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, population or cryptic species distinction) need to be externally validated to ensure a reliable performance across situations. In this study, we used hydrogen stable isotope ratios of feathers (δ^2H_f) to test the validity of morphological classification functions (MCFs) previously designed to assess the migratory behaviour of European Robins *Erithacus rubecula* wintering in southern Iberia. Our results showed that a great number of migrants (mostly females and juveniles) were erroneously assigned as sedentary, which could compromise the reliability of previous ecological studies that made use of these MCFs. The development of improved MCFs or the use of alternative differentiation methods (δ^2H_f) could help us to gain a more realistic insight into the habitat distribution and ecological interactions of sympatric migratory and sedentary robins overwintering in southern Iberia.

RESUMEN.- Los métodos morfológicos para distinguir grupos de aves con interés de investigación (*e.g.* distinción de sexos, poblaciones o especies crípticas) requieren de validación independiente para asegurar su funcionamiento adecuado de forma consistente. En este estudio, usamos la relación de isótopos estables del hidrógeno en las plumas (δ^2H_f) para comprobar la validez de las funciones de clasificación morfológicas (MCFs) diseñadas con anterioridad para identificar el comportamiento migratorio de los petirrojos *Erithacus rubecula* invernantes en el sur ibérico. Los resultados revelaron que un gran número de migrantes (sobre todo hembras y jóvenes) fueron clasificados erróneamente como 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 diferenciación alternativos (δ^2H_f) podrían ayudarnos a obtener una idea más realista acerca de la distribución entre hábitats e interacciones ecológicas de los petirrojos migratorios y sedentarios que invernan en simpatría en el sur ibérico.

Discriminant function analyses (DFA) -and other similar statistical approaches- based on avian morphological traits are a readily accessible method to separate morphologically 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- species (Arizaga *et al*., 2008; Bertolero *et al*., 2016). With mixed success, they have also 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 traits (Wennerberg *et al*., 2002; Maggini *et al*., 2016). In many cases, morphology is now 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; Bensch *et al*., 2002), while methods based on bird morphology are normally subject to 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 alternative techniques do not substantially improve the classification potential of 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 potential uncertainty associated with the use of morphology, it is essential to validate the reliability of morphological methods using independent approaches, which can be very useful to reveal previously unnoticed flaws in their performance.

The study of the sympatric interactions between local sedentary birds and overwintering conspecific migrants in southern Iberia has greatly benefitted from the use of morphological classification functions (MCFs) that are one of the outcomes of DFA (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 variation is sometimes large enough for developing effective MCFs to distinguish each other. 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 Eurasian Blackcap (*Sylvia atricapilla*) and European Robin (*Erithacus rubecula*), 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 constitute only a small fraction of the wintering population occurring in Southern Iberia. Consequently, whether these MCFs can successfully be applied to distinguish among wintering birds of unknown origin needs to be explicitly corroborated (Ellrich *et al*., 2010).

Most of the migratory blackcaps and robins wintering in Iberia originate from further Northeast in Europe, so they would have a more migratory-like morphology than any Iberian 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 sympatric populations wintering in Southern Iberia. This has been confirmed for blackcaps (De la Hera *et al*., 2007) and validated using a well-known pattern of stable isotope variation for the Palaearctic region (De la Hera *et al*., 2012). However, the validity of the MCFs designed for distinguishing between migratory and sedentary robins remains to be tested using an independent control. Unlike blackcaps, robins show a great within-population variation in wing morphology, with male and adult robins having on average longer wings than females and juveniles, respectively (Ellrich *et al*., 2010; De la Hera *et al*., 2014). In this 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, 1990) and hence more likely to reach southern Iberia for overwintering, where their short wings might overlap in size with those of local sedentary robins, particularly with males (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-Tris *et al*., 2000), which raises the possibility that the error rate would change if testing a wintering population with a different population composition.

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 southern Iberia, we took advantage of the predicted geographic variation in the hydrogen stable isotope signals of robin feathers (δ^2H_f; Catry *et al*., 2016). We first characterized isotopically the sedentary robin population of research interest in southern Iberia, as well as one migratory population in northern Iberia. We then made predictions on how the δ^2H_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 worked well (see premises 1 and 2 below).

We determined the δ^2H_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).

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 place in the two abovementioned woodland localities, as well as in two nearby shrubland areas (36°09'03"N, 5°37'54"W and 36°05'11"N, 5°42'09"W) that host robins only during the winter period (Tellería *et al*., 2001). Each trapped robin was aged as adult or juvenile using plumage characteristics (Jenni & Winkler, 1994). We also recorded the eighth primary (P8) 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: P10). 'Primary distance' was defined as the distance from the tip of each primary to the tip of 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 ringers in 2006-07 (IdH, JP-T) and only by one of these in 2013-14 (IdH). Additionally, we 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 breeding and wintering sampled birds had grown in the same season: the previous summer (either 2006 or 2013), providing thus comparable feather samples with their corresponding winter.

Feather samples were sent to the Colorado Plateau Stable Isotope Laboratory (http://www.isotope.nau.edu/), where their hydrogen isotopic ratios were measured by coupled pyrolysis/isotope-ratio mass spectrometry. δ^2H_f values were expressed in units per 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 δ^2H_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 reliability of obtained δ^2H_f measurements.

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

these MCFs by comparing the δ^2H_f values of the wintering robins assigned as migratory ($Pc_{mig.} > 0.5$) or sedentary ($Pc_{mig.} < 0.5$) with the δ^2H_f values of robins captured in Álava (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 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) wintering robins assigned as sedentary by the MCFs and breeders captured in Gibraltar would have similar δ^2H_f scores; and 2) the δ^2H_f values of wintering robins assigned as migratory should be at least similar to Álava breeders or smaller (reflecting a more Northern origin; Hobson *et al*., 2004). This last assumption is based on the observation that most of the migratory robins wintering in Gibraltar should come from farther North-Northeast than Álava (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 populations, and sedentariness is to be expected in many Iberian populations (Purroy, 2003; Tellería, 2012).

δ^2H_f values greatly varied among the four groups of robins compared , (Fig. 1). Thus, robins captured in Gibraltar during the breeding period showed higher δ^2H_f scores than conspecifics captured in Álava ($t_{40} = -8.64$, $P < 0.001$; Fig. 1), with their ranges of values overlapping only very marginally (δ^2H_f range for Gibraltar: [-40.4, -16.3]; δ^2H_f range for 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 migratory by the MCFs showed the most negative δ^2H_f scores of the four groups for comparison (Fig. 1). These values were significantly lower than those displayed by the wintering robins assigned as sedentary ($t_{147} = -2.76$, $P = 0.006$), Gibraltar breeders ($t_{75} = 10.26$, $P < 0.001$) or Álava breeders ($t_{69} = 2.54$, $P = 0.013$; Fig. 1). However, birds classified as sedentary by the MCFs also differed markedly in their δ^2H_f values from the local birds captured during summer in Gibraltar ($t_{118} = 5.42$, $P < 0.001$), contrary to what would be expected if the MCFs were operating correctly. In contrast, their δ^2H_f values were similar to the ones displayed by the robins breeding in Álava ($t_{112} = 0.15$, $P = 0.877$; Fig. 1).

Given the marginal overlap in the δ^2H_f values between robins breeding in Álava and Gibraltar, we decided to use -40‰ as an arbitrary δ^2H_f threshold to separate sedentary from migratory robins in our study site during winter and to analyse in further detail the performance of the MCFs. This -40‰ threshold should tell apart most of the sedentary population in our study site (mean ± SD for Gibraltar breeders, -27.59 ± 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 the MCFs was 92% for sedentary birds (3 erroneous assignations out of 38 birds with δ^2H_f >-40‰; see right quadrant in Fig. 2) and a 45% for migrants (61 errors out of 111 birds with δ^2H_f <-40‰; left quadrant in Fig. 2), with significant differences in the error rate between populations (Chi-squared: $\chi^2_1 = 25.6$, $P < 0.001$). Thus, the MCFs worked better than random detecting sedentary birds (Chi-squared: $\chi^2_1 = 26.95$, $P < 0.001$), but did not perform differently from chance for migrants (Chi-squared: $\chi^2_1 = 1.09$, $P = 0.296$). Our data also revealed clear age and sex-related biases in the distribution of the MCFs errors. The three 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 females or juveniles (only 7 males within the 40 errors made on juveniles, and none of the 17 migratory adult males was misclassified; see Fig. 2). Among the migrants wrongly assigned as sedentary (n = 61) errors were not homogeneously distributed between sex and age categories (Chi-squared: $\chi^2_3 = 17.11$, $P < 0.001$).

Our results showed that the mean δ^2H_f values of wintering robins assigned as sedentary by the MCFs were lower than those shown by Gibraltar breeders (Fig. 1), which refuted one of the main assumptions that supported the validity of these MCFs. In general, this classification method worked well to identify sedentary robins (92% of correct assignations), but its performance was virtually random on migrants (55% of them were 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 populations show marked sex and age-related variation in these characteristics that caused a relatively large morphological overlap between populations. This situation was further aggravated by the fact that the migratory population occurring during winter in Campo de Gibraltar region is overrepresented by juveniles (Chi-squared: $\chi^2_1 = 6.19$, $P = 0.013$) and females (Chi-squared: $\chi^2_1 = 5.23$, $P = 0.022$) when compared to the sedentary fraction, and these two population groups have more chances of being misclassified as 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.

Supporting this idea, updated MCFs obtained from a new DFA that considered the sex 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 respective groups (Wilks' Lambda: 0.39; $F_{11,137} = 19.7$, $P < 0.001$). This result supports the 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. However, we discourage a broad use of these newly proposed MCFs before their 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 sampling of Iberian Robins would be necessary to extrapolate the classification method to other wintering areas of Robin sympatric occurrence and to have a better characterization of the isotopic signals of Iberian migrants.

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 ones used to develop the technique (Ellrich *et al*., 2010). Isotopic signatures revealed that the 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 birds. This suggests that previously described between-habitat patterns of sedentary robins in 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 distributed during winter in southern Iberia (Pérez-Tris *et al*., 2000). Values of δ^2H_f seem to be a more reliable method than morphology to assess the migratory behaviour of robins (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 arriving migratory counterparts (as MCFs initially suggested; Tellería *et al*., 2001; Tellería & Pérez-Tris, 2004) or, alternatively, they are able to remain in their breeding habitats year-round as it is the case for other species (i.e. Blackcaps; Pérez-Tris & Tellería, 2002). This is

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

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Table 1. Classification functions obtained from a Discriminant Function Analysis that considered the 149 wintering robins whose migratory behaviour was estimated using δ^2H_f values. New individuals will be assigned to the group (migratory or sedentary) for which the corresponding function provides the highest value. For each individual, equations are solved by adding the value of the constant to the sum of products of each coefficient multiplied by its morphological trait. Males and females were coded as 1 and 2, respectively.

Tabla 1. Funciones de clasificación obtenidas a partir de un Análisis de Funciones Discriminantes que consideró 149 petirrojos invernantes cuyo comportamiento migratorio fue estimado empleando valores de δ^2H_f. Los nuevos individuos serán asignados al grupo (migratorio o sedentario) para el que su función correspondiente proporciona el valor más alto. Para cada individuo, las ecuaciones se resuelven sumando los valores de la constante a la suma de los productos de cada coeficiente multiplicado por el valor correspondiente del rasgo morfológico. Machos y hembras fueron codificados como 1 y 2, respectivamente.

	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

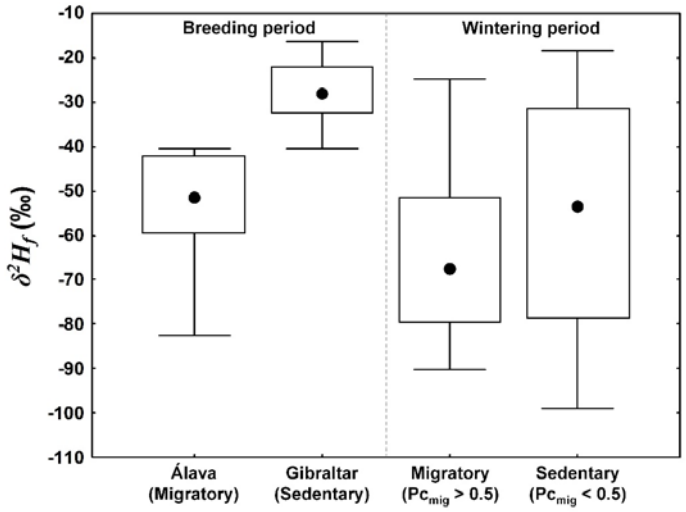
Figure legends.

Figure 1. Variation in δ^2H_f values between robins captured during breeding in Álava and Gibraltar that are known to be migratory and sedentary (left quadrant), respectively; and values for the wintering robins assigned as migratory ($Pc_{mig} > 0.5$) or sedentary ($Pc_{mig} < 0.5$) by their morphology (right quadrant). Graph shows medians (black dots), percentiles 25-75 (boxes) and percentiles 1-99 (whiskers).

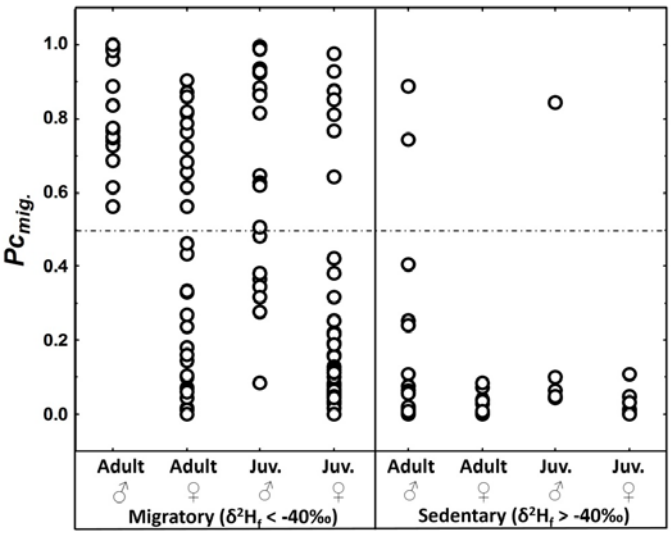
Figura 1. Variación en los valores de δ^2H_f entre petirrojos capturados durante la reproducción en Álava y Gibraltar para los que se sabe que son migratorios y sedentarios (cuadrante izquierdo), respectivamente; y valores para los petirrojos invernantes asignados como migratorios ($Pc_{mig} > 0.5$) y sedentarios ($Pc_{mig} < 0.5$) a partir de su morfología (cuadrante derecho). La gráfica muestra medianas (puntos negros), percentiles 25-75 (rectángulos) y percentiles 1-99 (segmento de líneas).

Figure 2. Variation in the posterior classification probabilities of being migratory ($Pc_{mig.}$) between different age and sex categories of wintering robins assigned as migratory or sedentary according to their δ^2H_f values. Individuals above the dashed line ($Pc_{mig.} > 0.5$) were assigned as migratory by the MCFs, while individuals below it ($Pc_{mig.} < 0.5$) were classified as sedentary.

Figura 2. Variación en las probabilidades posteriores de clasificación de ser migratorio ($Pc_{mig.}$) entre diferentes categorías de edad y sexo de los petirrojos invernantes asignados como migratorios o sedentarios de acuerdo a sus valores de δ^2H_f. Los individuos sobre la 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. δ^2H_f values and the migratory behaviour of robins.



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384 De la Hera et al. Figure 2. δ^2H_f values and the migratory behaviour of robins.

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