1   **Short communication:**

2   **Individual identification of endangered species using mosquito blood meals: a**

3   **proof-of-concept study in Iberian lynx**

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Abstract

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17   Host identification from mosquito blood meals has been routinely used to

18 identify the feeding preferences of insects in studies on transmission of vector borne

19 pathogens. Here, we identified for the first time the susceptibility of the endangered

20 Iberian lynx (*Lynx pardinus*) to the attack of a wild mosquito female, the mosquito

21 *Anopheles atroparvus*. Furthermore, we used eleven microsatellite markers to test for

22 the utility of vertebrate DNA isolated from insect blood meals for individual

23 identification of wildlife. Only the three smallest markers were successfully amplified,

24 however this genotype did not match with any of the previously genotyped individuals

25 in southern Spain. These results support the use of DNA from mosquito blood meals as

26 a non-invasive source of DNA and a powerful tool on epidemiological and conservation

27 biology studies. However, as may be the case of other non-invasive sampling methods,

28 the utility of this technique is probably limited by the quantity and quality of vertebrate

29 DNA.

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31 **Key-words:** *Anopheles atroparvus*; diseases; *Lynx pardinus*; non-invasive blood

32 sampling, parasites

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Introduction

Blood meals have been routinely used to identify the feeding preferences of mosquitoes in studies on pathogen transmission (i.e. Kilpatrick et al. 2006, Muñoz et al. 2012). Recently, it has been proposed as a powerful non-invasive source of genetic material for the study of unconspicuous species, species inventories, infectious agent distribution and population dynamics (Calvignac-Spencer et al. 2013a).

In addition to inter-specific differences in the susceptibility to insect attacks, individuals may also differ in their exposure to insect bites (Kelly 2001), affecting the evolution of host-pathogen interactions and epidemiology. Contrary to the case of mosquitoes feeding on humans, individual identifications of wild animals or livestock from mosquito blood meals are scarce especially for insects caught in pristine areas (Calvignac-Spencer et al. 2013a). Microsatellites, as individual DNA markers, could be used to identify hosts at the individual level as they may be easily amplified even from degraded blood meals due to their small length (Mukabana et al. 2002). Using this procedure under semi-controlled conditions, the individual hosts of mosquitoes captured in nest-boxes containing *Sialia sialis* were identified (Ligon *et al.* 2009, Burkett-Cadena *et al.* 2010). Also, Torr et al. (2001) identified the individual hosts of tsetse flies parasitizing cattle. However, its utility in the wild may be limited because the microsatellite loci from the host population under study needs to be characterized (Kent 2009).

Here, we used a molecular approach to identify, at the individual level, the origin of a mosquito blood meal derived from an Iberian lynx (*Lynx pardinus*). The Iberian lynx is one of the world's most endangered cat species with a decreasing population trend (Palomares et al. 2012). The 2012 census estimated a total of 84 and

186 individuals (25 and 52 territorial females) in the two main populations, Doñana and

Sierra Morena, separated by ca. 240 km

(http://www.lifelince.org/public/Informe_Censo_2012.pdf). A large fraction of the

Iberian lynx population has been characterized using microsatellite markers to evaluate

their genetic status (Casas-Marce et al. 2013), and as part of the ongoing monitoring of

this species.

Methods

As a part of an extensive study on the host-feeding pattern of mosquitoes from

southern Spain (Muñoz et al. 2012, Martínez-de la Puente et al. 2013), we collected

blood fed female mosquitoes resting at the Cañada de los Pájaros (Seville, Spain;

6°14'W, 36°57'N). Standard protocol used includes morphological identification of

mosquitoes using available keys (Brunhes et al. 2000, Schaffner et al. 2001) and DNA

isolation using the QIAGEN DNeasy Blood and Tissue® kit from the abdomen of

engorged mosquitoes. Vertebrate hosts were identified using a nested-PCR approach to

amplify a fragment of the COI gene (Alcaide et al. 2009). Positive amplifications were

sequenced in one direction and edited using the software Sequencher™ v4.9 (Gene

Codes Corporation) (see Martínez-de la Puente et al. 2013). Sequences were assigned to

vertebrate species by comparison with sequences deposited in GenBank DNA database

and/or the Barcode of Life Data System.

The only sample assigned to Iberian lynx was tested with eleven microsatellite

loci (Table 1, see Casas-Marce et al. (2013)) previously used to amplify DNA for

samples with a low amount of DNA as feces. We performed a multiplex PCR with all

fluorescently labeled markers and an independent post-PCR for each marker. All PCR

were performed containing a final concentration of 1x PCR buffer, 2mM $MgCl_2$,

82   0.25mM dNTPs, 0.01mg/mL of BSA, 0.2 μM of each primer, 0.4U of Taq polymerase

83   and 4μl of the DNA extraction or the preamplification product as template, in a total

84   volume of 20μl. PCRs were run at 92ºC for 2 min, followed for 40 cycles of 30´´at

85   92ºC, 30´´ at 55ºC, and 30´´ at 72ºC, and a final extension of 5 min at 75ºC. Products

86   were analyzed in an ABI 3130 Genetic Analyzer (Applied Biosystems). This process

87   was repeated three times independently to account for genotyping errors. Alleles were

88   scored using GENEMAPPER v3.7 (Applied Biosystems).

89   **Results**

90   After editing, a 411-bp fragment of the COI gene was obtained from an engorged

91   *Anopheles atroparvus* female captured the 5th of September of 2012 (sequence

92   deposited in Genbank: KP641612). The fragment quality was 90.8% according to

93   Sequencher™ (default conditions). Comparison of this fragment with sequences

94   deposited in public databases provided a >90% similarity with *Neofelis nebulosa* and

95   three *Lynx* species, *L. canadiensis*, *L. lynx* and *L. rufus*, all of them absent in the studied

96   area. Also, we compared our sequence with those unpublished COI sequences from *L.*

97   *pardinus* (J.A. Godoy, unpublished) showing a 100% match. *Canis lupus* (n=13),

98   *Equus caballus* (n=8), *Rattus norvegicus* (n=2), *Bos taurus* (n=1), *Turdus merula* (n=1)

99   and *Gallus gallus* (n=1) were the other hosts identified from 31 *An. atroparvus* captured

100   in the same trapping session. Host identification failed in 4 mosquitoes, probably due to

101   an advanced degree of blood meal digestion status.

102   A genotype was consistently obtained for the three replicates for the three

103   smallest markers, providing a reliable consensus genotype for these markers (sizes

104   ranging from 100 to 144 bp, Table 1). The other markers led us to inconclusive

105   genotype (sizes 148-190 bp) or did not amplify (sizes 137-242 bp). The obtained

106 consensus genotype did not match any of the previously genotyped individuals from the

107 study area and surroundings.

108 **Discussion**

109   To our knowledge this is the first record of a mosquito species feeding on the

110 Iberian lynx, although different ectoparasites, including ticks, fleas, lice and louse flies,

111 were recorded (Pérez et al. 2013). *An. atroparvus* usually feed on mammals, although

112 Iberian lynx DNA has not been previously isolated from this species in the study area

113 (Alcaide et al. 2009, Roiz et al. 2012, Martínez-de la Puente et al. 2013). The low host

114 density in addition to a differential host-susceptibility and/or an insect-preference for

115 this species may explain, at least in part, the low frequency of blood meals from the

116 Iberian lynx found in mosquitoes. *An. atroparvus* transmit pathogens causing tahyna,

117 myxomatosis, tularaemia, West Nile disease, malaria and dirofilariasis (Reusken et al.

118 2011). Further studies are necessary to identify the potential impact of mosquitoes on

119 the health status of the Iberian lynx and their potential role in the transmission of

120 infective agents.

121   Factors such as the quantity of blood meal and the degree of blood digestion

122 may affect the success of host identification (Martínez-de la Puente et al. 2013). Being

123 based in low copy number nuclear DNA, individual identification from suboptimal

124 DNA is more challenging than species identification, which is based on high copy

125 number mitochondrial DNA. Besides, shorter DNA fragments are more easily amplified

126 from blood meals than longer fragments (Mukabana et al. 2002). In agreement with this

127 expectation, only those microsatellite markers of a small size were consistently

128 amplified and yielded a reliable consensus genotype. It must also be noted that

129 individual identification by microsatellites is expected to be more difficult in mammals

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130 than in birds, because mammalian blood contains anucleated erythrocytes and,

131 therefore, a lower amount of nuclear DNA than avian derived mosquito blood meals.

132 The low quantity of nuclear DNA with respect to mitochondrial DNA in mammal blood

133 explains the high success in amplifying and sequencing a relatively large mitochondrial

134 DNA fragment but the lower success with microsatellite markers (see Table 1). The

135 design of shorter microsatellite amplicons or the use of single nucleotide

136 polymorphisms should thus increase the success rates of individual identification of

137 blood meals and other non-invasive sources of degraded DNA (Senge et al. 2011).

138   Individual host identification of mosquito blood meals may be useful for the

139 monitoring of endangered species by the identification of ranging habitats of particular

140 species and/or individuals for a particular period of time (Calvignac-Spencer et al.

141 2013b). Mosquitoes are able to digest the blood meal few days after feeding (Elizondo-

142 Quiroga et al. 2006) and disperse short distances, up to 4 km for the case of *An.*

143 *atroparvus* (Kauffman and Briegel 2004). However, as may be the case of other non-

144 invasive sampling methods, the utility of this technique is probably limited by the

145 quantity and quality of vertebrate DNA (Taberlet et al. 1999) and could be combined

146 with other non-invasive techniques (i.e. collection of feces; Palomares et al. 2002) for

147 individual identification.

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158 **References**

159 Alcaide M, Rico C, Ruiz S, Soriguer R, Muñoz J, Figuerola J (2009) Disentangling

160   Vector-borne transmission networks: A universal DNA barcoding method to

161   identify vertebrate hosts from arthropod bloodmeals. PLoS ONE 4:e7092.

162 Brunhes J, Rhaim A, Geoffroy B, Angel G, Hervy J-P (2000) Les moustiques de

163   l´Afrique mediterranéenne. IRD Editions: CD ROM PC; OpenURL.

164 Burkett-Cadena ND, Ligon RA, Liu M, Hassan HK, Hill GE, Eubanks MD, Unnasch

165   TR (2010) Vector-host interactions in avian nests: do mosquitoes prefer

166   nestlings over adults? Am J Trop Med Hyg 83:395-399.

167 Calvignac-Spencer S, Leendertz FH, Gilbert MTP, Schubert G (2013a) An invertebrate

168   stomach's view on vertebrate ecology. Bioessays 35:1004–1013.

169 Calvignac-Spencer S, Merkel K, Kutzner N, Kühl H, Boesch C, Kappeler PM, Metzger

170   S, Schubert G, Leendertz FH (2013b) Carrion fly-derived DNA as a tool for

171   comprehensive and cost-effective assessment of mammalian biodiversity.

172   Mol Ecol 22:915–924.

173 Casas-Marce M, Soriano L, López-Bao JV, Godoy JA (2013) Genetics at the verge of

174   extinction: insights from the Iberian lynx. Mol Ecol 22:5503-5515.

175   Elizondo-Quiroga A, Flores-Suarez A, Elizondo-Quiroga D, Ponce-Garcia G, Blitvich

176   BJ, Contreras-Cordero JF, Gonzalez-Rojas JI, Mercado-Hernandez R, Beaty

177   BJ, Fernandez-Salas I (2006) Gonotrophic cycle and survivorship of *Culex*

178   *quinquefasciatus* (Diptera: Culicidae) using sticky ovitraps in Monterrey,

179   northeastern Mexico. J Am Mosq Control Assoc 22:10-14.

180   Kauffman C, Briegel H (2004) Flight performance of the malaria vectors *Anopheles*

181   *gambiae* and *Anopheles atroparvus*. J Vector Ecol 29:140-153.

182   Kelly DW (2001) Why are some people bitten more than others? Trends Parasitol

183   17:578-581.

184   Kent RJ (2009) Molecular methods for arthropod bloodmeal identification and

185   applications to ecological and vector-borne disease studies. Mol Ecol Resour

186   9:4-18.

187   Kilpatrick AM, Kramer LD, Jones MJ, Marra PP, Daszak P (2006) West Nile Virus

188   epidemics in North America are driven by shifts in mosquito feeding

189   behavior. PLoS Biol 4:e82.

190   Ligon RA, Burkett-Cadena ND, Liu M, Hill GE, Hassan HK, Unnasch TR (2009)

191   Assessing mosquito feeding patterns on nestling and brooding adult birds

192   using microsatellite markers. Am J Trop Med Hyg 81:534-537.

193   Martínez-de la Puente J, Ruiz S, Soriguer R, Figuerola J (2013) Effect of blood meal

194   digestion and DNA extraction protocol on the success of blood meal source

195   determination in the malaria vector *Anopheles atroparvus*. Malaria J 12:109.

196   Mukabana WR, Takken W, Knols BGJ (2002) Analysis of arthropod bloodmeals using

197   molecular genetic markers. Trends Parasitol 18:505-509.

198   Palomares F, Godoy JA, López-Bao JV, Rodríguez A, Roques S, Casas-Marce M,

199   Revilla E, Delibes M (2012) Possible extinction vortex for a population of

200   Iberian lynx on the verge of extirpation. Conserv Biol 26:689-697.

201   Palomares F, Godoy JA, Piriz A, O'Brien SJ, Johnson WE (2002) Faecal genetic

202   analysis to determine the presence and distribution of elusive carnivores:

203   design and feasibility for the Iberian lynx. Mol Ecol 11:2171–2182.

204   Pérez JM, Sánchez I, Palma RL (2013) The dilemma of conserving parasites: the case of

205   *Felicola* (Lorisicola) *isidoroi* (Phthiraptera: Trichodectidae) and its host, the

206   endangered Iberian lynx (*Lynx pardinus*). Insect Conserv Diver 6:680-686.

207   Reusken C, De Vries A, Den Hartog W, Braks M and Scholte E-J (2011) A study of the

208   circulation of West Nile virus in mosquitoes in a potential high-risk area for

209   arbovirus circulation in the Netherlands, "De Oostvaardersplassen". Eur

210   Mosq Bull 29:66-81.

211   Roiz D, Roussel M, Munoz J, Ruiz S, Soriguer R, Figuerola J (2012) Efficacy of

212   mosquito traps for collecting potential West Nile mosquito vectors in a

213   natural mediterranean wetland. Am J Trop Med Hyg 86:642-648.

214   Schaffner E, Angel G, Geoffroy B, Hervy J-P, Rhaiem A, Brunhes J (2001) Les

215   moustiques d´Europe. CD ROM PC. IRD Editions: Logiciel d´identification

216   et d´enseignement

217   Senge T, Madea B, Junge A, Rothschild MA, Schneider PM (2011) STRs, mini STRs

218   and SNPs – A comparative study for typing degraded DNA. Legal Medicine

219   13:68-74.

220   Taberlet P, Waits LP, Luikart G (1999) Noninvasive genetic sampling: look before you

221   leap. Trends Ecol Evol 14:323-327.

222   Torr SJ, Wilson PJ, Schofield S, Mangwiro TNC, Akber S, White BN (2001)

223   Application of DNA markers to identify the individual-specific hosts of

224   tsetse feeding on cattle. Med Vet Entomol 15:78–86.

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226   **Table 1.** Microsatellite markers used in this study and genotype obtained for the

227   mosquito blood meal from the Iberian lynx.

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Marker	Expected size (bp)	Result
Fca571	100-102	100-102
BCG8 Tb	137-165	137-137
Fca082b	174-190	Inconclusive
Fca559b	226-242	No amplification
BCD8T	137-143	No amplification
Fca566b	148-156	Inconclusive
Fca115	223-239	No amplification
Fca519b	140-144	142-144
Fca453b	180-194	No amplification
Fca547b	214-226	No amplification
Fca424b	179-191	No amplification

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