

## Migratory orientation in a narrow avian hybrid zone

David Toews, Kira Delmore, Matthew Osmond, Phil Taylor, Darren Irwin

**BACKGROUND:** Areas of secondary contact between 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 myrtle and Audubon's warblers that is likely maintained by selection against hybrids. Allopatric populations of these two taxa show different broad scale patterns of migration, however it is unclear how hybrids between them orient. Therefore, this is an excellent system to quantify the orientation behaviour of hybrids.

**METHODS:** We used molecular genotyping and video-based monitoring of migratory restlessness of temporarily-held migrating warblers in the center of the hybrid zone. Using a set of species-specific diagnostic genetic markers, we then tested whether there was a relationship between the genetic background of an individual and fall migratory orientation.

**RESULTS:** There was large variation in orientation direction, with an overall mean towards the NE, surprising for birds on fall migration, but aligned with the mountain valley in which the study took place. We also found no association between orientation and genetic background.

**DISCUSSION:** While these results are consistent with selection against hybrids on migration and a migratory orientation that is based on local topographical features, such as mountain valleys, there are a number of alternative explanations. We discuss these alternatives and highlight steps future studies can take to differentiate between them.

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### 3 **Migratory orientation in a narrow avian hybrid zone**

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23 **Abstract:**

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42 valleys, there are a number of alternative explanations. We discuss these alternatives and  
43 highlight steps future studies can take to differentiate between them.

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46 **Introduction:**

47         The breeding and wintering ranges of many species are separated by thousands of  
48 kilometers. Long-distance seasonal migration exhibited by many taxa moving between these  
49 disjunct areas is a complex and energetically demanding task that has been studied for decades  
50 (Berthold 1996). However, the physiological mechanisms, controls and senses involved in  
51 navigation of many taxa are still unclear, as are the contributions of various cues to migratory  
52 behavior (Berthold and Terrill 1991; Alerstam 2006). Much of our understanding of migratory  
53 directionality and navigation comes from studies of blackcap warblers (*Sylvia atricapilla*) in  
54 Europe. In an influential series of studies, Helbig and colleagues (Helbig 1991; Berthold et al.  
55 1992; Helbig 1994; Helbig 1996) reared blackcaps from populations that exhibit different  
56 migratory routes in a common environment. They found that, even in captivity, individuals  
57 recapitulated their natural routes, as assayed by Emlen funnels (Emlen 1970), and this was  
58 strongly correlated with a birds' genetic background (Helbig 1996). These studies also found that  
59 lab-crossed hybrids between the parental types show an intermediate migratory orientation, on  
60 average.

61         Unfortunately, few studies have collected similar data in wild populations, primarily due  
62 to logistical challenges, and our understanding of how these migratory behaviours are expressed  
63 under natural settings is unclear. Such observations are particularly pertinent to studies of  
64 migratory divides, where populations that differ in migratory directionality come into contact  
65 and interbreed (e.g. Irwin and Irwin 2005; Bensch et al. 2009; Rohwer and Irwin 2011). For  
66 example, if hybrids between divergent populations have a mixture of alleles responsible for  
67 migration and exhibit an intermediate migratory orientation, as has been observed in lab-raised  
68 blackcap warblers (Helbig 1991; Helbig 1994; Helbig 1996), such a novel phenotype may be

69 inferior and represent an important fitness detriment. This is because, in a number of systems,  
70 intermediate routes have been suggested to take hybrid individuals over unsuitable or  
71 inhospitable environments (e.g., large bodies of water or mountains), although empirical  
72 examples are rare (Rohwer and Irwin 2011).

73         To date, the only study to assay the migratory behaviour of wild individuals differing in  
74 their genetic constitution across a migratory divide is in a study of Swainson's thrushes  
75 (*Catharus ustulatus*; Delmore et al. 2012; Delmore and Irwin 2014). Two populations at the  
76 center of a hybrid zone between inland and coastal Swainson's thrushes were tracked using light-  
77 level geolocators; hybrids exhibited greater variability in their routes than parentals did, with  
78 some taking routes that were intermediate to parental forms and took them over the arid regions  
79 of the southwestern USA. There are a number of constraints that make geolocators impractical in  
80 some study systems, including the limited capacity of many small songbirds to carry such a  
81 device and high costs preventing large sample sizes. In addition, tracking the patterns of recently  
82 hatched birds is difficult, giving the low return rate of young birds. Indirect methods, such as  
83 genetic markers and stable isotopes, can be used to examine the orientation of birds that are too  
84 small to carry tags, but studies using these methods are uncommon. One example is from Ilieva  
85 et al.'s (2012) research of willow warblers (*Phylloscopus trochilus*) in Europe. In this case,  
86 individuals were captured in SE or SW Sweden during fall migration and their orientation was  
87 assayed using Emlen funnels (Emlen 1970). Individuals were genotyped at loci that differ  
88 between the two subspecies which, based on ring recoveries and isotopic data, differ in their fall  
89 migratory directionality (Bensch et al. 2009). The mean orientation of birds from each  
90 subspecies was consistent with expectations (i.e., *P. t. trochilus* SE and *P. t. acredula* SW).  
91 While this was one of the first studies to assay migratory orientation of wild individuals across a

92 migratory divide, it had a number of important limitations. First, individuals that differed in their  
93 genetic composition were captured in differing locations (i.e. SE versus SW Sweden), therefore  
94 it is unknown whether individuals were responding to more local, geographic factors as opposed  
95 to innate genetic tendencies. Second, there was only a small sample of hybrids, and it was  
96 unclear whether those birds that had intermediate genotypes were from the contact zone, so that  
97 it was not possible to infer the behaviour of hybrids more generally.

98         We attempted to address a number of these limitations here with a large-scale study of  
99 birds sampled on fall migration from a hybrid zone between Audubon's / myrtle warblers  
100 (*Setophaga auduboni* and *S. coronata*) in western North America. The narrowness of this hybrid  
101 zone suggests that some form of moderately strong selection maintains it (Brelsford and Irwin  
102 2009). Assortative mating and other pre-mating reproductive barriers are unlikely to be strong  
103 between these taxa (Brelsford and Irwin 2009; Brelsford et al. 2011; Toews et al. 2014a),  
104 implying a potentially sizeable role for post-mating selection against hybrids, possibly based on  
105 inferior migratory behaviour. Band recovery and isotopic data from birds outside the hybrid zone  
106 suggests the two parental taxa differ in their migratory movements (Figure 1; Toews et al.  
107 2014a). Hence, if migratory traits are genetically controlled and inherited additively, as the  
108 studies of blackcaps and Swainson's thrushes suggest, we might expect a correlation between a  
109 bird's orientation and its ancestry in the hybrid zone. In particular, we predicted that individuals  
110 more genetically Audubon's-like would orient SSW, individuals more myrtle-like would orient  
111 SE, and hybrids would orient intermediate between these two (i.e. south).

112         To study the orientation of these warblers on migration we used a video-based orientation  
113 cage method, initially developed by Fitzgerald and Taylor (2008). These orientation cages are  
114 similar in many respects to traditional Emlen funnels (Emlen 1970), but in this case the

115 behaviour of birds was scored using video cameras. As compared to the scratch marks quantified  
116 with Emlen funnel experiments, this method has a number of benefits, including: (1) scoring of  
117 the videos can be automated, ensuring objectivity, (2) a longer period of observation can be  
118 obtained for each individual, (3) specific time periods can be analyzed in isolation and (4) any  
119 behavioural changes over the course of the trial can be quantified. We designed our study to  
120 assay the orientation of individuals in the evening of the day they were captured, which previous  
121 research suggests is predictive of later orientation (Ilieva et al. 2012). We then genotyped each  
122 individual with species diagnostic genetic markers and tested whether there was a correlation  
123 between orientation and genetic background.

## 124 **Methods**

### 125 *Study site and orientation trials*

126       Between August 15<sup>th</sup> and September 12<sup>th</sup> of 2011 we captured migratory yellow-rumped  
127 warblers ( $n = 181$ ) near Kananaskis, Alberta, a site at the center of the Audubon's / myrtle hybrid  
128 zone (Brelsford and Irwin 2009). Our sampling was concentrated in two areas: along the  
129 southern edge of Barrier Lake (51.0235°N, 115.0608°W) and within the hamlet of Lac Des Arc  
130 (51.0536°N, 115.1589°W; Figure 1C). We set up mist-nets before dawn and used passive netting  
131 along with song playback to increase our likelihood of catching individuals of our target species.  
132 Immediately after capturing each individual we took morphometric measurements (bill, tarsus,  
133 wing and tail length), photographs, and a blood sample (10-40 $\mu$ L), applied unique aluminum  
134 USFWS bands and aged the birds according to Pyle (1997). Birds were then transported  
135 approximately three kilometers from the capture site to the location where the orientation trials  
136 were performed (51.0286°N, 115.0242°W). This site is a large, recently clear-cut field ( $\sim 400\text{m}^2$ ;  
137  $<10$  years old) near the Kananaskis Biogeoscience Institute and has clear views in all directions.

138           Each individual was placed into one of 12 outdoor holding / orientation cages (Figure 2)  
139 and given water and small mealworms throughout the day. The cages were a modified design  
140 based on Fitzgerald and Taylor (2008), who also used them to study orientation in yellow-  
141 rumped warblers (Figure 2). The cages were leveled, oriented with a compass, and spaced  
142 approximately 3-5 meters apart. The cage frames were made out of pine boards, with the top and  
143 bottom of the cages made from composite plywood. The perch was made with a 9” plastic  
144 embroidery hoop, held up by four 7/16” dowels, and placed horizontally such that birds could  
145 perch on the hoop (Figure 1C). Since research suggests that birds may use magnetic cues for  
146 navigation, the wood pieces were joined with non-magnetic brass screws and stainless steel  
147 staples were used to affix screen mesh to the top and sides of the cage (Alerstam 1993; Muheim  
148 et al. 2006). During the day the sides of the cages were covered with a blue, opaque tarp for sun  
149 shelter, with part of the tarp rolled up to provide sufficient airflow (Figure 2A). Using the same  
150 cages to both hold the individuals during the day and run the orientation trials in the evening has  
151 a number advantages over traditional the traditional use of Emlen funnels (Emlen 1970).  
152 Importantly, this likely reduces the stress inherent in holding individuals indoors in artificial  
153 environments and moving individuals between holding and assay cages shortly before the trials,  
154 as is necessary with funnels (Emlen 1970). We were careful in assessing the welfare of the birds:  
155 if individuals did not immediately fly to the perch, showed any signs of stress (e.g. panting), or  
156 were not eating properly they were released during the day (less than 15% of birds captured).

157           Before we started the orientation trials we removed the food and water dishes and closed  
158 the tarp around the sides of the cages, allowing individuals to see only out of the top of the cage,  
159 which had a full view of the sky. Through a hole in the bottom of each cage we attached a D-  
160 Link Wireless Network Camera (DCS-932L) pointing directly up, with the top of the camera

161 oriented northwards. This method has advantages over some other recently described video-  
162 based orientation methods (e.g. Muheim et al. 2014), as the cameras do not obstruct a bird's view  
163 of the sky. The cameras we used have an infrared LED light for illumination during low light  
164 conditions. There is no evidence to suggest that the birds can see into the infrared spectrum; if  
165 there was an effect of the light it would not explain any differences found between birds because  
166 the light was applied consistently across all of the trials. The cameras were set such that the  
167 video image was recorded with the right side of the video representing the west side of the cage  
168 and the left side of the video representing the east side of the cage. We recorded 320p x 240p 30  
169 frame-per-second video from each cage simultaneously using the D-ViewCam software on a PC  
170 laptop via a D-Link router. We began recording approximately one hour before sunset and ran  
171 the trials until approximately 30 minutes past sunset. Each evening we ran the trials until the last  
172 individual stopped moving, after which we released all of the individuals. During the trials we  
173 recorded wind speed, wind direction and amount of cloud cover (on a scale from 0 to 8). We  
174 tested each bird once and recorded the behaviour of 1 to 10 individuals each evening, averaging  
175 6 birds per evening, with a total of 124 individuals for which we collected complete orientation  
176 data. All animal care and experimentation was conducted according to the University of British  
177 Columbia protocol Nos. A11-0054 (Project title: Orientation in migratory songbirds) and A09-  
178 0131 (Project title: Geographic variation in birds of western Canada). Field permits were  
179 provided by the Canadian Wildlife Service, Prairie and Northern Region office  
180 AB Scientific Permit 11-AB-SC023 and Alberta Parks 11-107.

### 181 *Video analysis*

182 From the D-ViewCam software we exported each video in “.asf” format, noting the start  
183 and end time of each trial. We analyzed the video data with radR, an open source platform

184 developed for acquiring and analyzing radar data (Taylor et al. 2010) that was more recently  
185 adapted to analyze video files. In brief, radR uses contrast to score individual pixels, and then  
186 uses movement, area and intensity to define an object. We sampled the videos at three frames per  
187 second. For each detected object we extracted the X and Y coordinates of its centroid, as  
188 weighted by the area of the object. Given that the light conditions change over the evening, we  
189 used three groups of parameters to accurately and consistently identify the bird as the primary  
190 object relative to the background (see supplementary material for the parameter used). We  
191 defined the center of the circular perch as the center of our analytical coordinate system and  
192 created an exclusion zone within the circular perch, such that the program would identify the bird  
193 only in the region from the perch outwards (Figure 3). We did this to reduce points where the  
194 individual was flying between sides of the perch; this also makes our results more comparable to  
195 previous funnel studies, which only recorded jumps where the individual contacts the side of the  
196 funnel (Fitzgerald and Taylor 2008). From this data radR generates a list of time-stamped X and  
197 Y coordinates for points where it identified an object (see supplementary material for an example  
198 of data extracted for a single individual).

199       Using R 3.0.3 (R-core Development Team 2013) we applied a number of additional  
200 filters, primarily to remove noise (i.e. objects that were not the bird) and remove times when the  
201 bird was not moving (i.e. sitting on the perch). To remove potential noise, we first removed all  
202 data where there were three or more objects identified at a single time-stamp, as three or more  
203 objects were invariably an artifact of background noise. Sometimes radR identified the bird as  
204 two separate objects, primarily when the individual was above one of the four perch dowels,  
205 such that parts of the bird stuck out on either side of the dowel. For time-stamps with two  
206 objects, we averaged the XY values that were less than 500 pixels apart to include only those

207 times that an individual was above the dowels (i.e. the dowels are slightly less than 500 pixels  
208 wide in the video image) and visually inspected the data to confirm this was accurate. To ignore  
209 points where the bird was sitting motionless on the perch we removed consecutive time points  
210 that had a lower XY distance than a predetermined threshold, which we calculated by studying  
211 videos of individuals sitting quietly on the perch (50 pixels for the current analysis). Each of the  
212 points that passed these filters were then transformed into an angle relative to North ( $N=0^\circ$ ,  
213  $E=90^\circ$ ,  $S=180^\circ$  and  $W=270^\circ$ ) and given a timestamp (in seconds) relative to the time of sunset for  
214 that evening.

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

224         To assess robustness of estimating the mean orientation for each individual using the data  
225 generated from radR we chose five orientation videos at random and scored them by eye, blind  
226 to the output from radR. For this we visually estimated the angle of the bird (if present in the  
227 frame) every 30 seconds, over the entire video, and also recorded whether the bird was on the  
228 perch or in mid-flight. We then calculated the difference in angle between this estimate and that  
229 obtained from radR for the full dataset and also from only those points where the bird was

230 observed to be in flight. Our data filtering appeared to be effective as our data from a random  
231 selection of videos analyzed by eye (for 5 birds) was consistent with the output from radR. When  
232 we compared all of the points observed by eye, including those where the individual was sitting  
233 on the perch, the resultant mean angles were within  $\pm 22^\circ$  relative to the output from radR after  
234 filtering. If we included only those points where the individual was in flight (by excluding those  
235 times we observed by eye for which the individual was on the perch), which our filters within  
236 radR were designed to remove, the resultant mean angles were within  $\pm 11^\circ$  of the radR output.  
237

### 238 *Molecular analysis*

239 Blood samples, taken using a small needle and capillary tube from the brachial vein, were  
240 stored in Queen's lysis buffer (Seutin et al. 1991) and left at ambient temperature until returned  
241 to the laboratory. DNA was extracted using a phenol-chloroform protocol and resuspended with  
242 50 – 200  $\mu$ L of buffer (depending on the size of the pellet) containing 10 mM Tris-HCl and 1  
243 mM EDTA, at pH 8.0, and stored at 4°C. We sexed individuals molecularly using the procedure  
244 described in Fridolfsson and Ellegren (1999). We genotyped individuals at three molecular  
245 markers. The full PCR and genotyping protocol for two of the three nuclear markers (*CHDIZ*  
246 and *numt-Dco1*) was presented in a previous publication (Brelsford and Irwin 2009). For the  
247 third nuclear marker, an 850 base pair fragment of *RIOK2*, we used the forward primer  
248 ATGGGTGTTGGCAAAGAATC and the reverse primer GCTCCTCTTCRTTWGCAACA and  
249 used a PCR annealing temperature of 60°C. An allele common in Audubon's warblers is cut by  
250 the enzyme *XmnI*, whereas an allele common in myrtle warblers remains intact. To generate a  
251 genetic hybrid index we added each myrtle allele for the three markers and divided this by the

252 total number of alleles (6 for males, 4 for females) resulting in an index that ranges between 0  
253 (all Audubon's alleles) to 1 (all myrtle alleles).

#### 254 *Combining genotype and migratory behaviour*

255 We tested whether birds in our sample, on average, tended to orient in a specific direction  
256 and whether there was association between genetic background and orientation direction. To test  
257 whether there was a significant mean orientation of all of individuals considered together,  
258 regardless of their genetic background, we used a Rayleigh test. This is a procedure to test the  
259 null hypothesis that the orientation angles are distributed randomly, with the alternative being  
260 that the distribution is clumped in certain direction(s). The test statistic is  $r$ , or rho, and is the  
261 magnitude of the mean vector (Fitzgerald and Taylor 2008).

262 To test whether orientation is associated with genetic background, we used two statistical  
263 approaches. First, we used a circular linear model to test whether orientation varied linearly with  
264 hybrid index (Agostinelli and Lund 2007). Second, we used a circular ANOVA to test whether  
265 mean orientation angle differed in any way (not necessarily linearly) among these five genetic  
266 groups: those with all Audubon's alleles (h-index = 0; Group A), those with mostly Audubon's  
267 alleles ( $0 < \text{h-index} < 0.5$ ; Group AH), those with mixed genotypes (h-index = 0.5; Group H),  
268 those with mostly myrtle alleles ( $0.5 < \text{h-index} < 1$ ; Group MH), and those with all myrtle alleles  
269 (h-index = 1; Group M).

## 270 **Results**

### 271 *Molecular data*

272 We obtained genotypes from 166 of the 181 individuals captured, including 123  
273 individuals with orientation data. Based on the three genetic markers, individual yellow-rumped

274 warblers migrating through the Kananaskis area had a mixture of myrtle and Audubon's alleles,  
275 suggesting a mixture of hybrids and pure-type individuals. The daily composite allele  
276 frequencies varied over the study period although the daily proportion of myrtle alleles was  
277 usually between 0.3 to 0.6, with an average over the study period of 0.4 (Figure 4A). Our  
278 molecular sexing of individuals indicated that we captured an excess of male birds with 73% of  
279 all of the individuals identified as males (Figure 4B). This was likely due to our use of song  
280 playback during mist netting.

### 281 *Orientation trials*

282 In the orientation trials, initial observations of yellow-rumped warbler behaviour  
283 suggested that their activity begins to increase approximately one hour before sunset. During this  
284 time of increased activity their behavior also changed, from primarily sitting on the perch and  
285 flying to the bottom of the cage, to performing more short flights from the perch to the top of the  
286 cage, consistent with *zugunruhe* (i.e. migratory restlessness; Emlen 1970). The activity of the  
287 birds increased over this period, peaking 20-30 minutes before sunset (Figure 5). Following  
288 sunset their activity sharply declined, such that we recorded virtually no movements after 40  
289 minutes post-sunset. At the end of most evenings individuals usually stopped moving within 5-  
290 10 minutes of each other with remarkable consistency (the steep decline to the right of Figure 5).

291 Of the 123 individuals where we had genotype and orientation data, we found that 96  
292 birds showed evidence of strong orientation (i.e., within-individual  $r > 0.1$ ; total activity  $> 500$ ).  
293 We used all 123 individuals in analyses; analyses using only those individuals with strong  
294 orientation did not qualitatively change results. Of the five genetic groups, our sample contained  
295  $n = 6$  for Group A (i.e., genetically Audubon's),  $n = 45$  for Group AH,  $n = 41$  for Group H,  $n =$   
296  $22$  for Group MH and  $n = 9$  for Group M (i.e., genetically Myrtle). While there was considerable

297 variability in orientation among individuals, there was a significant mean orientation towards 25°  
298 or NNE (Figure 6A;  $n = 123$ , among-individual  $r = 0.320$ ,  $P < 0.01$ ), with a 95% confidence  
299 interval between 5° and 46°. Separating the birds by their genetic hybrid index, each group had  
300 mean orientations similar to N or NE, although only Groups M (h-index = 1) and H (h-index =  
301 0.5) showed evidence of significant mean orientations compared to the null of an even  
302 distribution of orientation angles: Group A, 38° ( $n = 6$ ,  $r = 0.634$ ,  $P = 0.09$ ); Group AH, 3° ( $n =$   
303 45,  $r = 0.230$ ,  $P = 0.09$ ); Group H, 54° ( $n = 41$ ,  $r = 0.373$ ,  $P < 0.01$ ); Group MH, 359° ( $n = 22$ ,  $r$   
304 = 0.321,  $P = 0.10$ ); and Group M, 15° ( $n = 9$ ,  $r = 0.733$ ,  $P < 0.01$ ). There was no significant  
305 difference in mean orientation between the genetic groups ( $F = 1.23$ ,  $P = 0.09$ ) and there was no  
306 significant linear relationship between mean orientation and genetic hybrid index ( $t = 0.779$ ,  $P =$   
307 0.22).

## 308 Discussion

309 Our use of video-based orientation trials meant we could sample a large number of  
310 individuals during fall migration while also gathering high resolution and objective orientation  
311 data for each bird, a benefit over previous Emlen funnel methods. With these methods we found  
312 1) large variation in the orientation of birds with an overall average orientation towards the NNE,  
313 and 2) no significant association between genetic background and orientation.

314 Among individuals we found a lot of scatter in the bird's directional tendencies, with a  
315 mean migratory orientation that was non-randomly directed towards the NNE (26°). A mean  
316 northwards orientation is a surprising result given that these birds are expected to be heading  
317 south for the winter months. Reverse migration is possible explanation for northward  
318 orientations in the fall, especially if the mountains constitute an ecological barrier, but this  
319 behaviour is expected to be restricted to those individuals with insufficient fat loads (Smolinsky

320 et al. 2013). Other authors have suggested that such northward movements during fall might be  
321 due to non-migratory, post-fledgling movements (Matthews 1961). However, the stereotyped  
322 movements of the birds were qualitatively similar to previous descriptions of *zugunruhe*,  
323 suggesting that the yellow-rumps in our sample were expressing behaviours consistent with  
324 migration.

325         Another possible explanation for the orientation results is that the warbler's orientation  
326 tendencies may change over the migratory period and that our data reflect a small portion of their  
327 overall movements. In this case, individuals may be moving northeast, out of the valleys in the  
328 Rocky Mountains, to later turn south. Indeed, the orientation of the valley near the capture  
329 location and orientation experiment area is approximately 23° (NNE; estimated using Google  
330 Earth™), very close to the observed orientation of the birds when grouped together (26° NNE).  
331 Eastward movement is consistent with the results from isotope values in feathers sampled from  
332 breeding birds: most warblers in the hybrid zone overlap with the isotopic distribution of myrtle  
333 warblers for feathers growing on the winter grounds, although the results also suggest that some  
334 birds may be wintering in the Audubon's wintering area (Toews et al. 2014a).

335         While previous orientation studies have tested the effect of ecological barriers on  
336 migratory behaviour (i.e. water bodies; Sandberg and Moore 1996; Ilieva et al. 2012), this is one  
337 of the first studies to assay orientation in and around mountainous areas. Using high-resolution  
338 radar technology, Williams et al. (2001) found evidence that nocturnal migrants responded to  
339 local topological features by changing their orientation during fall migration, especially those  
340 birds migrating below 300m, as is assumed with yellow-rumped warblers. Given the type of data  
341 these methods collect, however, it is challenging to assign these types of observations to specific  
342 species or even species groups (Williams et al. 2001). For the warblers in our study, it may be

343 that individuals have a memory of the axis of the valley at the time of capture, which they  
344 recapitulate in the orientation cage. We recommend that future orientation studies around  
345 mountainous areas should consider including additional orientation localities in valleys of  
346 varying orientations. It would also be useful to assay individuals each evening over a longer  
347 period (i.e. 1-2 weeks) to test whether this orientation is maintained or dissipates with time. This  
348 could provide a robust test of the role of topological features in influencing migratory  
349 movements. More generally, our orientation data suggest that tracking studies using recent  
350 technology such as radio towers and geolocator tags, which are becoming increasingly  
351 miniaturized, may be more useful for studying large-scale migratory movements (Taylor et al.  
352 2011; Delmore et al. 2012; Veen 2013; Delmore and Irwin 2014).

353         Mean orientation direction did not differ between the different genotype classes. In their  
354 study of migratory willow warblers (*Phylloscopus trochilus*) in Sweden, Ilieva et al. (2012)  
355 found that groups differing in their genetic composition differed significantly in their orientation,  
356 in the expected directions based on ringing recovery and isotope data (Bensch et al. 2009). In  
357 their case, however, groups of differing genetic constitutions were also sampled in different sites,  
358 confounding geography and genetics. Our site in the Audubon's / myrtle hybrid zone sampled a  
359 wide variety of genetic classes, allowing for a more robust test of a correlation between genetics  
360 and behaviour. In our case, we found no predictive power between genotype and orientation.

361         In conclusion, we used molecular genetic methods and a video-based orientation assay  
362 that provided objective, high resolution temporal data for many individuals in a semi-natural  
363 setting. Our data were consistent with selection against hybrids in the non-breeding season and  
364 an orientation that aligned with local topographical features. We suggest that, with future  
365 modifications, these methods could be a powerful tool for understanding migratory orientation in

366 many species. In particular, when replicated in multiple locations, one could use these orientation  
367 assays to examine the effect of local topographical features. Or, if assayed orientations were  
368 verified by geolocators, one could identify potentially maladaptive migratory tendencies in  
369 hybrid individuals. We hope these methods will contribute to the understanding of migratory  
370 divides, and of migration more generally.

371

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381

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501 **Figure Captions:**

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

507 **Figure 2** Holding and orientation cages as modified from Fitzgerald and Taylor (2008).

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

511 **Figure 4 (A)** Average hybrid index and sex-ratio fluctuations over the fall migratory period for  
512 the three nuclear genetic markers. Hybrid index is relative to the myrtle allele (i.e. 0 – all  
513 Audubon's alleles; 1 – all myrtle alleles). **(B)** Proportion of daily sample that were male, as  
514 determined by molecular sexing.

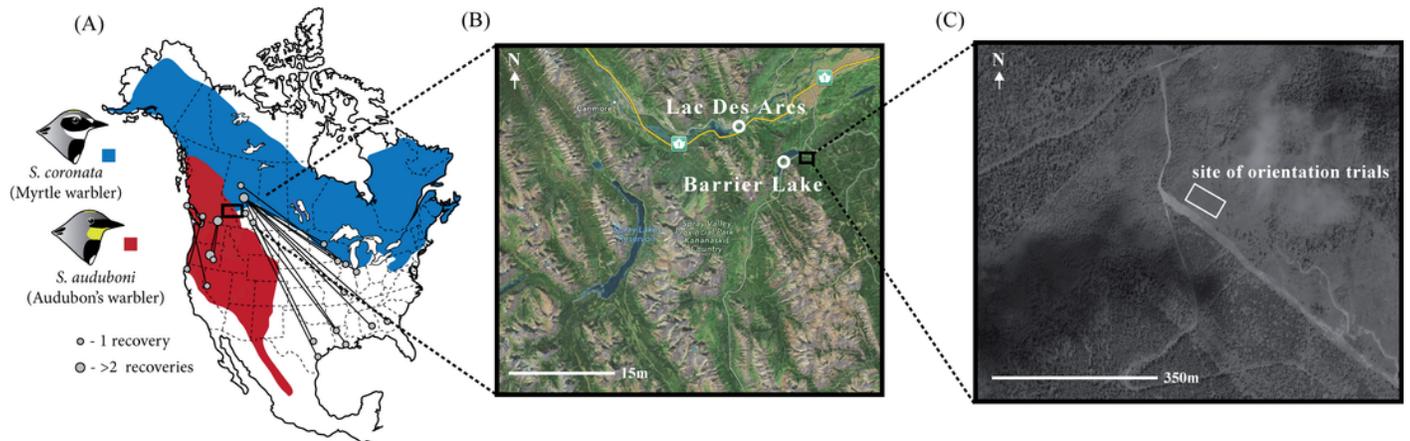
515 **Figure 5** Activity of birds over the evening during the orientation trials. Each point is the  
516 number of objects identified by radR over a 5-minute time period with the time relative to sunset.  
517 The grey points are the raw data from all individuals. The connected, filled circles are the  
518 averaged points over a 20-minute window. Peak activity occurs approximately 20 minutes before  
519 sunset.

520 **Figure 6** Mean orientation of all individuals as **(A)** grouped together and **(B-F)** as distinguished  
521 by genetic hybrid index.

## 1

## Distribution of myrtle and Audubon's warblers with location of study site

Distribution and band recoveries of Audubon's and myrtle warblers. (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 that is not distinguished on this map. (B) Sites of capture for migratory yellow-rumped warblers with the site of the orientation assays (C).



## 2

## Schematic of orientation holding cages

Holding and orientation cages as modified from Fitzgerald and Taylor (2008).

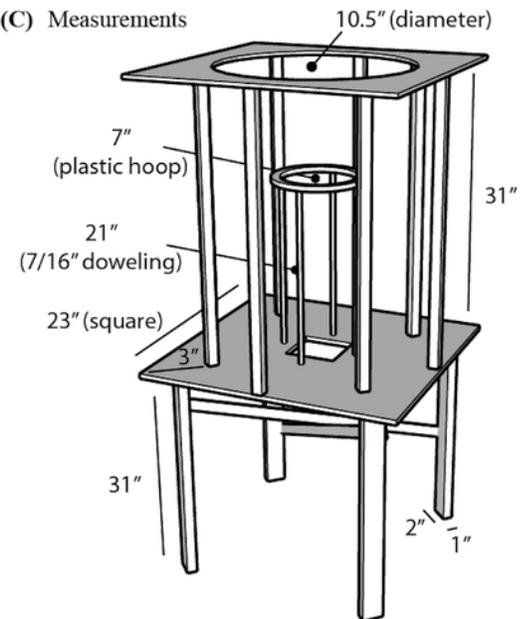
(A) Orientation cage with tarp sheild



(B) Orientation cage without tarp sheild



(C) Measurements

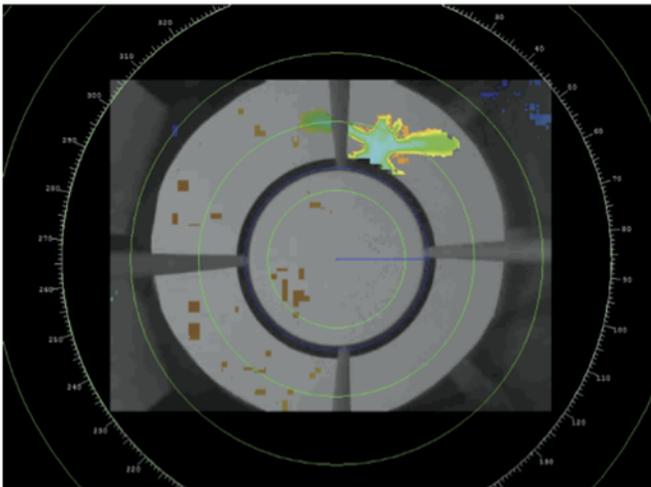


## 3

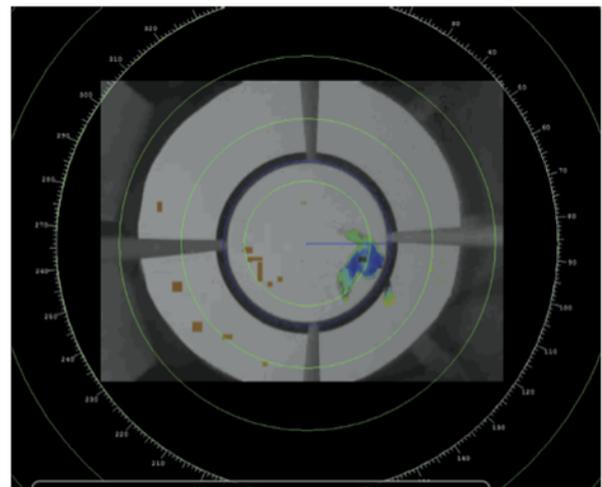
## Example of radR interface

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

(A) Bird identified by radR during flight; cendroid XY point recorded



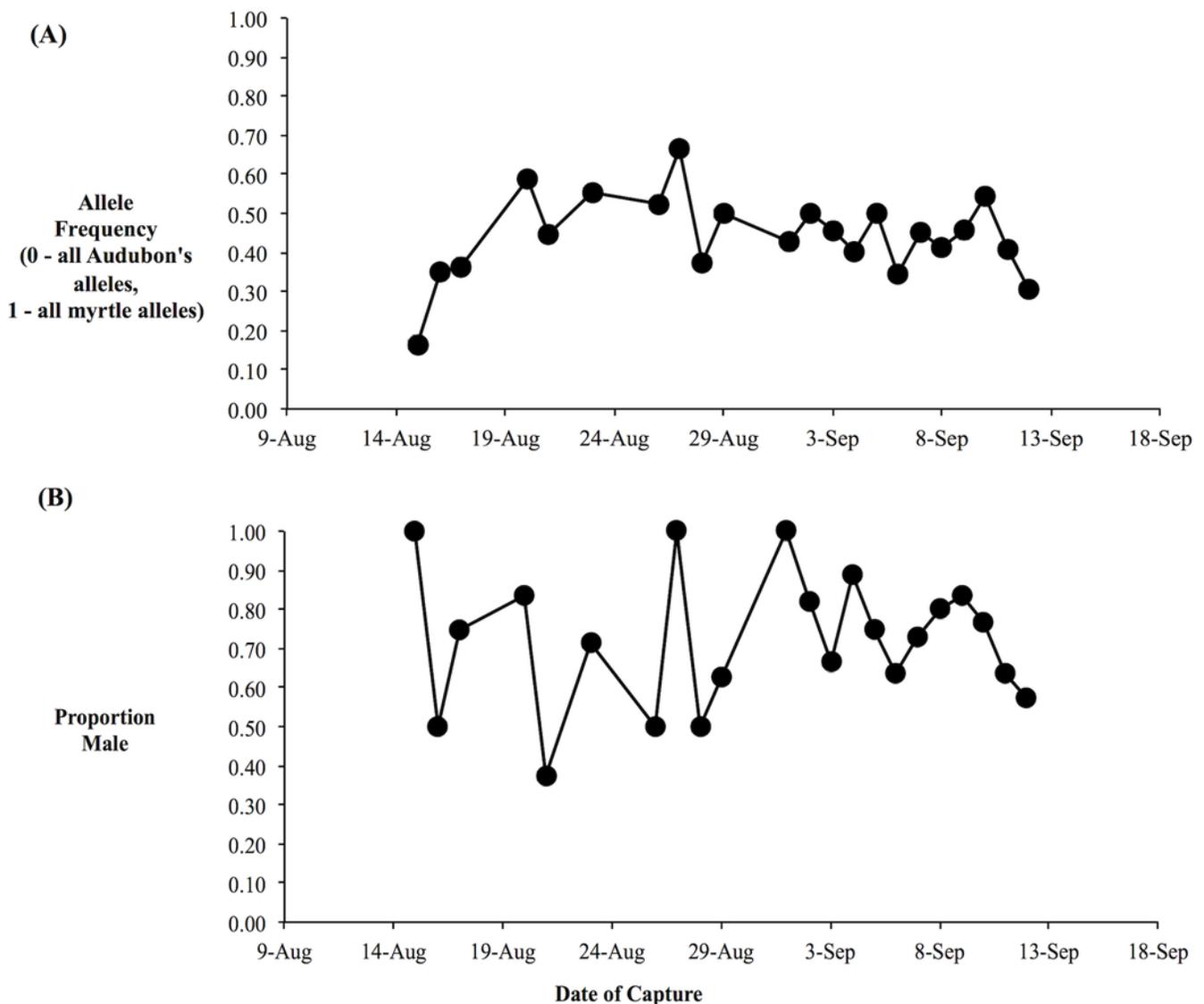
(B) Bird identified by radR within perch exclusion zone; XY point not recorded



## 4

## Proportions of hybrid classes and sexes over migratory period

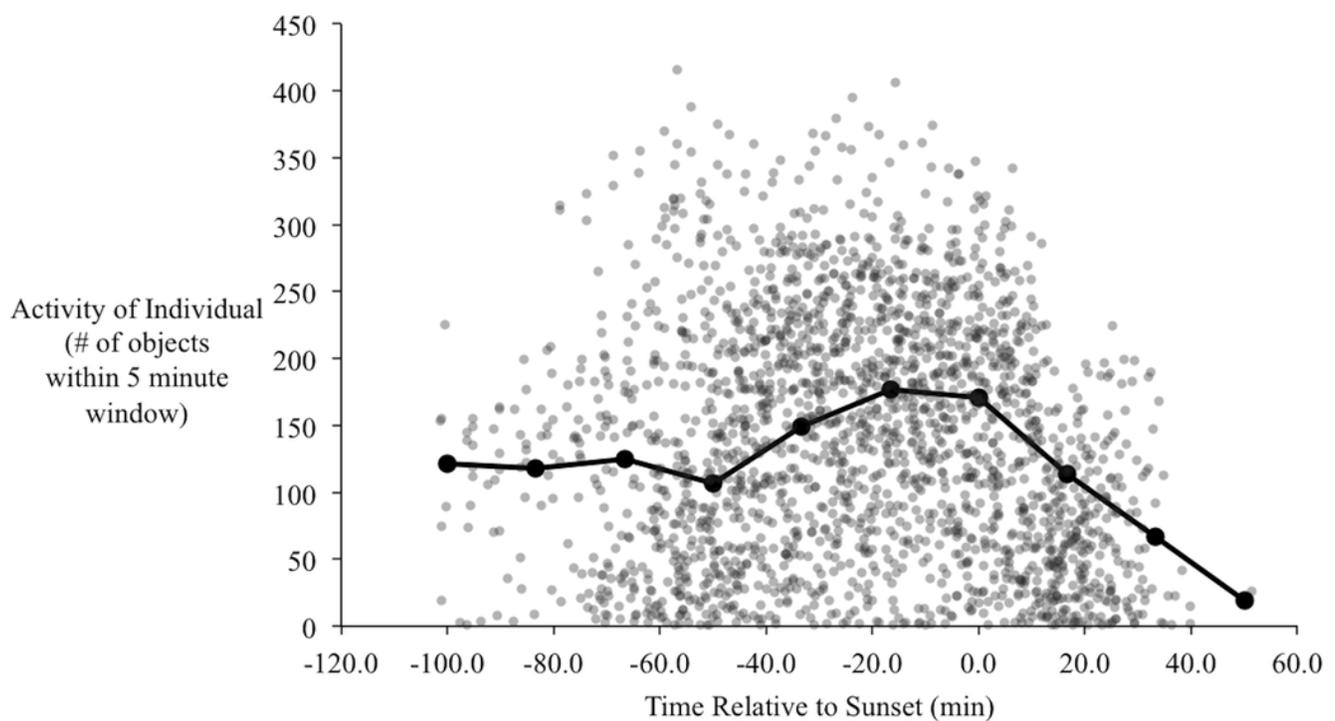
(A) Average hybrid index and sex-ratio fluctuations over the fall migratory period for the three nuclear genetic markers. Hybrid index is relative to the myrtle allele (i.e. 0 - all Audubon's alleles; 1 - all myrtle alleles). (B) Proportion of daily sample that were male, as determined by molecular sexing.



## 5

## Activity of birds over the evenings during the study

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

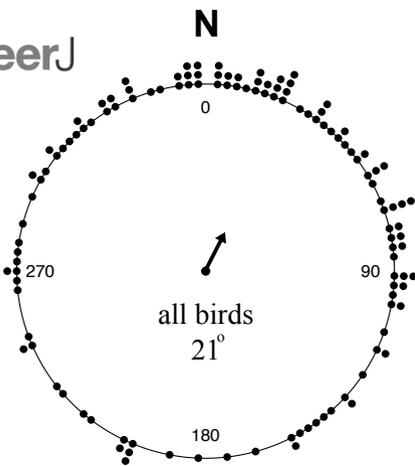


**Figure 6** (on next page)

Mean orientation of birds on migration

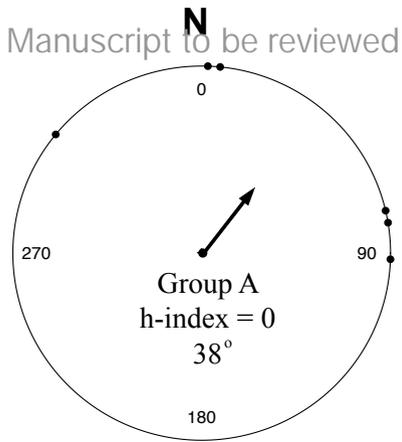
Mean orientation of all individuals as (A) grouped together and (B-F) as distinguished by genetic hybrid index.

(A) PeerJ

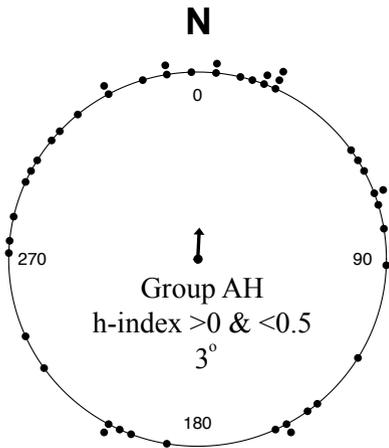


(B)

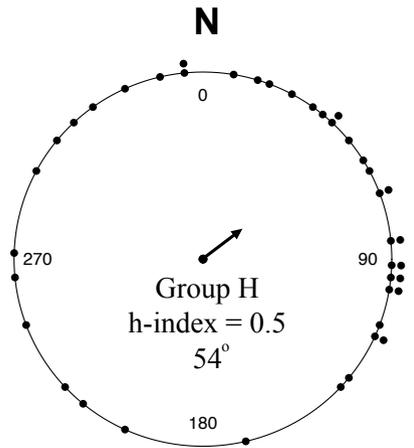
Manuscript to be reviewed



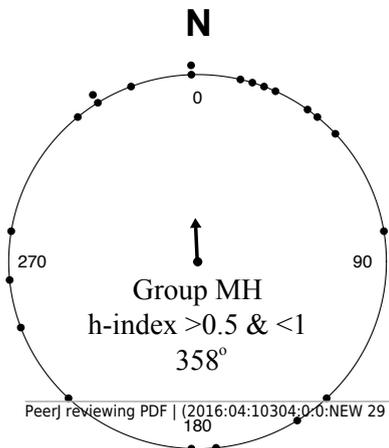
(C)



(D)



(E)



(F)

