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Toxic and Essential Element Concentrations in Iberian Ibex (*Capra pyrenaica*) from the Sierra Nevada Natural Park (Spain): Reference Intervals in Whole Blood

Arián Ráez-Bravo[1] · José Enrique Granados[2,3] · Francisco Javier Cano-Manuel[2,3] · Ramón C. Soriguer[4] · Paulino Fandos[5] · Jesús M. Pérez[6] · Igor Y. Pavlov[7] · Diego Romero[1]

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Abstract Iberian ibex (*Capra pyrenaica*) blood samples from the Sierra Nevada Natural Park (Spain) were analyzed to establish concentrations of toxic and essential elements. Samples (whole blood from 32 males and 34 females) were taken from wild animals and the concentrations of inorganic elements considered as (1) non-essential and toxic (Pb, Cd and As), (2) essential but potentially toxic (Cu, Zn and Mn) and (3) occasionally beneficial (B, Cr, Al and Ni), as well as (4) essential minerals (Ca, Na, K, P, Mg, S, Co and Fe), were analyzed. The low concentration of Pb and Cd indicated that there is no heavy metal contamination in this geographical area for these elements. The concentration of elements in this ibex population was defined for different genders and ages. Significant differences between genders were only found for Mg and Cu, while significant differences in concentrations of Ca, Cr, Fe, Mn, P, S and Zn were found between ages.

Keywords Iberian ibex · Blood · Spain · Elements · Metals

✉ Diego Romero
diegorom@um.es

1 Área de Toxicología, Facultad de Veterinaria, Universidad de Murcia, Campus Espinardo, 30100 Murcia, Spain

2 Parque Nacional y Parque Natural de Sierra Nevada, Ctra. Antigua de Sierra Nevada Km 7, 18071 Pinos Genil, Granada, Spain

3 Agencia de Medio Ambiente y Agua, Consejería de Agricultura, Pesca y Alimentación, Gerencia de Granada, 18013 Granada, Spain

4 Estación Biológica de Doñana, Avd. María Luisa s/n, 41013 Seville, Spain

5 Agencia de Medio Ambiente y Agua, Consejería de Agricultura, Pesca y Alimentación, Servicios Centrales, 41013 Seville, Spain

6 Dpto. Biología Animal, Biología Vegetal y Ecología, Universidad de Jaén, Campus Las Lagunillas s/n, 23071 Jaén, Spain

7 ARUP Institute for Clinical and Experimental Pathology, Department of Pathology, University of Utah School of Medicine, Salt Lake City, UT, USA

Wildlife species can be used to test or monitor the quality of the environment via analysis of concentrations of xenobiotic heavy elements (Baroni et al. 2000). In environmental studies, high concentrations of contaminants in ecosystems (such as Pb, Cd or As) can be monitored by analysing the blood of the animals that occupy these systems, thereby providing valuable information about possible risks. The occasionally beneficial elements such as B, Cr, Al and Ni can be regarded as essential in ultratrace concentrations (<1 mg kg^{-1}), although the same can be said for potentially toxic elements (Nielsen 1996; Suttle 2010). Other elements such as Cu, Zn and Mn may be toxic in some cases. High essential mineral concentrations may arise through exposure to plants and concentrates in food supplements, and the use of fertilizers in the soil. Animals have degrees of tolerance and homeostatic mechanisms to regulate excesses of elements such as Ca, Co, Mg and P. However, the reference intervals for hematic components are unknown for many species (Poppenga et al. 2012).

Information about essential minerals is very important because animals need these elements to develop their normal life processes (McDowell 2003) and reference interval information will greatly help the diagnosis of deficiencies. According to Kinkaid (1999), trace element deficiencies can cause serious health problems in animals, even before clinical signs become apparent. Thus, this knowledge will also provide information about animals' nutritional status and the presence of diseases, which is necessary for the proper management of wildlife populations (Montané et al. 2002; Pérez et al. 2003; López-Olvera et al. 2006).

The Iberian ibex (*Capra pyrenaica,* Schinz 1838) is endemic to the Iberian Peninsula and is potentially a good indicator of the quality of mountainous habitats. Over the years, numerous studies of the Iberian ibex populations in Andalusia (Spain) have been performed to establish specific conservation measures aimed at improving their health and status. Studies have been conducted aimed at characterizing the ibex population in the Sierra Nevada Natural Park (Pérez et al. 1999, 2003, 2006; Serrano et al. 2007, 2008; Alasaad et al. 2012; Lorca-Oró et al. 2012), whose main problems are genetic isolation and its specialized habitat requirements (Shackleton 1997). Other factors that hamper the conservation of this species in the Sierra Nevada include human activities that reduce and fragment habitat, poaching, the absence of predators, the abundance of other herbivores, the lack of knowledge of the species (Pérez et al. 2002; Granados et al. 2007, 2009) and environmental pollution.

To date, no studies of the concentrations of heavy metals in wild mammals in Sierra Nevada have ever been carried out, and no study has ever analysed the toxic and essential elements in whole blood from the Iberian ibex. The aim of this research was to (1) in this species to analyze 18 essential elements and elements that have been shown to be toxic, (2) assess heavy metal concentrations, (3) characterize hematologic element values and (4) establish corresponding reference intervals. This information will henceforth be highly useful for research on the conservation of this species in Andalusia.

Materials and Methods

The animals were captured in the Sierra Nevada Natural Space (36°00′–37°10′N, 2°34′–3°40′W) in a corral trap (100 × 50 m) baited with food the day before sampling. Animals were physically restrained and their eyes covered. The samples (5 mL whole blood, n = 66) were collected from clinically normal animals according to annual check-ups (32 males and 34 females) by jugular venipuncture with a syringe and transferred into heparinized tubes. Samples were stored at –20 °C until analysis.

Samples were analyzed for their content of (1) toxic (Pb, Cd, As), (2) essential but potentially toxic (Cu, Zn, Mn), (3) occasionally beneficial (B, Cr, Al, Ni) and (4) essential (Ca, Na, K, P, Mg, S, Co and Fe) elements. All chemical elements studied were analyzed by inductively coupled plasma optical emission spectrometry (ICP-OES, ICAP 6500 Duo, Thermo Scientific, with One Fast System). Prior to analysis, samples (0.1 g) were treated with trace mineral grade nitric acid (69 % Suprapure, Merck) and 33 % H_2O_2 (Suprapure, Merck) in a microwave digestion system (UltraClave-Microwave Milestone) for 20 min at 220 °C, and finally diluted to 25 mL with double deionized water (MilliQ) in Teflon reaction vessels. All concentrations were expressed as microgram per gram of wet weight of whole blood. The detection limit for most of the elements analyzed was 0.01 µg g^{-1} but was 0.1 µg g^{-1} for Ca, K, Mg, P and S. Two readings for each sample were performed and the obtained concentration values were taken as the mean of these readings. To check for possible contamination, one blank sample (reagents only or reference samples) for every 11 samples was also analysed.

The reference interval for each inorganic element was calculated with EP Evaluator® software as the central 95 % range, using either parametric methods – if the element concentrations had a Gaussian distribution – or transformed parametric methods utilizing Box-Cox transformation in other cases. Statistical analyses for each sex and age group were performed using SPSS v15.0 for Windows. Non-parametric statistical methods were used to compare groups. The Mann–Whitney Test was used to look for significant differences in concentrations of each element between males and females and between sub-adults (≤2-years old) and adults (>2-years old). The significance level for all tests was 0.05.

Results and Discussion

The reference intervals for the inorganic elements analyzed are shown in Table 1. In all the studied animals (n = 66), concentrations above the detection limit were detected for all elements except Al (n = 64), Pb (n = 30), Ni (n = 13), Cd (n = 3) and As (n = 0). The latter three elements were not included in the statistical analysis. The descriptive statistics for each sex and age are summarized in Table 2. Significant differences between males and females were found in concentrations of Cu ($p = 0.000$) and Mg ($p = 0.001$), with females having lower concentrations than males. Between age groups, significant differences were found in concentrations of Ca ($p = 0.004$), Cr ($p = 0.028$), Fe ($p = 0.034$), Mn ($p = 0.029$), P ($p = 0.001$), S ($p = 0.001$) and Zn ($p = 0.019$), with higher concentrations of Ca, P and Zn in subadult animals (≤2 years) and higher concentrations of Cr, Fe, Mn and S in adults (>2 years).

A number of authors have established reference ranges for certain hematological and biochemical parameters in the Iberian ibex in Andalusia (Pérez et al. 1999, 2003, 2006; González et al. 2002) and Catalonia (Peinado et al. 1993, 1995; Casas-Díaz et al. 2008). Inorganic elements

Table 1 Reference intervals (central 95 % interval) of inorganic elements in whole blood (μg g^{-1}) in Iberian ibex from Andalusia

	n	Method	Lower limit		Upper limit	
			Value	90 % CI	Value	90 % CI
Al	64	TP	0.04	0.00–0.08	0.77	0.69–0.85
B	66	TP	0.06	0.05–0.06	0.21	0.19–0.23
Ca	66	TP	43.29	41.97–44.66	61.43	59.55–63.37
Co	66	P	0.05	0.04–0.05	0.11	0.10–0.11
Cr	66	P	0.15	0.14–0.16	0.28	0.27–0.29
Cu	66	TP	0.38	0.34–0.43	1.33	1.19–1.49
Fe	66	P	300.00	274.79–325.20	584.14	558.94–609.35
K	66	P	837.83	785.19–890.47	1431.19	1378.55–1483.83
Mg	66	P	16.74	15.59–17.89	29.67	28.52–30.81
Mn	66	P	0.05	0.05–0.06	0.13	0.13–0.14
Na	66	TP	1759.55	1715.80–1804.41	2336.95	2278.85–2396.54
P	66	P	110.26	103.86–116.66	182.44	176.04–188.85
Pb	30	P	n/a	n/a	0.02	0.02–0.02
S	66	P	1103.98	1060.17–1147.79	1597.76	1553.96–1641.57
Zn	66	TP	1.33	1.21–1.46	3.76	3.43–4.12

CI confidence interval, *P* parametric, *TP* transformed parametric, *n/a* not available

Table 2 Descriptive statistics (mean ± SD) of inorganic elements in whole blood (μg g^{-1}) in male/female and subadult (≤2 years old)/adult (>2 years old) Iberian ibex from Andalusia

	Male		Female		Sub-adults		Adults	
	N	Mean ± SD	N	Mean ± SD	N	Mean ± SD	N	Mean ± SD
Al	30	0.38 ± 0.14	34	0.37 ± 0.21	13	0.31 ± 0.17	51	0.39 ± 0.18
B	32	0.11 ± 0.04	34	0.12 ± 0.05	13	0.11 ± 0.05	53	0.12 ± 0.04
Ca	32	50.93 ± 3.93	34	52.57 ± 5.58	13	54.99 ± 4.73	53	50.99 ± 4.63
Co	32	0.08 ± 0.01	34	0.08 ± 0.02	13	0.07 ± 0.02	53	0.08 ± 0.01
Cr	32	0.22 ± 0.02	34	0.21 ± 0.04	13	0.20 ± 0.03	53	0.22 ± 0.03
Cu	32	0.88 ± 0.28	34	0.63 ± 0.17	13	0.66 ± 0.17	53	0.77 ± 0.27
Fe	32	457.92 ± 52.56	34	427.16 ± 85.33	13	415.24 ± 60.09	53	448.65 ± 74.23
K	32	1149.71 ± 112.11	34	1120.20 ± 181.35	13	1110.32 ± 124.76	53	1140.44 ± 157.69
Mg	32	24.50 ± 3.02	34	21.99 ± 3.11	13	23.57 ± 4.87	53	23.11 ± 2.84
Mn	32	0.09 ± 0.02	34	0.10 ± 0.02	13	0.08 ± 0.02	53	0.10 ± 0.02
Na	32	2005.47 ± 115.04	34	2059.39 ± 184.92	13	2064.89 ± 152.03	53	2025.49 ± 157.70
P	32	147.28 ± 16.14	34	145.48 ± 20.53	13	161.07 ± 20.81	53	142.74 ± 16.01
Pb	12	0.01 ± 0.00	18	0.01 ± 0.01	5	0.01 ± 0.00	25	0.01 ± 0.01
S	32	1339.35 ± 111.89	34	1361.71 ± 138.73	13	1241.38 ± 128.46	53	1377.73 ± 110.88
Zn	32	2.44 ± 0.95	34	2.23 ± 0.66	13	2.62 ± 0.99	53	2.26 ± 0.76

such as Ca, K and Fe were included in some of these studies, of which some were performed on dead (Pérez et al. 2006) or anesthetized (Pérez et al. 1999) animals, or with only a small sample size (Peinado et al. 1993, 1995). Furthermore, most of these studies were performed on serum or plasma and not on whole blood as in this study. Ca, Mg and Na concentrations are much greater in serum or plasma than in erythrocytes, although these cells do have greater Fe, K, Mn and Zn concentrations. Erythrocytes in cattle have an average life of 160 days (Schalm 1980) and so the concentration of trace elements in whole blood changes more slowly than in plasma due to changes in intakes of trace minerals (Kinkaid 1999). Thus, erythrocytes reflect presumed dietary intake for a much longer period of time (Robinson et al. 1978; Rea et al. 1979), which is a key issue in wildlife management. Several further factors such as blood infestation or technical problems during extraction and conservation procedures can lead to haemolysis in wild animal blood samples. The high concentrations of many elements in erythrocytes can generate artifacts in the outcome of assays that are to date not well understood and difficult to solve in released animals (e.g. hyperpotassemia caused by stress or haemolysis). Thus, the characterization of metal concentrations in whole blood may help diagnoses in wild animals. Finally, to date there are no studies of concentrations of toxic elements in wildlife from Sierra Nevada.

Samples were collected in early November, before the mating season (Granados et al. 2001). At this time male horn growth is not rapid, but males fight other males, and they physically deteriorate due to the energy they invest in mating activities. On the other hand, females may also have deficiencies in body reserves due to the maternal transfer of trace minerals and so knowledge of normal trace element concentrations in both males and females is relevant if appropriate population management is to be conducted. Animals' ages also have a great influence on trace element absorption since juveniles grow rapidly and have greater mineral requirements. Thus, information regarding trace metal concentrations is important in juvenile Iberian ibex since malnutrition and hypocaloric diets can reduce the supply of trace elements (Forrer et al. 2001).

In our study, a sample size (66 animals) was selected that would give statistically meaningful information. According to Walton (2001), with this sample size there is a 99 % probability that 90 % of the population will be contained between the lower and upper limits of the observed values. A tolerance interval is an interval with a specified chance of covering a prescribed proportion of reference population values, and is generated simply by using the extremes of an observation set as the lower and upper limits (Mood et al. 1974). Solberg (2006) states that, to establish reference values, the animals used for sampling must be healthy and be representative of the population in terms of sex, age and physiological state. The habitat and the time when samples are collected must also be taken into account because some parameters may change during the year due to differences in diet, stress and physiological state (Sartorelli et al. 1997). Strict exhaustive controls of Iberian ibex have been carried out in El Toril natural reserve in the Sierra Nevada for many years, which makes it an ideal site for studying the reference values of toxic and essential elements in whole blood. Clinical examinations and diagnostic tests such as serum biochemistry and haematology (data not shown) in this population were normal (i.e. similar to previous years) and so, in line with several authors (e.g. Bailey et al. 1989; Klinkhoff et al. 1988; Pattinson and Theron 1989; Pérez et al. 1999, 2003; Theodossi et al. 1981), this population can be regarded as healthy. Furthermore, the coefficient of variation in the majority of essential minerals (Ca, Fe, K, Mg, Na, P and S) was below 20 %; thus, we considered that there were no deficient concentrations in any of the tested animals.

In terms of toxic elements, blood Pb concentrations were found to be close to the detection limit (0.01 $\mu g\ g^{-1}$); Cd only was detected in three samples (0.01 $\mu g\ g^{-1}$) and As in none. According to some authors, there are several possible sources of pollutants in a specific natural area: its natural geology, weather (via the uptake of metals by the soil), food availability and industrial development and other human activities such as mining (Wren et al. 1994; Hogstad 1996; Reglero et al. 2008). In Andalusia, there is little mining activity in the Protected Natural Areas and Sites of Community Importance. Although there is some mineral extraction in the Sierra Nevada Natural Park, the quarries extract marble, limestone, dolomite and gravel do not generate these pollutants in El Toril natural reserve. The public roads surrounding park are mainly secondary roads, while inside the protected area there are few roads and mainly dirt tracks, which, in combination with the prohibition on leaded petrol (Real Decreto 785/2001), could explain the low lead concentrations found in the ibex blood.

Of the essential but potentially toxic elements, Zn, Cu and Mn were detected at concentrations above 1 $\mu g\ g^{-1}$ (Zn), near to 1 (Cu) and near to 0.1 $\mu g\ g^{-1}$ (Mn). Cu and Mn have never previously been evaluated in Iberian ibex blood, although some data exists for whole blood from goats and other ruminants. Cu values were similar to those described as clinically normal in goat whole blood (Swarup et al. 2006), while the concentration of Mn was similar to that described by Kinkaid (1999) for whole blood from cattle and by Paul et al. (2005) in goat plasma. Zn is one of the most important trace elements in the body and it is involved in essential organic activities (Stefanidou et al. 2006). However, we have found no references as to the measurement of this element in the blood of Iberian ibex. The Zn concentrations found in this study (1.33–3.76 $\mu g\ g^{-1}$) were similar to those described in whole blood from goats by Swarup et al. (2006) but higher than those in goat serum (0.4–0.6 mg L^{-1}, Suttle 2010) and plasma (0.69–0.93 mg L^{-1}) (Puchala et al. 1999; Paul et al. 2005; Pechova et al. 2009). Concentrations of this element were higher in sub-adults ($p < 0.05$), a finding that is similar to the results obtained by Ahmed et al. (2001) in goat plasma. Therefore, we believe that these are reference values for this species and should be used in Iberian ibex management.

The occasionally beneficial elements analyzed (Al, B, Cr and Ni) were detected at concentrations below 0.1 $\mu g\ g^{-1}$. Although little is known about the bioavailability and metabolism of these elements in ruminants, some authors have described a B concentration in cattle plasma that is higher than that obtained in our analysis of

Table 3 Concentrations of inorganic elements in Iberian ibex (converted to mg/L)

References	Type of samples and capture method	Ca	Fe	K	Mg	Na	P
Peinado et al. (1993)	Serum Physically restrained	116.23 ± 32.06 (n = 14)	n/a	230.74 ± 50.84 (n = 14)	26.76 ± 4.86 (n = 14)	3264.37 ± 114.94 (n = 14)	105.26 ± 27.86 (n = 14)
Peinado et al. (1993)	Serum Anaesthetised	88.18 ± 8.02 (n = 18)	n/a	187.72 ± 46.93 (n = 18)	21.89 ± 9.73 (n = 18)	3425.29 ± 160.92 (n = 18)	83.59 ± 24.76 (n = 18)
Peinado et al. (1995)	Serum Physically restrained	141.48 ± 43.69 (n = 7)	n/a	246.38 ± 39.11 (n = 7)	28.69 ± 5.84 (n = 7)	3241.38 ± 68.97 (n = 7)	118.88 ± 23.83 (n = 7)
Pérez et al. (2003)	Serum Animals captured (corral traps)	106 ± 20 (n = 175)	1.612 ± 6.48 (n = 201)	70 ± 26 (n = 137)	30 ± 8 (n = 202)	1452 ± 82 (n = 137)	n/a
Pérez et al. (2006)	Serum Animals culled	107 ± 18 (n = 50)	1.855 ± 8.00 (n = 36)	154 ± 81 (n = 24)	42 ± 11 (n = 49)	1386 ± 82 (n = 24)	n/a
Casas-Díaz et al. (2008)	Serum Animals captured (drive-net)	n/a	n/a	211.18 ± 54.36 (n = 26)	n/a	3552.64 ± 196.09 (n = 24)	n/a
Casas-Díaz et al. (2008)	Serum Animals captured (box-trap)	n/a	n/a	181.85 ± 49.28 (n = 43)	n/a	3566.67 ± 171.03 (n = 44)	n/a
This study	Whole blood Animals captured (corral traps)	43.29–61.43 (n = 66)	300.00–584.14 (n = 66)	837.83–1431.19 (n = 66)	16.74–29.67 (n = 66)	1759.55–2336.95 (n = 66)	110.26–182.44 (n = 66)

n/a not available

whole blood (Fry et al. 2011). Likewise, in our study the concentrations of Al, Cr and Ni were higher than the concentrations of these elements in plasma and serum described from several other ruminant species (Paul et al. 2005; Mora et al. 2006; Haldar et al. 2009). Further investigation and monitoring of this population are still needed if we are to fully understand the requirements, essentiality and toxic concentrations of these elements.

Ca, P and Mg are the most abundant minerals in the animal body (Suttle 2010) and abnormalities in their homeostasis are common. These abnormalities are collectively referred to as disorders of mineral metabolism (Moe 2008). P concentrations (110.26–182.44 $\mu g\ g^{-1}$) were slightly higher than those reported in serum by Peinado et al. (1993, 1995) and the phosphates described by Pérez et al. (2003, 2006). However, the concentration of Ca (43.29–61.43 $\mu g\ g^{-1}$) was lower than those reported by other authors in the serum of domestic goats (Matthews 2009) and Iberian ibex (Peinado et al. 1993, 1995; Pérez et al. 2003, 2006), while the concentration of Mg was similar. The Ca:Mg ratio obtained in our study was 2:1, while Peinado et al. (1993, 1995) report a ratio of 4:1 and Pérez et al. (2003, 2006) 2.5–3.5:1. A comparison of the results between age groups reveals that the concentrations of Ca and P were higher in sub-adults than in adults ($p < 0.05$), which could be related to the high concentrations of these elements needed for growth (Liesegang et al. 2003).

Fe concentrations (300.00–584.14 $\mu g\ g^{-1}$) show that Fe is also a major element in blood. Van Miert (1985) reports a physiological value of Fe in goat serum of $24.1 \pm 1.2\ \mu mol\ L^{-1}$ ($1.35 \pm 0.07\ mg\ L^{-1}$), while Pérez et al. (2003, 2006) give values from Iberian ibex of 1.61 and 1.86 $mg\ L^{-1}$. In mammals, up to 60 % of organic Fe resides in hemoglobin (Linder 1988), which would explain the concentration of this element found in whole blood. Comparing age groups, Fe concentrations were found to be higher in adults than in sub-adults ($p < 0.05$). When we analyzed values of erythrocytes, hemoglobin, hematocrit, MCV, MCH and MCHC (data not shown), we found that hematocrit and MCV were higher in adults than in sub-adults ($p < 0.05$), which could explain the difference in Fe concentrations between age groups.

Three of the essential elements analyzed had concentrations that were near or above 1000 $\mu g\ g^{-1}$ (Na = 1759.55–2336.95, S = 1103.98–1597.76 and K = 837.83–1431.19). Previous analyses (Table 3) of the serum of Iberian ibex captured in Andalusia with the same system (Pérez et al. 2003) or of shot animals (Pérez et al. 2006) have given Na concentrations that are lower than those found in this study. However, serum concentrations of this element in Iberian ibex captured in Catalonia (Peinado et al. 1993, 1995; Casas-Díaz et al. 2008) and in other wild ruminants such as bighorn sheep *Ovis canadensis* (Borjesson et al. 2000; Poppenga et al. 2012) and southern chamois *Rupicapra pyrenaica* (López-Olvera et al. 2006) were higher than those found in our study. K concentrations were lower than Na concentrations, which is normal in the erythrocytes of ruminants such as goats (Bernstein 1954). Furthermore, the concentrations of K found in whole blood were higher than those described in serum because this is the one element that is controlled intracellularly. S is an essential element in metabolic functions related to health and livestock yield (Dias et al. 2013). Moreover, this element is also found in amino acids such as methionine, cysteine, homocysteine and taurine, which play critical roles in protein synthesis, structure and function. We found significant differences ($p < 0.05$) in S concentrations between age groups: S concentrations were found to be higher in adults (>2 years) than in sub-adults (≤2 years old). However, this element is not usually measured in plasma or serum and so we have found no literature with which to compare our results.

Finally, the assessment of Co often centres on measures of B12 status. However, Swarup et al. (2006) found Co concentrations in whole blood from goats to be slightly higher than in whole blood from Iberian ibex.

We conclude that the Iberian ibex from the Sierra Nevada Natural Space are not exposed to toxic metals such as Pb, Cd and As in their environments, and that concentrations of essential but potential toxic elements (Cu, Zn and Mn) are similar to concentrations reported in other ungulate species. The values and concentrations of occasionally beneficial and essential elements in whole blood should be considered as reference intervals for the Iberian ibex, and will be useful for the control and management of this species.

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