PD. 2008.** Migratory orientation of juvenile yellow-rumped warblers
453 (*Dendroica coronata*) following stopover: sources of variation and the importance of
454 geographic origins. *Behavioral Ecology and Sociobiology* **62**:1499–1508.

455 **Fridolfsson A, Ellegren H. 1999.** A simple and universal method for molecular sexing of non-
456 ratite birds. *Journal of Avian Biology* **30**:116–121.

457 **Helbig A. 1991.** Inheritance of migratory direction in a bird species: a cross-breeding experiment
458 with SE- and SW-migrating blackcaps (*Sylvia atricapilla*). *Behavioral Ecology and*
459 *Sociobiology* **28**:9–12.

460 **Helbig A. 1994.** Genetic basis and evolutionary change of migratory directions in a European
461 passerine migrant *Sylvia atricapilla*. *Ostrich* **65**:151–159.

462 **Helbig A. 1996.** Genetic basis, mode of inheritance and evolutionary changes of migratory
463 directions in Palaearctic warblers (Aves: Sylviidae). *Journal of Experimental Biology*
464 **199**:49–55.

465 **Hunt PD, Flaspohler DJ. 1998.** Yellow-rumped warbler (*Setophaga coronata*). In: Poole A,
466 Gills F, eds. *The birds of North America*, No. 376. Ithaca: Cornell Lab of Ornithology.

467 **Ilieva M, Toews DPL, Bensch S, Sjoholm C, Åkesson S. 2012.** Autumn migratory orientation
468 and displacement responses of two willow warbler subspecies (*Phylloscopus trochilus*
469 *trochilus* and *P. t. acredula*) in South Sweden. *Behavioural Processes* **91**:253–261.

470 **Irwin DE, Irwin JH. 2005.** Siberian migratory divides: the role of seasonal migration in
471 speciation. In: Greenberg R, Marra PP, eds. *Birds of two worlds: the ecology and*
472 *evolution of migration.* Baltimore, Maryland: Johns Hopkins University Press, 27–40.

473 **Irwin DE, Irwin JH, Smith T. 2011.** Genetic variation and seasonal migratory connectivity in
474 Wilson's warblers (*Wilsonia pusilla*): species-level differences in nuclear DNA between
475 western and eastern populations. *Molecular Ecology* **20**:3102–3115.

476 **Liedvogel M, Åkesson S, Bensch S. 2011.** The genetics of migration on the move. *Trends in*
477 *Ecology and Evolution* **26**:561–569.

478 **McGregor, RC. 1899.** The Myrtle Warbler in California and description of a new race. *Condor*
479 1:31–33.

480 **Muheim R, Moore FR, Phillips JB. 2006.** Calibration of magnetic and celestial compass cues
481 in migratory birds: a review of cue-conflict experiments. *Journal of Experimental Biology*
482 **209**:2–17.

483 **Muheim R, Henshaw I, Sjöberg S, Deutschlander ME. 2014.** BirdOriTrack: a new video-
484 tracking program for orientation research with migratory birds. *Journal of Field*
485 *Ornithology* **85**:91–105.

486 **Paxton KL, Yau M, Moore FR, Irwin DE. 2013.** Differential migratory timing of western
487 populations of Wilson's Warbler (*Cardellina pusilla*) revealed by mitochondrial DNA
488 and stable isotopes. *The Auk* **130**:689–698.

489 **R Development Core Team. 2013.** R: A language and environment for statistical computing.
490 Vienna, Austria: R Foundation for Statistical Computing.

491 **Rohwer S, Irwin DE. 2011.** Molt, orientation, and avian speciation. *The Auk* **128**:419–425.

492 **Rolshausen G, Segelbacher G, Hobson KA, Schaefer HM. 2009.** Contemporary evolution of
493 reproductive isolation and phenotypic divergence in sympatry along a migratory divide.
494 *Current Biology* **19**:2097–2101.

495 **Rolshausen G, Segelbacher G, Hermes C, Hobson KA, Schaefer HM. 2013.** Individual
496 differences in migratory behavior shape population genetic structure and microhabitat
497 choice in sympatric blackcaps (*Sylvia atricapilla*). *Ecology and Evolution* **3**:4278–4289.

498 **Ruegg K. 2007.** Genetic, morphological, and ecological characterization of a hybrid zone that
499 spans a migratory divide. *Evolution* **62**:452–466.

500 **Sandberg R, Moore FR. 1996.** Fat stores and arrival on the breeding grounds: reproductive
501 consequences for passerine migrants. *Oikos* **77**:577–581.

Seutin G, White BN, Boag PT. 1991. Preservation of avian blood and tissue samples for DNA analyses. *Canadian Journal of Zoology* **69**:82–90.

Sullivan KA. 1989. Predation and starvation: age-specific mortality in juvenile juncos (*Junco phaenotus*). *The Journal of Animal Ecology* **58**:275–286.

Taylor PD, Brzustowski JM, Matkovich C, Peckford ML, Wilson D. 2010. radR: an open-source platform for acquiring and analysing data on biological targets observed by surveillance radar. *BMC Ecology* **10**:22.

Taylor PD, Mackenzie SA, Thurber BG, Calvert AM, Mills AM, McGuire LP, Guglielmo CG. 2011. Landscape movements of migratory birds and bats reveal an expanded scale of stopover. *PloS ONE* **6**:e27054.

Toews DPL, Brelsford A, Irwin DE. 2014a. Isotopic variation across the Audubon's and myrtle hybrid zone. *Journal of Evolutionary Biology* **27**:1179–1191.

Toews DPL, Mandic M, Richards JG, Irwin DE. 2014b. Migration, mitochondria and the yellow-rumped warbler. *Evolution* **68**:241–255.

Toews DPL. 2017. Habitat suitability and the constraints of migration in New World Warblers. *Journal of Avian Biology*, in press.

Van Doren BM, Liedvogel M, Helm B. 2017. Programmed and flexible: long-term *Zugunruhe* data highlight the many axes of variation in avian migratory behavior. *Journal of Avian Biology* **48**:155–172.

Veen T. 2013. Unraveling migratory connections: the next level. *Molecular Ecology* **22**:4144–4146.

Williams TC, Williams JM, Williams PG, Stokstad P. 2001. Bird migration through a mountain pass studied with high resolution radar, ceilometers, and census. *The Auk* **118**:389–403.

Figure Captions:

Figure 1 Distribution and band recoveries of Audubon's and myrtle warblers, and location of study site. **(A)** Banding data obtained from Brewer et al. (2006) and the Canadian Bird Banding Office (2013). Note that there is a distinct subspecies of Audubon's warbler, the black-fronted warbler, that occurs in Mexico and is not distinguished on this map. (**B**) Sites of capture for migratory yellow-rumped warblers with (**C**) the site of the orientation assays. Map data: Google, DigitalGlobe.

Figure 2 Holding and orientation cages as modified from Fitzgerald and Taylor (2008). (A) Orientation cage with tarp shield. (B) Orientation cage without tarp shield. (C) Dimensions.

Figure 3 Example of the radR interface. The image shows two frames from a video taken from the bottom center of the cage. The perch exclusion zone is the area within the circular perch.

Figure 4 Average hybrid index and sex proportion was relatively constant over the migratory period. (**A**) Average hybrid index over the fall migratory period. Hybrid index was based on three nuclear genetic markers and was equivalent to the proportion of myrtle alleles (i.e. 0 = all Audubon's alleles; 1 = all myrtle alleles). (**B**) Proportion of daily sample that were male, as determined by molecular sexing.

Figure 5 Activity of birds over the evening during the orientation trials. Each point is the number of objects identified by radR over a 5-minute time period with the time relative to sunset.

545 The grey points are the raw data from all individuals. The connected, filled circles are the
546 averaged points over a 20-minute window. Peak activity occurs approximately 20 minutes before
547 sunset.

548 **Figure 6** Orientation of yellow-rumped warblers in relation to genetic ancestry. (**A**) Orientation
549 of all individuals in the study. (**B-F**) Orientation of individuals grouped according to genetic
550 hybrid index.

Figure 1

Distribution and band recoveries of Audubon's and myrtle warblers, and location of study site.

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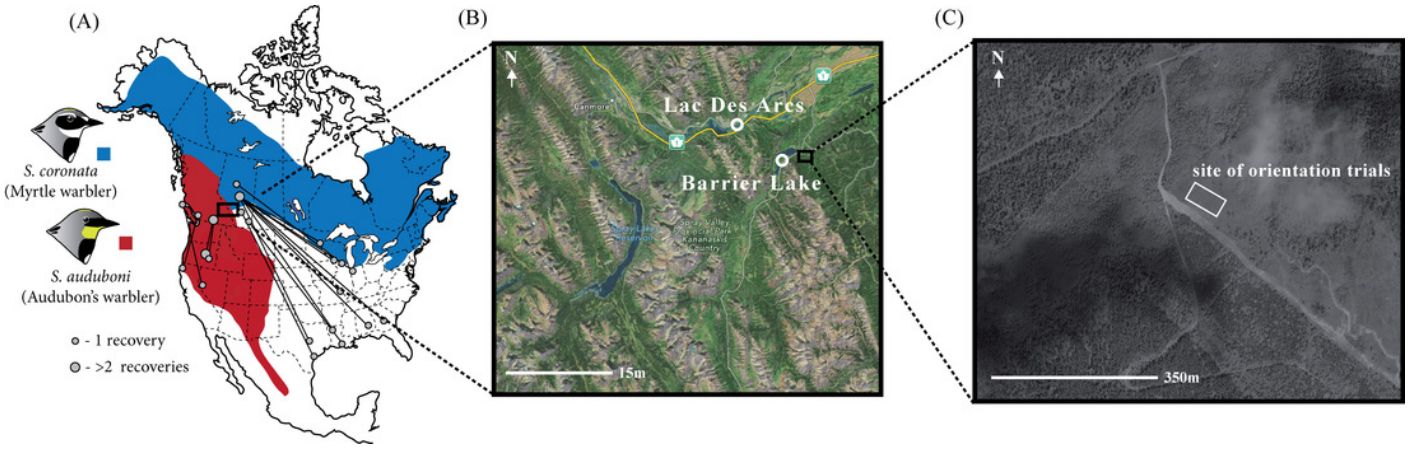

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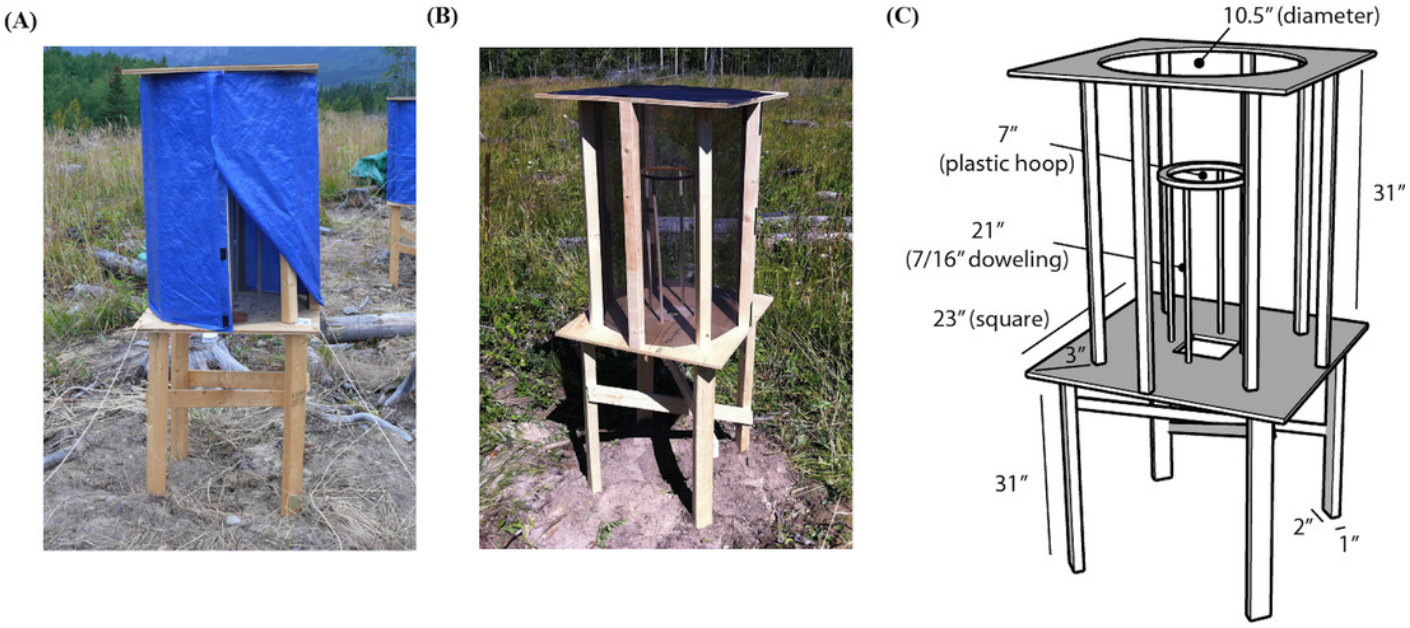

Figure 3

Example of the radR interface.

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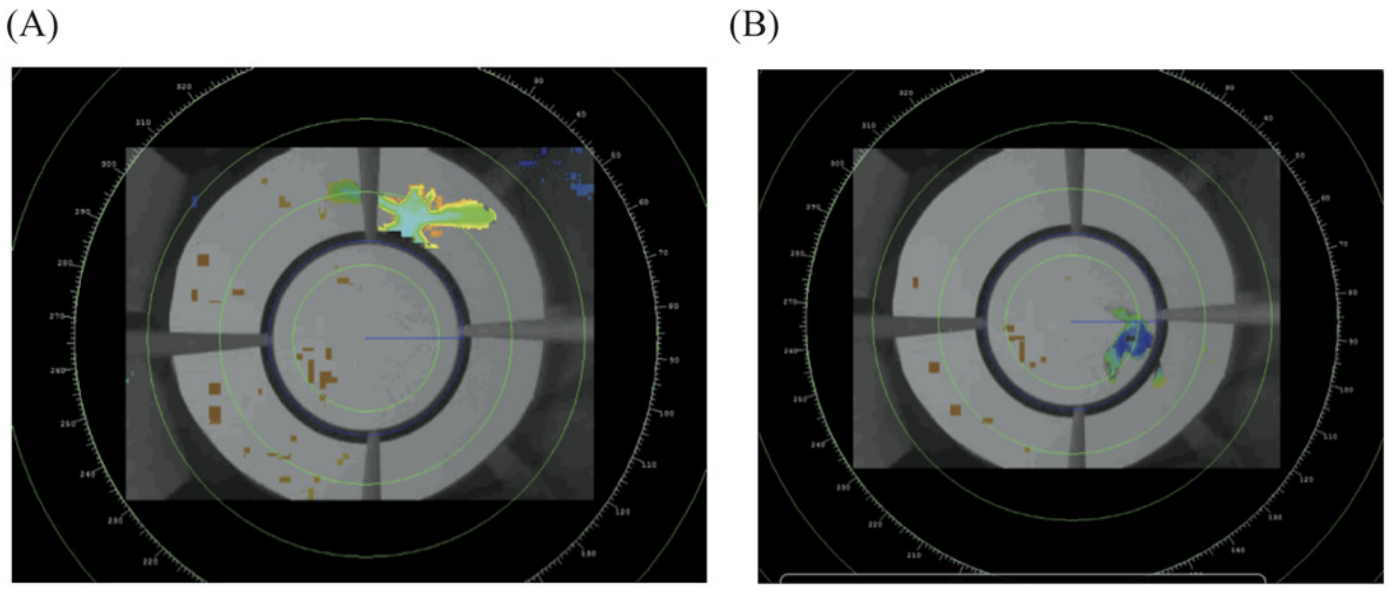

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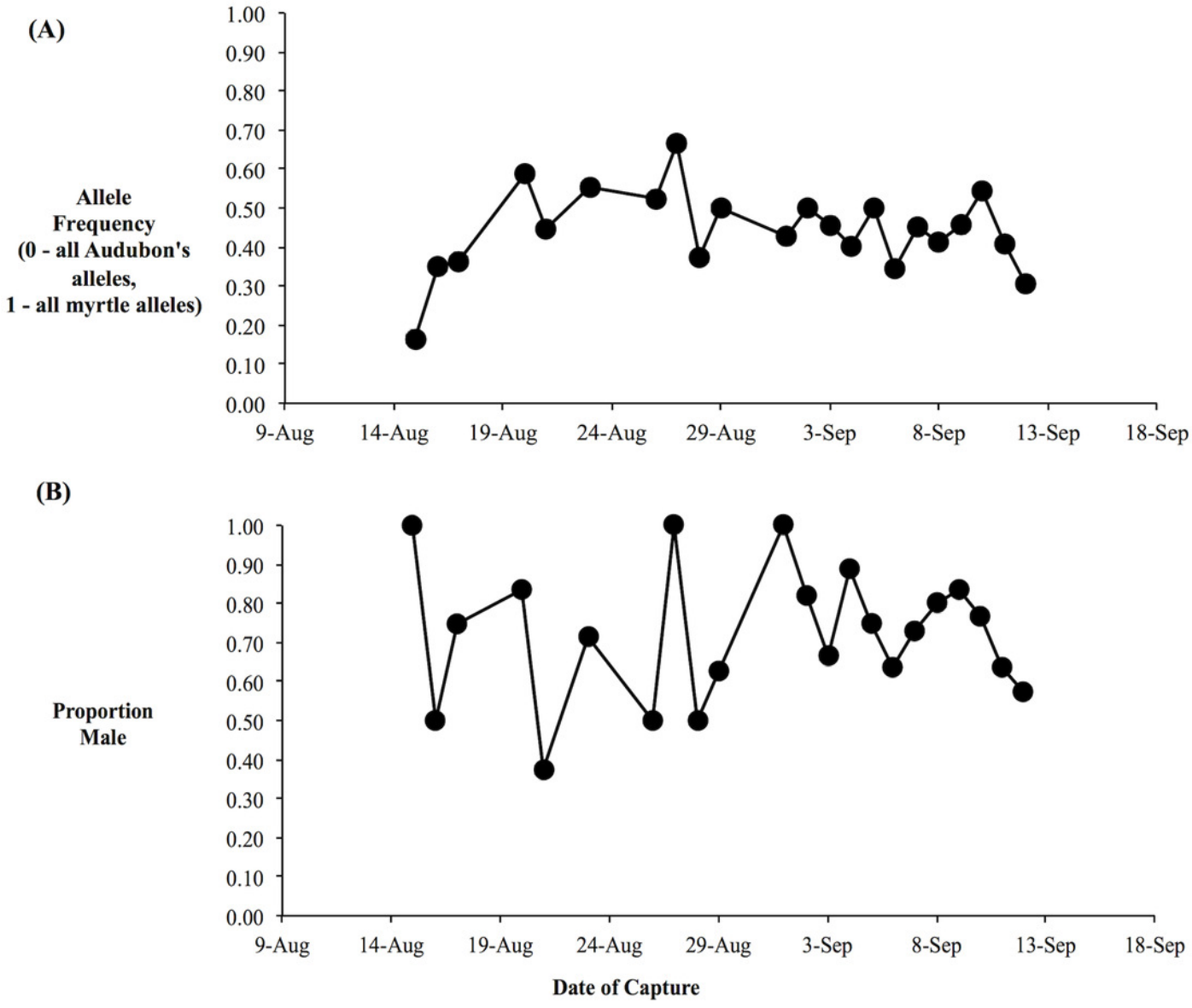

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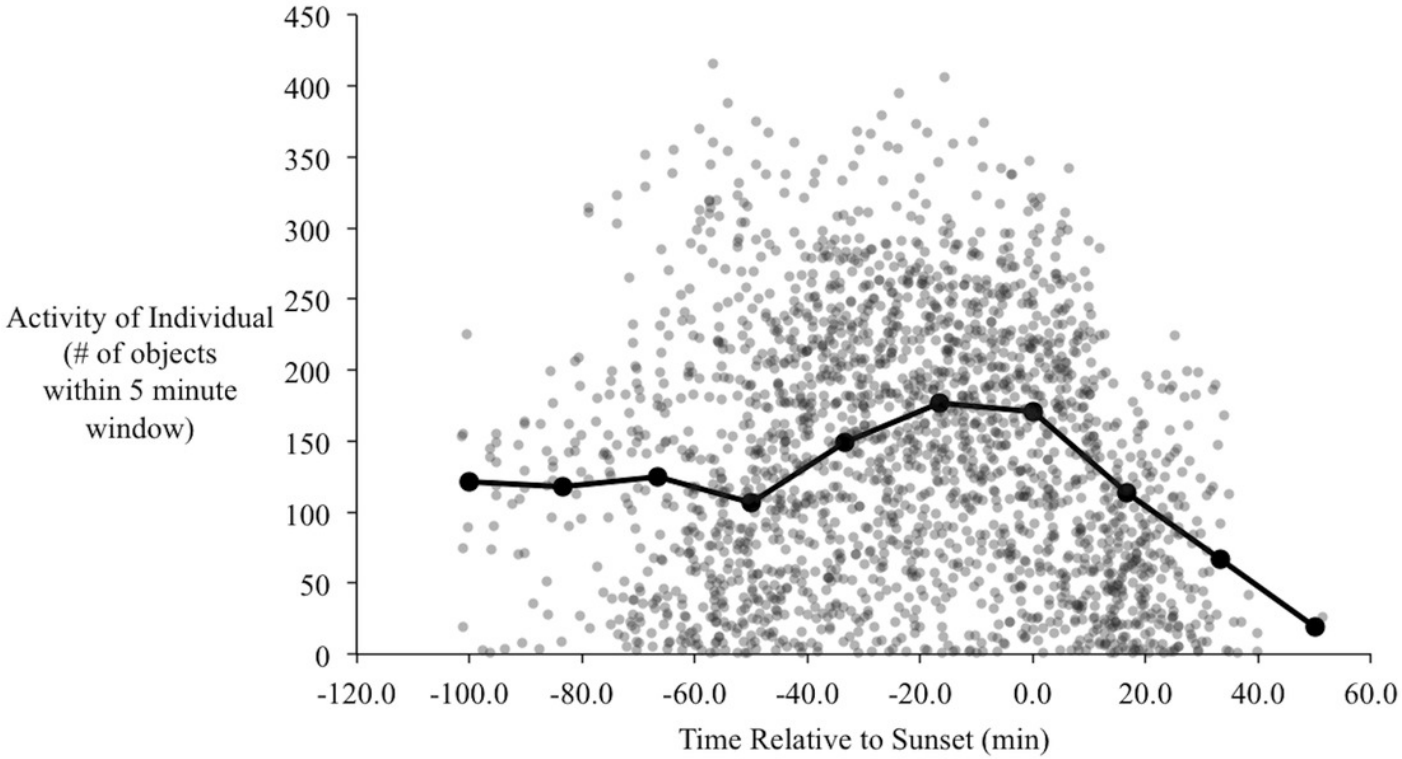

Figure 6(on next page)

Orientation of yellow-rumped warblers in relation to genetic ancestry.

(**A**) Orientation of all individuals in the study. (**B-F**) Orientation of individuals grouped according to genetic hybrid index.

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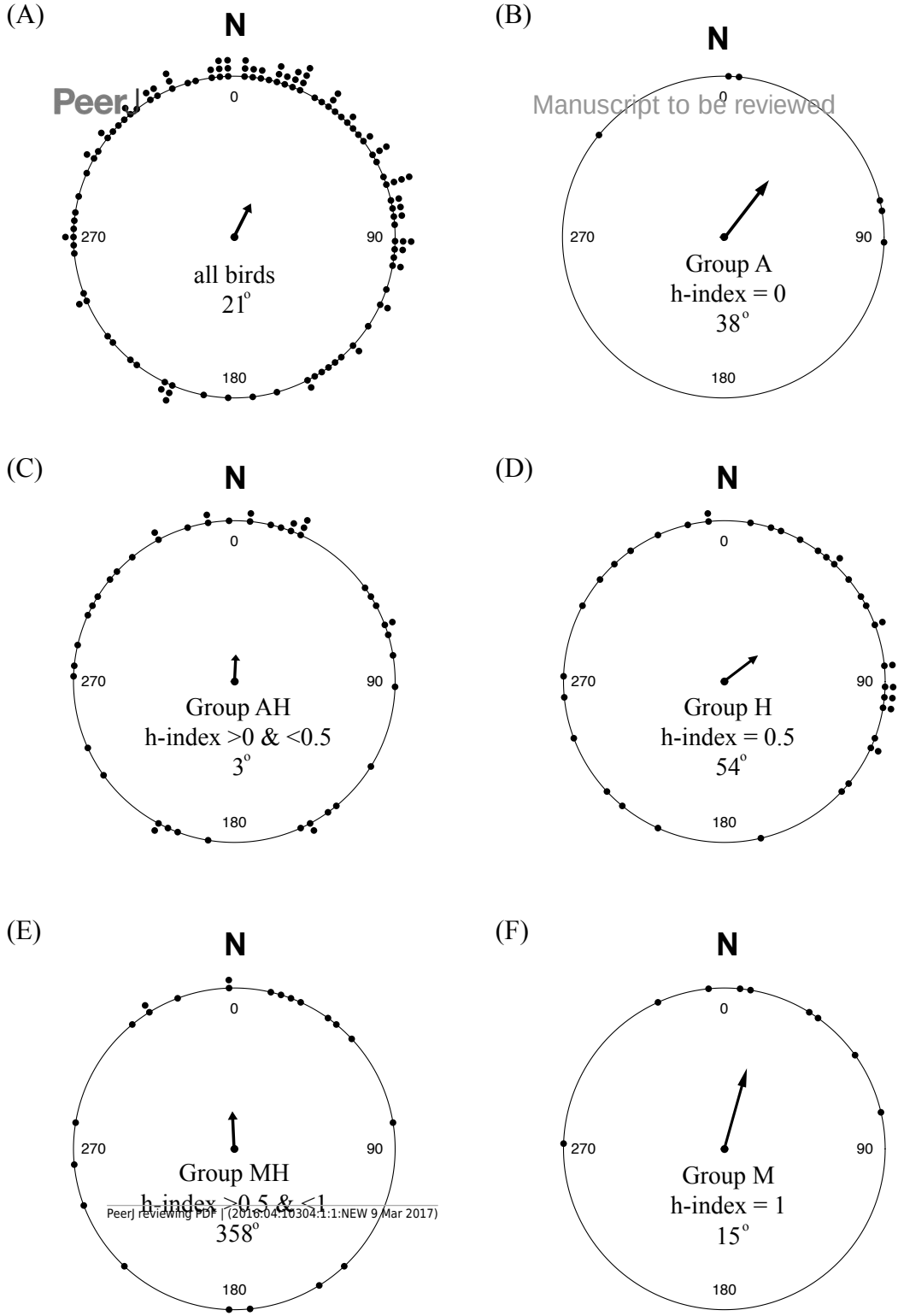

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