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STABLE ISOTOPE ANALYSIS REVEALS BIASES IN THE PERFORMANCE OF A MORPHOLOGICAL METHOD TO DISTINGUISH THE MIGRATORY BEHAVIOUR OF EUROPEAN ROBINS *ERITHACUS RUBECULA*

EL ANÁLISIS DE ISÓTOPOS ESTABLES REVELA SESGOS EN EL FUNCIONAMIENTO DE UN MÉTODO MORFOLÓGICO PARA DIFERENCIAR EL COMPORTAMIENTO MIGRATORIO DE LOS PETIRROJOS ERITHACUS RUBECULA

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SUMMARY.- Morphological methods to distinguish avian groups of research interest (e.g. sex, 1 population or cryptic species distinction) need to be externally validated to ensure a reliable 2 3 performance across situations. In this study, we used hydrogen stable isotope ratios of feathers $(\delta^2 H_f)$ to test the validity of morphological classification functions (MCFs) 4 previously designed to assess the migratory behaviour of European Robins Erithacus 5 rubecula wintering in southern Iberia. Our results showed that a great number of migrants 6 (mostly females and juveniles) were erroneously assigned as sedentary, which could 7 compromise the reliability of previous ecological studies that made use of these MCFs. The 8 9 development of improved MCFs or the use of alternative differentiation methods ($\delta^2 H_f$) could help us to gain a more realistic insight into the habitat distribution and ecological interactions 10 11 of sympatric migratory and sedentary robins overwintering in southern Iberia. 12 RESUMEN.- Los métodos morfológicos para distinguir grupos de aves con interés de 13 14 investigación (e.g. distinción de sexos, poblaciones o especies crípticas) requieren de 15 validación independiente para asegurar su funcionamiento adecuado de forma consistente. En este estudio, usamos la relación de isótopos estables del hidrógeno en las plumas ($\delta^2 H_f$) para 16 comprobar la validez de las funciones de clasificación morfológicas (MCFs) diseñadas con 17 anterioridad para identificar el comportamiento migratorio de los petirrojos Erithacus 18 19 rubecula invernantes en el sur ibérico. Los resultados revelaron que un gran número de 20 migrantes (sobre todo hembras y jóvenes) fueron clasificados erróneamente como 21 sedentarios, lo que podría comprometer la fiabilidad de los estudios ecológicos previos que han hecho uso de estas MCFs. El desarrollo de MCFs mejoradas o el uso de métodos de 22 diferenciación alternativos ($\delta^2 H_f$) podrían ayudarnos a obtener una idea más realista acerca de 23 la distribución entre hábitats e interacciones ecológicas de los petirrojos migratorios y 24 25 sedentarios que invernan en simpatría en el sur ibérico. 26

Discriminant function analyses (DFA) -and other similar statistical approaches- based on 27 avian morphological traits are a readily accessible method to separate morphologically 28 29 discrete groups of birds (Ellrich et al., 2010; Tellería et al., 2013). They are particularly useful to identify males and females in monochromatic -but still morphologically dimorphic-30 31 species (Arizaga et al., 2008; Bertolero et al., 2016). With mixed success, they have also 32 been implemented in the identification of cryptic species (Wilson et al., 2012; Gordo et al., 2017), as well as to identify populations within the same species differing in morphological 33 traits (Wennerberg et al., 2002; Maggini et al., 2016). In many cases, morphology is now 34 35 clearly outweighed by more novel techniques (Webster et al. 2002). For example, molecular genetics can provide unambiguous sex and species identifications (Griffiths et al., 1998; 36 37 Bensch et al., 2002), while methods based on bird morphology are normally subject to 38 variable degree of uncertainty in their assignments. In any case, under budget constraints, logistical limitations (e.g. permits for biological samples collection) and/or when these 39 40 alternative techniques do not substantially improve the classification potential of 41 morphology, the latter still can be the most cost-effective way to satisfactorily differentiate among avian groups of research interest (De la Hera et al., 2012). In any case, given the 42 potential uncertainty associated with the use of morphology, it is essential to validate the 43 reliability of morphological methods using independent approaches, which can be very useful 44 45 to reveal previously unnoticed flaws in their performance. 46 The study of the sympatric interactions between local sedentary birds and overwintering conspecific migrants in southern Iberia has greatly benefitted from the use of 47 morphological classification functions (MCFs) that are one of the outcomes of DFA 48 49 (StatSoft, 2004). It is well known that natural selection favours longer and more pointed wings in migrants compared to sedentary counterparts (Piersma et al., 2005), and this 50 51 variation is sometimes large enough for developing effective MCFs to distinguish each other. 52 For instance, MCFs built from Iberian breeding populations of known migratory behaviour provided a 90 and an 80 percent of correct assignations of the migratory behaviour for the 53 54 Eurasian Blackcap (Sylvia atricapilla) and European Robin (Erithacus rubecula), 55 respectively (Pérez-Tris et al., 1999; Pérez-Tris et al., 2000). However, these MCFs have only been optimized for distinguishing among a few Iberian breeding populations, and these 56 constitute only a small fraction of the wintering population occurring in Southern Iberia. 57 Consequently, whether these MCFs can successfully be applied to distinguish among 58 wintering birds of unknown origin needs to be explicitly corroborated (Ellrich et al., 2010). 59

Most of the migratory blackcaps and robins wintering in Iberia originate from further 60 Northeast in Europe, so they would have a more migratory-like morphology than any Iberian 61 62 counterpart (Cramp, 1992; Korner-Nievergelt et al., 2014). This should ensure an even better performance of these MCFs for the migratory group when they are applied to seasonally 63 sympatric populations wintering in Southern Iberia. This has been confirmed for blackcaps 64 (De la Hera et al., 2007) and validated using a well-known pattern of stable isotope variation 65 for the Palaearctic region (De la Hera et al., 2012). However, the validity of the MCFs 66 designed for distinguishing between migratory and sedentary robins remains to be tested 67 using an independent control. Unlike blackcaps, robins show a great within-population 68 variation in wing morphology, with male and adult robins having on average longer wings 69 70 than females and juveniles, respectively (Ellrich et al., 2010; De la Hera et al., 2014). In this 71 respect, there are two main concerns that could affect the performance of MCFs during winter. First, female and juvenile robins are more prone to migrate (Adriaensen & Dhondt, 72 73 1990) and hence more likely to reach southern Iberia for overwintering, where their short 74 wings might overlap in size with those of local sedentary robins, particularly with males 75 (Ellrich et al., 2010). On the other hand, juveniles were overrepresented in the Iberian robin sample used to develop these MCFs, and the sex ratio of the sample was unknown (Pérez-76 Tris et al., 2000), which raises the possibility that the error rate would change if testing a 77 78 wintering population with a different population composition. 79 To clarify the accuracy of the MCFs proposed by Pérez-Tris et al. (2000) for distinguishing between sedentary and migratory robins during the wintering period in 80 81 southern Iberia, we took advantage of the predicted geographic variation in the hydrogen stable isotope signals of robin feathers ($\delta^2 H_f$; Catry *et al.*, 2016). We first characterized 82 isotopically the sedentary robin population of research interest in southern Iberia, as well as 83 84 one migratory population in northern Iberia. We then made predictions on how the $\delta^2 H_f$ of

wintering robins, classified as migratory or sedentary by the MCFs, should vary in relation to
the values of these two breeding populations of known migratory behaviour if the MCFs

87 worked well (see premises 1 and 2 below).

We determined the $\delta^2 H_f$ signature of the sedentary population occurring in the Campo de Gibraltar (Cádiz, South Spain) by sampling one tail feather (one rectrix number 5; Jenni & Winkler, 1994) from robins captured during August 2006 (after moulting period) and May 2014 (before moulting period). Robins were trapped in two woodland sites (36°09'48"N, 5°34'56"W and 36°09'54"N, 5°34'55"W) located in 'Los Alcornocales' Natural Park. In parallel, we also sampled feathers from robins breeding in Álava (Northern Spain 42°54'02"N, 2°32'07"W), where the robin population is considered migratory (De la Hera *et al.*, 2014).

96 On the other hand, wintering robins in Gibraltar were trapped between mid-November and mid-February during two different winters (2006-07 and 2013-14). Winter sampling took 97 place in the two abovementioned woodland localities, as well as in two nearby shrubland 98 areas (36°09'03"N, 5°37'54"W and 36°05'11"N, 5°42'09"W) that host robins only during the 99 winter period (Tellería et al., 2001). Each trapped robin was aged as adult or juvenile using 100 plumage characteristics (Jenni & Winkler, 1994). We also recorded the eighth primary (P8) 101 102 length (being P1 the innermost primary) and the so-called wing formula (Svensson, 1992): the primary distances of the 9 longest primaries (excluding the vestigial outermost primary: 103 104 P10). 'Primary distance' was defined as the distance from the tip of each primary to the tip of 105 the longest primary with the wing folded, with a value of zero for the primary (or primaries) constituting the wingtip. All morphological measurements were taken by two standardized 106 107 ringers in 2006-07 (IdH, JP-T) and only by one of these in 2013-14 (IdH). Additionally, we 108 used a syringe to extract a sample of blood from the jugular vein that was used for molecular sexing (Griffiths et al., 1998), and collected one rectrix number 5. Note that feathers of both 109 breeding and wintering sampled birds had grown in the same season: the previous summer 110 (either 2006 or 2013), providing thus comparable feather samples with their corresponding 111 112 winter. 113 Feather samples were sent to the Colorado Plateau Stable Isotope Laboratory (http://www.isotope.nau.edu/), where their hydrogen isotopic ratios were measured by 114 coupled pyrolysis/isotope-ratio mass spectrometry. $\delta^2 H_f$ values were expressed in units per 115

mil (‰), and normalized according to the VSMOW-SLAP scale using the values obtained for three keratin standards (Keratin-SC Lot SJ, Caribou hoof and Kudo horn). The $\delta^2 H_f$ values of 20 individuals were measured a second time to estimate analytical repeatability (Lessells & Boag, 1987), which was highly significant (r_i = 0.98; F_{19,20} = 130.9; P < 0.001) supporting the

120 reliability of obtained $\delta^2 H_f$ measurements.

121 We used the same DFA that gave rise to the MCFs detailed in Pérez-Tris *et al.* (2000) 122 to classify as either migratory or sedentary the 149 robins captured during winters 2006-07 123 and 2013-14. From this DFA, we obtained, for each wintering individual, the probability of 124 being migratory ($Pc_{mig.}$) or sedentary ($Pc_{sed.}$) according to its morphology (StatSoft, 2004). 125 The sum of $Pc_{mig.}$ plus $Pc_{sed.}$ equals 1, so that the migratory behaviour assigned to each 126 particular robin will be that for which the Pc is higher, which accurately matches with the

127 outcome of MCFs assignations (Pérez-Tris *et al.*, 2000). We then tested the reliability of

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these MCFs by comparing the $\delta^2 H_f$ values of the wintering robins assigned as migratory 128 $(Pc_{mig} > 0.5)$ or sedentary $(Pc_{mig} < 0.5)$ with the $\delta^2 H_f$ values of robins captured in Álava 129 130 (migratory) and Gibraltar (sedentary) during the breeding period. Given the lack of homogeneity of variances between the four groups of birds, we used Welch t-tests with 131 132 separate variance estimates to make comparisons among them (Fig. 1). We predicted that a good performance of the MCFs will be supported by the fulfilment of two premises: 1) 133 wintering robins assigned as sedentary by the MCFs and breeders captured in Gibraltar would 134 have similar $\delta^2 H_f$ scores; and 2) the $\delta^2 H_f$ values of wintering robins assigned as migratory 135 should be at least similar to Álava breeders or smaller (reflecting a more Northern origin; 136 Hobson et al., 2004). This last assumption is based on the observation that most of the 137 138 migratory robins wintering in Gibraltar should come from farther North-Northeast than Álava 139 (Bueno, 1998; Korner-Nievergelt, et al. 2014), since the breeding densities of the species south of Álava are relatively low compared to North and Central European migratory 140 141 populations, and sedentariness is to be expected in many Iberian populations (Purroy, 2003; Tellería, 2012). 142 $\delta^2 H_f$ values greatly varied among the four groups of robins compared, (Fig. 1). Thus, 143

robins captured in Gibraltar during the breeding period showed higher $\delta^2 H_f$ scores than 144 conspecifics captured in Álava ($t_{40} = -8.64$, P < 0.001; Fig. 1), with their ranges of values 145 overlapping only very marginally ($\delta^2 H_f$ range for Gibraltar: [-40.4, -16.3]; $\delta^2 H_f$ range for 146 147 Álava: [-82.5, -40.3]). Out of the 149 wintering robins captured in Gibraltar region, 53 were assigned as migratory and 96 as sedentary by the MCFs. Wintering robins assigned as 148 migratory by the MCFs showed the most negative $\delta^2 H_f$ scores of the four groups for 149 comparison (Fig. 1). These values were significantly lower than those displayed by the 150 wintering robins assigned as sedentary ($t_{147} = -2.76$, P = 0.006), Gibraltar breeders ($t_{75} =$ 151 152 10.26, P < 0.001) or Álava breeders (t₆₉ = 2.54, P = 0.013; Fig. 1). However, birds classified as sedentary by the MCFs also differed markedly in their $\delta^2 H_f$ values from the local birds 153 captured during summer in Gibraltar ($t_{118} = 5.42$, P < 0.001), contrary to what would be 154 expected if the MCFs were operating correctly. In contrast, their $\delta^2 H_f$ values were similar to 155 156 the ones displayed by the robins breeding in Álava ($t_{112} = 0.15$, P = 0.877; Fig. 1). Given the marginal overlap in the $\delta^2 H_f$ values between robins breeding in Álava and 157 Gibraltar, we decided to use -40‰ as an arbitrary $\delta^2 H_f$ threshold to separate sedentary from 158 migratory robins in our study site during winter and to analyse in further detail the 159

160 performance of the MCFs. This -40% threshold should tell apart most of the sedentary

population in our study site (mean \pm SD for Gibraltar breeders, -27.59 \pm 6.62, N = 24), but it

is likely to assign erroneously to the sedentary group some North and Central Iberian
migrants that would show similar or less negative values than Alava breeders (mean ± SD for
Álava breeders, -53.75 ± 12.75, N = 18). According to the probability density functions of
Álava and Gibraltar breeders, we would expect that 14 of the wintering robins with less
negative values than -40‰ were actually migrants. Accordingly, the analyses shown below
should be taken with caution..

Using abovementioned criteria, we estimated that the rate of correct classifications of 168 the MCFs was 92% for sedentary birds (3 erroneous assignations out of 38 birds with $\delta^2 H_f$ >-169 170 40%; see right quadrant in Fig. 2) and a 45% for migrants (61 errors out of 111 birds with $\delta^2 H_f < 40\%$; left quadrant in Fig. 2), with significant differences in the error rate between 171 populations (Chi-squared: $\chi^2_1 = 25.6$, P < 0.001). Thus, the MCFs worked better than random 172 detecting sedentary birds (Chi-squared: $\chi^2_1 = 26.95$, P < 0.001), but did not perform 173 differently from chance for migrants (Chi-squared: χ^2_1 = 1.09, P = 0.296). Our data also 174 175 revealed clear age and sex-related biases in the distribution of the MCFs errors. The three 176 sedentary birds classified erroneously as migrants were all males (two adults and one juvenile; Fig. 2), while the 61 migrants incorrectly assigned to the sedentary group were all 177 females or juveniles (only 7 males within the 40 errors made on juveniles, and none of the 17 178 migratory adult males was misclassified; see Fig. 2). Among the migrants wrongly assigned 179 as sedentary (n = 61) errors were not homogeneously distributed between sex and age 180 categories (Chi-squared: $\chi^2_3 = 17.11$, P < 0.001). 181 Our results showed that the mean $\delta^2 H_f$ values of wintering robins assigned as 182

sedentary by the MCFs were lower than those shown by Gibraltar breeders (Fig. 1), which 183 184 refuted one of the main assumptions that supported the validity of these MCFs. In general, 185 this classification method worked well to identify sedentary robins (92% of correct 186 assignations), but its performance was virtually random on migrants (55% of them were 187 incorrectly classified as sedentary). MCFs are based on the existing differences in wing size and shape between migratory and sedentary robins (Pérez-Tris et al., 2000), but both 188 populations show marked sex and age-related variation in these characteristics that caused a 189 190 relatively large morphological overlap between populations. This situation was further aggravated by the fact that the migratory population occurring during winter in 191 Campo de Gibraltar region is overrepresented by juveniles (Chi-squared: $\chi^2_1 = 6.19$, P = 192 0.013) and females (Chi-squared: $\chi^2_1 = 5.23$, P = 0.022) when compared to the sedentary 193 fraction, and these two population groups have more chances of being misclassified as 194 195 sedentary according to their wing morphology (Fig. 2). Such circumstance, in combination

with a potential bias in the representation of the different age and sex categories in the sample
of Iberian breeders used to develop the MCFs, might have led to an unrealistic 80% of correct
assignations (Pérez-Tris *et al.*, 2000) that is effectively less when applied to the wintering
population. New MCFs that incorporated the sex of the individuals in their construction –
something initially overlooked in the development of the original MCFs– would significantly
increase their ability to distinguish migratory and sedentary robins in their sympatric
wintering grounds.

203 Supporting this idea, updated MCFs obtained from a new DFA that considered the sex 204 of the individuals and was developed using the -supposedly- 111 migratory and 38 sedentary robins from our winter sample (Table 1), assigned correctly over 93% of individuals to their 205 respective groups (Wilks' Lambda: 0.39; $F_{11,137} = 19.7$, P < 0.001). This result supports the 206 207 view that morphological characterisation can still be a useful tool for discriminating between migratory and sedentary robins, under the condition that individuals need to be sexed first. 208 209 However, we discourage a broad use of these newly proposed MCFs before their 210 performance is properly tested using independent samples. Likewise, we acknowledge that our MCFs are based on the study of only two breeding populations, so that a more extensive 211 sampling of Iberian Robins would be necessary to extrapolate the classification method to 212 other wintering areas of Robin sympatric occurrence and to have a better characterization of 213

the isotopic signals of Iberian migrants.

215 Our study is another good example of the potential problems that researchers can find when applying morphology-based differentiation methods on populations different from the 216 ones used to develop the technique (Ellrich et al., 2010). Isotopic signatures revealed that the 217 218 MCFs available to distinguish migratory and sedentary robins in sympatric wintering grounds of southern Iberia did not work properly, but overestimated the number of local sedentary 219 220 birds. This suggests that previously described between-habitat patterns of sedentary robins in 221 Gibraltar region might be biased by the misclassification of many migratory females and juveniles as sedentary, providing a misleading picture of how these birds are spatially 222 distributed during winter in southern Iberia (Pérez-Tris *et al.*, 2000). Values of $\delta^2 H_f$ seem to 223 224 be a more reliable method than morphology to assess the migratory behaviour of robins 225 (although this is not the case in other species; De la Hera et al., 2012), and could be used to re-assess whether sedentary robins are really outcompeted from woodlands during winter by 226 arriving migratory counterparts (as MCFs initially suggested; Tellería et al., 2001; Tellería & 227 Pérez-Tris, 2004) or, alternatively, they are able to remain in their breeding habitats year-228 229 round as it is the case for other species (i.e. Blackcaps; Pérez-Tris & Tellería, 2002). This is

230	an important question in areas where individuals with different migratory behaviour occur in
231	sympatry during winter, since it can help us to assess the vulnerability of some of these
232	wintering populations that are currently facing a drastic decline as a consequence of global
233	warming and other anthropogenic alterations (Herrero & Zavala, 2015; Tellería, 2015).
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336 116.

337 Table 1. Classification functions obtained from a Discriminant Function Analysis that

considered the 149 wintering robins whose migratory behaviour was estimated using $\delta^2 H_f$

339 values. New individuals will be assigned to the group (migratory or sedentary) for which the

340 corresponding function provides the highest value. For each individual, equations are solved

341 by adding the value of the constant to the sum of products of each coefficient multiplied by

342 its morphological trait. Males and females were coded as 1 and 2, respectively.

343 Tabla 1. Funciones de clasificación obtenidas a partir de un Análisis de Funciones

344 Discriminantes que consideró 149 petirrojos invernantes cuyo comportamiento migratorio

345 fue estimado empleando valores de $\delta^2 H_{f.}$ Los nuevos individuos serán asignados al grupo

346 (migratorio o sedentario) para el que su función correspondiente proporciona el valor más

347 alto. Para cada individuo, las ecuaciones se resuelven sumando los valores de la constante a

348 la suma de los productos de cada coeficiente multiplicado por el valor correspondiente del

349 rasgo morfológico. Machos y hembras fueron codificados como 1 y 2, respectivamente.

350

	Migratory	Sedentary
Constant	-1036.74	-939.15
Sex	89.11	83.86
P8 length	35.30	33.58
Primary distance to P9	-4.14	-3.36
Primary distance to P8	21.88	21.63
Primary distance to P7	3.85	4.05
Primary distance to P6	4.90	7.83
Primary distance to P5	-5.87	-6.02
Primary distance to P4	13.72	11.72
Primary distance to P3	-4.57	-5.02
Primary distance to P2	-4.93	-5.05
Primary distance to P1	2.41	3.21

351

352

354 Figure legends.

356	Figure 1. Variation in $\delta^2 H_f$ values between robins captured during breeding in Álava and
357	Gibraltar that are known to be migratory and sedentary (left quadrant), respectively; and
358	values for the wintering robins assigned as migratory $(Pc_{mig} > 0.5)$ or sedentary $(Pc_{mig} < 0.5)$
359	by their morphology (right quadrant). Graph shows medians (black dots), percentiles 25-75
360	(boxes) and percentiles 1-99 (whiskers).
361	Figura 1. Variación en los valores de $\delta^2 H_f$ entre petirrojos capturados durante la
362	reproducción en Álava y Gibraltar para los que se sabe que son migratorios y sedentarios
363	(cuadrante izquierdo), respectivamente; y valores para los petirrojos invernantes asignados
364	como migratorios ($Pc_{mig} > 0.5$) y sedentarios ($Pc_{mig} < 0.5$) a partir de su morfología
365	(cuadrante derecho). La gráfica muestra medianas (puntos negros), percentiles 25-75
366	(rectángulos) y percentiles 1-99 (segmento de líneas).
367	
368	Figure 2. Variation in the posterior classification probabilities of being migratory (Pcmig.)
369	between different age and sex categories of wintering robins assigned as migratory or
370	sedentary according to their $\delta^2 H_f$ values. Individuals above the dashed line (Pc _{mig} > 0.5) were
371	assigned as migratory by the MCFs, while individuals below it (Pc_{mig} < 0.5) were classified as
372	sedentary.
373	Figura 2. Variación en las probabilidades posteriores de clasificación de ser migratorio
374	(Pc _{mig.}) entre diferentes categorías de edad y sexo de los petirrojos invernantes asignados
375	como migratorios o sedentarios de acuerdo a sus valores de $\delta^2 H_f$. Los individuos sobre la
376	línea discontinua ($Pc_{mig.} > 0.5$) fueron asignados como migradores por las MCFs, mientras

que los individuos bajo esa misma línea ($Pc_{mig.}$ < 0.5*) fueron clasificados como sedentarios.*



De la Hera et al. Figure 1. $\delta^2 H_f$ values and the migratory behaviour of robins.



De la Hera et al. Figure 2. $\delta^2 H_f$ values and the migratory behaviour of robins.