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Changes in blood pesticide levels in booted eagle (*Hieraaetus pennatus*) associated with agricultural land practices☆

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ABSTRACT

We estimated the degree of exposure to lindane and endosulfan in the blood of booted eagle nestlings (*Hieraaetus pennatus*) (1999–2003), in order to assess the usefulness of these samples as a unit for monitoring changes in exposure as a result of shifts in agricultural practices and the implementation of legal measures. The highest blood lindane concentrations were obtained 1 year prior to its prohibition by the European Union. Subsequent to that year, the drop in blood concentrations was dramatic. Furthermore, endosulfan blood concentrations follow a progression coinciding with an increase in olive, grape and plum-tree crops. We conclude that concentrations of organochlorine pesticides in the blood of booted eagle nestlings may be used to monitor the use of those pesticides over a particular agricultural region and alert the authorities of possible environmental or health risks.

1. Introduction

Organochlorine pesticides have been identified as one of the main groups of environmental contaminants which, as a result of human agricultural activities, have spread across all levels, both in land-based and aquatic ecosystems (Herrera et al., 1996; Van Wyk et al., 2001). Organochlorine compounds (OC) are well known as toxic and persistent contaminants that accumulate in the upper trophic levels of the food chain, due to their persistence and lipophilic properties. In recent years there has been increasing concern about these chemicals, mainly with regard to their oestrogenic properties (Gómara and González, 2006; Martínez-López et al., 2007). In order to estimate human health risks, identify food-chain contaminants, determine levels of environmental contamination and identify the adverse effects on animals associated with these compounds, in 1991, the Committee on Animals as Monitors of Environmental Hazards, from the National Research Council (NRC), put forward a series of recommendations for the use of sentinel animals in risk assessment.

According to the NRC (1991) a sentinel species should have a measurable response to the agent or class of agents in question, a territory or home range that overlaps the area to be monitored, be easily enumerated and captured, and must have a sufficient population size and density to enable enumeration.

The blood from the booted eagle (*Hieraaetus pennatus*), which nests in areas of Mediterranean forest surrounded by and interspersed with arable land, was chosen for this study. This species meets the characteristics for a sentinel species as required by the NRC (1991). Lastly, the information obtained could also be of great interest in assessing risks for endangered species of raptors.

Bird tissues are often used to assess the contamination to organochlorine insecticides. Many papers provide data on predatory birds, which are considered particularly useful for this purpose due to their trophic position in food webs. However, the natural scarcity of large predators in Spain sometimes restricts the availability of samples for analysis. Also, ethical and legal reasons make it difficult to obtain internal tissue samples from free-roaming specimens and therefore only certain types of samples, such as blood, are available for such studies. In humans, the levels of organochlorides in blood have been revealed as a valid indicator of the total body burden (Radomski et al., 1971) and that of fatty tissue (Stellman et al., 1998; Botella et al., 2004). There are very few bibliographic references regarding concentrations of these compounds in the blood of wild birds. However, high levels of

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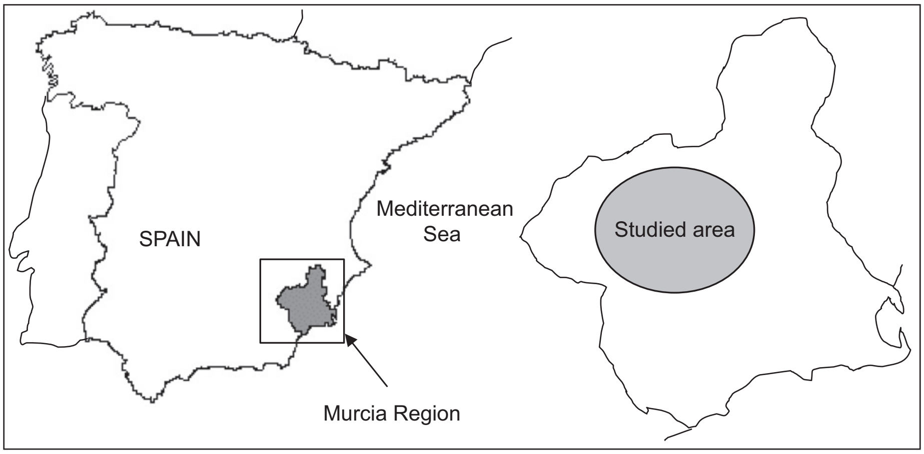

Fig. 1. Maps showing the geographical location of the Region of Murcia (SE Spain) and the areas studied.

blood residues of different organochlorines have been linked to impaired behaviour, reduced reproductive performance, poor survival, and other adverse effects in glaucous gulls (Sagerup et al., 2000; Bustnes et al., 2001, 2003, 2005; Verreault et al., 2004).

Furthermore, the measuring of organochlorides in plasma has proven to be an effective monitor for residual levels (Donaldson et al., 1999), which may even be useful for identifying exposure in migratory species, as concluded by Johnstone et al. (1996). Likewise, blood plasma is shown to be a good, non-destructive indicator, which makes it particularly valid for studying species of a delicate conservation status (Donaldson et al., 1999).

The study area has one of the highest sales rates of pesticides in Spain (10.14% of the nation-wide total) (AEPLA, 2003). Since 1999 a change in the surface area dedicated to cereal production towards other, more productive crops has occurred in this area, such as olives, grapes and plum trees, thus changing the use of pesticides. Most organochloride insecticides have been banned in Spain, endosulfan was the last organochlorine to be prohibited in Spain, the use of which was permitted until June 2007 (Decision, 2005/864/EC). Furthermore, the use of lindane for agriculture was banned by the European Union in 2000 (Decision, 2000/801/EC). Endosulfan is an insecticide and acaricide of the cyclodiene subgroup, which acts as a poison against a wide variety of insects and mites on contact. It is used primarily for a wide variety of food crops like citrus, fruit, vegetables and cereals (Barberá, 1989). Lindane, on the other hand, was used for treating the soil against plagues of insects in crops such as onion, potato, kale and cereals. It was also used for treating seeds.

The aim of this study was to assess the usefulness of blood samples from booted eagle chicks as a unit for monitoring changes in exposure as a result of shifts in agricultural practices in breeding areas and the implementation of legal measures banning the use of pesticides.

2. Material and methods

2.1. Species

The booted eagle is a medium-sized raptor species and a trans-Saharan migrant, which usually nests in trees and, more rarely on cliffs (del Hoyo et al., 1994). Although it is a common species of the forests and woodland areas of the Iberian Peninsula, several authors have shown that booted eagles select areas with a mixture of woodlands and open lands, suggesting the importance of extensive crops adjacent to the nesting woodland patches as foraging habitats for the species (Sánchez-Zapata and Calvo, 1999; Suárez et al., 2000). In terms of its conservation, one of the threats highlighted for this species is the potential accumulation of organochloride pesticides, possibly affecting reproductive success, as has been described for other migratory species (Muñoz and Blas, 2006).

2.2. Study area and sampling

This study was carried out in the northwest of the Region of Murcia which is located in southeastern Spain (Fig. 1). The study area covers about 250,000 ha and ranges from 550 to 1521 m. The climate is Mediterranean with a mean annual rainfall of 400 mm. The landscape is characterized by mountain slopes covered by pine forests (*Pinus halepensis*) interspersed with farming crops (cereals, vineyards, olive and woody crops) and far from any urban, industrial or mining zones. The nearest industrial area, urban area (population $>$350,000 h), and highway are situated 70, 60, and 70 km away, respectively. The study area has one of the highest densities of booted eagles and short-toed eagles (*Circaetus gallicus*) in Europe (Martínez, 2002) with one pair per 3–4 km^2 (Martínez et al., 2006a, b). The booted eagle population in the area was estimated at 50 breeding pairs between 1999 and 2004 (Martínez et al., 2006a; our own data).

From the 1999–2003 breeding seasons, the reproductive behaviour of the booted eagle was observed from the beginning of courtship (March–April) until nestlings left their nests (June–July). Close to the predicted hatching date, nests were visited twice weekly. The same nest was visited each year. A total of 62 nestlings were studied and blood samples (2.0 ml) were taken by puncturing the radial vein using a hypodermic needle and syringe and taken immediately to the laboratory in refrigerated conditions and frozen at $-40\,^{\circ}$C until processing. To carry out the sampling it was necessary to obtain permits from the Environmental Office of the Autonomous Regional Government of Murcia. Nestlings were sampled at between 35 and 45 days old in order to obtain sufficient blood samples without damage the nestlings' health. Careful steps were taken in order to avoid stressing the nestlings. The head of the nestlings was covered before the animal was put into a bag and brought down to the ground, where a blood sample was taken by the veterinarian from the "Santa Faz" Wildlife Recovery Centre (Alicante, Spain), who also evaluated the health status of the nestlings. All individuals were marked with a numbered steel band. Finally, nestlings were returned to the nest. These nests were monitored until the chicks commenced flying. No chicks were injured during the sampling and all were able to fly satisfactorily.

2.3. Organochlorine analysis

Blood samples were analysed for a series of organochlorine pollutants including 1,2,3,4,5,6-hexachlorocyclohexane (HCH) isomers, α-HCH, β-HCH, γ-HCH (lindane), δ-HCH, endosulfan I, endosulfan II and endosulfan sulphate.

2.4. Reagents and standards

All reagents used for the analysis were of a trace analysis grade. Hexane, acetone, petroleum ether, diethyl ether were supplied by Lab-scan Analytical Sciences and anhydrous sodium sulphate by Merck Co. (Darmstadt). SepPak® Florisil columns, were supplied by Waters®. Pesticide standard (Pesticide Mix 4-8858 dissolved in methyl alcohol–methylene chloride 98:2) was procured from Supelco (USA). Prior to analytical procedures, all glassware was rinsed several times with acetone and hexane.

2.5. Analytical procedure

Samples were analysed according to a slightly modified version of the method described by María-Mojica et al. (2000). A volume of 200 μl blood was sonicated and homogenized using hexane:acetone (3:1 v/v) as an extract solvent.

Table 1
Blood concentrations of organochlorine insecticides (μg/l) for booted eagle nestlings over five breeding seasons (1999–2003)[a]

	1999 N = 10	2000 N = 18	2001 N = 14	2002 N = 10	2003 N = 10	1999–2003 N = 62	% not detected
α-HCH	0.52 ± 0.43 nd(7)–1.30	0.95 ± 0.89 nd(9)–3.00	3.08 ± 5.97 nd(7)–20.00	2.38 ± 4.15 nd(6)–12.00	1.41 ± 1.71 nd(6)–4.82	1.67 ± 3.43 nd(35)–20.00	52.42
β-HCH	0.78 ± 0.98 nd(7)–2.80	1.09 ± 1.23 nd(10)–4.00	7.96 ± 19.61 nd(2)–75.00	17.73 ± 20.66 nd(2)–48.00	6.78 ± 5.29 nd(3)–15.66	6.19 ± 13.59 nd(24)–75.00	38.71
δ-HCH	4.40 ± 8.76 nd(8)–22.00	8.37 ± 8.11 nd(6)–28.00	5.81 ± 13.07 nd(8)–50.00	12.58 ± 11.48 nd(3)–30.00	5.60 ± 7.71 nd(6)–21.27	7.38 ± 10.08 nd(31)–50.00	50
Lindane	38.41 ± 37.21 5.80–123.00	11.04 ± 9.56 nd(4)–32.00	0.71 ± 0.81 nd(8)–3.00	1.48 ± 2.35 nd(3)–8.00	1.89 ± 2.49 nd(5)–6.92	10.10 ± 20.16 nd(18)–123.00	60.64
α-Endosulfan	27.84 ± 15.55 7.40–53.00	18.07 ± 9.24 nd(1)–37.00	33.50 ± 59.24 nd(4)–203.00	50.65 ± 53.31 nd(2)–135.00	109.04 ± 75.42 40.56–239.03	43.06 ± 55.07 nd(7)–239.03	11.29
β-Endosulfan	9.94 ± 6.24 3.40–24.00	9.25 ± 3.87 nd(1)–16.00	18.94 ± 24.02 1.00–90.00	26.53 ± 22.64 nd(1)–68.00	21.42 ± 19.60 nd(2)–61.80	16.30 ± 17.59 nd(4)–90.00	6.45
Endosulfan sulfate	47.3 ± 26.38 20.00–103.0	27.14 ± 17.71 nd(2)–74.00	19.85 ± 28.21 nd(1)–100.00	50.90 ± 63.78 7.00–216.00	9.59 ± 26.59 nd(8)–84.88	29.75 ± 35.76 nd(11)–216.00	17.74

Range (min–max)
nd: not detected in parenthesis.
[a] Values area presented as the mean ± standard deviation.

The samples were filtered using anhydrous sodium sulphate and then the solvent collected was evaporated until dry. After redisolution in 5 ml hexane, samples were cleaned up via Florisil column chromatography (SepPak, Waters®) using a petroleum ether–diethyl ether mix (21:4) as the elution. The solvent collected was evaporated until dry.

The final volume was adjusted to 1 ml with *n*-hexane. One microlitre was injected into a gas chromatograph with electron capture (GC-ECD 17 Shimadzu) for the detection of OC. The SPB-5 capillary column (Supelco®) was 30 m long, 0.25 mm i.d. with a 0.25 μm-thick coating. Helium was used as the carrier gas. The injector was set at the splitless mode; the injector temperature was 220 °C. The column programme was: 2 min 50 °C, from 50 to 150 °C at 40 °C/min, 2 min 150 °C, from 150 to 290 °C at 8 °C/min, 10 min 290 °C. The detector temperature was 300 °C and the gas make up was nitrogen. Quantification was based on an external standard. The standard solution marked in mixture was prepared by dissolving the references substances in *n*-hexane (1/50) at the following concentrations: 10 μg/ml for α-HCH, β-HCH, δ-HCH and lindane; and 20 μg/ml for endosulfan I, endosulfan II and endosulfan sulphate. Detection limits ranged from 0.20 to 0.50 μg/l. The percentage of variability between duplicates varied from 0.8% to 12% and mean recovery in spiked samples ranged from 85.8% to 146.0%. Quality assurance criteria were based on the application of quality controls which included the analysis of blank and duplicate samples covering the complete analytical procedure. Concentrations of OC were expressed as μg/l.

2.6. Statistical analyses

All analyses were carried out using the SPSS v.11.5 statistical package. Reported OC values represent the mean ± standard deviation. Since residues were not normally distributed the non-parametric Kruskal–Wallis test was used in order to detect differences between sampling years, followed by Mann–Whitney tests when differences were found. Since a great number of non-detected values for some compounds were observed (Table 1), they were assigned $\frac{1}{2}$ the detection limit established when mean comparison tests were performed. In the case of lindane and β-HCH, differences in blood concentrations were observed before and after 2001, therefore statistical tests were performed in order to compare both time periods. In these cases, concentrations in each period were distributed normally and therefore parametric *T* tests for independent samples were used to examine these differences. The level of significance for these tests was set at $\alpha = 0.05$.

3. Results

3.1. Organochlorine concentrations

Blood organochlorine concentrations are shown in Table 1. Taking data for all years into consideration, lindane concentrations were the highest HCH isomers ranging between non-detectable values ($n = 24$) and 123 μg/l, with a mean concentration of 10.10 ± 20.16 μg/l. Furthermore, endosulfan was the most frequently detected organochlorine insecticide (Table 1). The metabolite endosulfan sulphate was present at a high percentage (82.3%) of the total blood sample examined with a mean concentration in the study period of 29.75 ± 35.76 μg/l. α-endosulfan was detected in 88.7% of blood samples with a mean of 43.06 ± 55.07 μg/l for this period. β-endosulfan levels were four times lower than those described for α-endosulfan (16.30 ± 17.59 μg/l).

3.2. Differences between years

An increase was observed in total endosulfan concentrations in the booted eagle (Table 1). In the case of HCH isomers, mean concentrations dropped from 1999 ($\chi^2 = 9.40$; $p = 0.05$), due mainly to large decreases in lindane use (Table 1).

Regarding the sum of HCH isomers, the highest mean concentration was obtained in 1999, being significant ($p = 0.013$) with respect to the year 2001. Analyzing these compounds separately, only lindane and β-HCH are seen to display significant differences between years. The mean lindane concentration was higher in 1999 and 2000, while that of β-HCH was higher in the period from 2001 to 2003 (Table 2).

4. Discussion

Birds, reptiles, and mammals constitute the main types of prey in the booted eagle's diet, with rabbits (*Oryctolagus cuniculus*) and ocellated lizards being the most important prey species in terms of biomass (Martínez and Calvo, 2005). Booted eagles are considered to be forest raptors, however, the booted eagles in the study zone turn to cultivated areas in search of prey, with which they feed their chicks (Sánchez-Zapata and Calvo, 1999). Studies carried out in part of the study area showed a variable percentage of home ranges (45.2–81.3%) lay outside the forest limit in zones with agricultural systems (Martínez et al., 2007). Therefore, changes in the crops grown and the increased use of agrochemical products could affect the nesting success of the

Table 2
Blood concentrations (μg/l) and the frequency of non-detection for β-HCH and lindane in booted eagle nestlings, prior to and following the ban on lindane sales for agricultural uses in the European Union

	1999–2000 ($n = 28$)		2001–2003 ($n = 34$)	
	Mean ± SD (min–max)	N.d. (%)	Mean ± SD (min–max)	N.d. (%)
β-HCH	0.94 ± 1.16 (nd–4.00)	17 (60.71)	10.47 ± 17.28 (nd–75.00)	7 (20.59)
Lindane	20.80 ± 26.41 (nd–123.00)	4 (14.29)	1.22 ± 1.96 (nd–8.00)	16 (47.06)

booted eagle population in the study area, due to changes in biodiversity (Ruiz-Pérez, 1990; Martínez et al., 2007). The booted eagle is a trans-Saharan migrant; nonetheless, contaminants, such as cadmium and lead, in blood are a good indicator of recent exposure (Martínez-López et al., 2004, 2005). Therefore, variations in adjacent agricultural fields could be reflected in the blood of booted eagle nestlings. According to Tucker and Heath (1994) it is likely that organochloride pesticides used in agriculture are accumulated in Booted Eagles on ingesting their prey, which according to the latter authors could have adverse effects on the reproductive success of this species as has been demonstrated in other bird-hunting raptors. This fact could provide information on the exposure of nestlings to contaminants via their diet (immediate dietary intake). A few years ago, the main crop in this area was cereals, however, in recent years, the low yields of these crops has caused it to be replaced by other, more productive crops for the type of climate, such as olives, grapes and woody crops (CARM, 2005). According to data gathered by CARM (2005) from 1999 to 2003, the surface area occupied by cereal crops in the study area went from 44,200 to 33,370 ha, whilst that of woody crops went from 40,150 ha to more than 46,000 ha, among which the plum tree is to be highlighted. This fact, together with the restriction imposed on the use of pesticides, such as lindane (Decision, 2000/801/EC) has lead to changes in the pesticides used with greater effects over the infestations affecting these crops.

Ninety-five percent of the samples contained traces of HCHs, with lindane and beta isomers being the most frequent (67.7% and 61.3%, respectively). Normally, the exposure to lindane is accompanied by the exposure to other isomers, of which β-HCH is the most persistent (Li et al., 1998). In the present study, precisely these two isomers are the only ones whose concentrations in blood showed differences between years ($p < 0.05$).

In a study of the temporal trends of OC in Black Guillemot (*Cepphus grylle*) from 1976 to 1996, the levels for lindane were lower than those of β-HCH. This is despite the fact that lindane was the only registered organochlorine pesticide in use in Iceland in that period (Ólafsdóttir et al., 2005). Van Wyk et al. (2001) found that β-HCH was, in relation to lindane, the organochlorine with the highest concentration in blood samples from African whitebacked vultures (*Pseudogyps africanus*). According to Heeschen et al. (1980) β-isomer accumulated in fatty tissue 10–30 time more than lindane and its metabolization was slower. This is likely the reason explaining the increase in β-HCH concentrations over recent years compared to the decrease in lindane in the present study.

The lindane values in our study (Table 1) are 10–100 times higher than those described by Maroni et al. (2000) for human beings. Furthermore, Van Wyk et al. (2001) detected lower lindane levels in the blood of African whitebacked vultures from two locations in South Africa far from any urban zones. These authors found means of 5.32 and 2.10 μg/l depending on the area. There are no established background lindane levels for raptors. In a study on human beings, concentrations in blood from non-exposed individuals were close to 0.1 μg/l and the mean blood lindane levels of the exposed group was 11.9 μg/l (Milby and Samuels, 1971). Slight neurological changes were associated with a blood level of 20–34 μg/l in human (Czegledi-Janko and Avar, 1970). On the other hand, rat blood levels exceeding 20 μg/l have been associated with neurologic effects (Dick et al., 1997). Herrera et al. (2000) detected high levels of lindane (from 1 to 18 μg/g) in fat from some partridges (*Alectoris rufa*) collected in Spain. These authors suggested that these concentrations are expected to cause adverse effects in these birds based on results of field and laboratory studies conducted with other avian species (Jansen, 1996). In rats, the fat/blood ratio of organochlorine concentrations ranged from 150 to 200 (WHO, 1991). Using this ratio on the concentrations cited above reported by Herrera et al. (2000) blood levels in partridges should be between 5 and 90 μg/l. In the present study, the highest blood lindane concentrations were obtained in the year 1999, one year prior to its prohibition by the European Union (Decision, 2000/801/EC). Subsequent to that year, the drop in blood concentrations was dramatic, reaching mean levels in blood at 1 μg/l in the year 2001, the year following prohibition. As such, if one were to take into account the levels in blood from samples subsequent to prohibition alone (2001–2003) (Table 2), one would arrive at a mean blood lindane concentration in chicks of 1 μg/l, similar to those values described by Maroni et al. (2000) for human beings and below those cited above associated to adverse effects.

As the number of hectares of olive, grape and plum plantations increased and the number of hectare of cereals decreased (CARM, 2005), the concentrations of lindane in the blood of booted eagles in the area decreased (Fig. 2). The cereal crops in the area (wheat, barley and oats) does not require the use of insecticides, from which the similar tendency observed in the graphs is due more to a coincidence in time for both parameters (change in crops and banning of lindane). However, olive, grape and plum production does require the use of insecticides, among which lindane was utilized prior to being banned. The graphs show that the increase in surface area dedicated to crops has not generated a higher demand for lindane, which could be explained by the use of alternative insecticides such as endosulfan.

In the case of endosulfan, certain studies have found endosulfan sulphate to be the main metabolite of endosulfan in soil samples (Antonious and Byers, 1997). We detected endosulfan sulphate concentrations similar to those found by Van Wyk et al. (2001) in African whitebacked vultures, under temporary confinement in a Wildlife Rehabilitation Centre located on a private farm (22.86–31.67 μg/l). The α-isomer values were higher than those for endosulfan sulphate (Table 1) and four times higher than β-endosulfan, which represents a percentage of 93.5%. Technical grade endosulfan is a mixture of alpha and beta isomers at a proportion of 70/30. The proportion of the two isomers in blood samples is the same as those for technical grade endosulfan (Table 1). According to Gorbach (1988), endosulfan is not persistent in warm-blooded organisms and is partly converted by hydrolysis to dialcohol and partly oxidized to endosulfan sulphate. However, we found endosulfan sulphate levels in blood samples to be lower than endosulfan (α and β) concentrations. This fact could be explained if booted eagle nestlings have

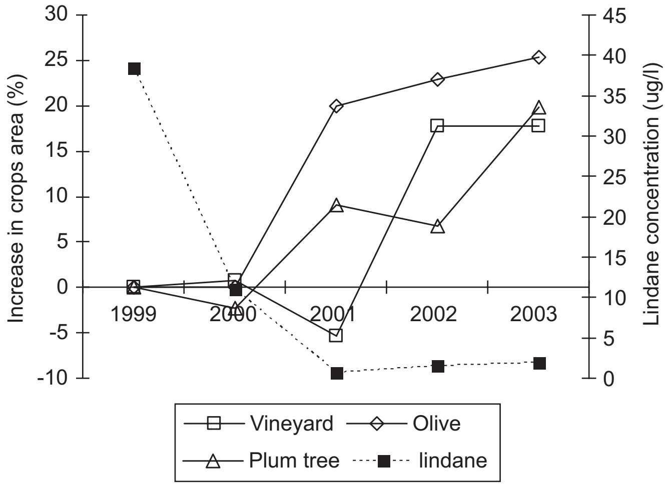

Fig. 2. Mean yearly blood lindane concentrations (μg/l) in booted eagle nestlings matched with the increase in surface area of a range of crops in the Region of Murcia.

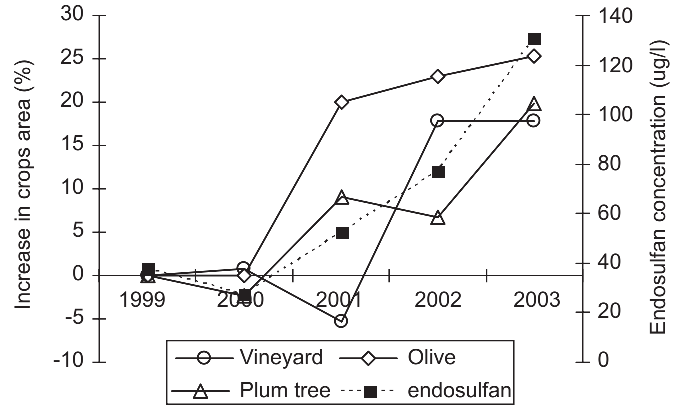

Fig. 3. Mean yearly endosulfan concentrations (the sum of isomers α, β and endosulfan sulphate) in the blood of booted eagle nestlings matched with the increase in surface area of a range of crops in the Region of Murcia.

suffered recent and continuous exposure. In spite of the high blood concentrations of endosulfan detected, nestlings did not show symptomatology of acute poisoning. No references relating endosulfan blood levels and effects in birds has been found. However, Repetto et al. (1995), in survival humans exposed between 0.29 and 0.67 mg/l associated to neurological alterations. These concentrations are very much higher than ours.

Endosulfan was a constituent of 71 products registered by the Ministry of Agriculture and Foodstuffs as pesticides for agricultural applications in the period studied (MAPA, 2005). Almost half (46.55%) of the products which contained endosulfan in high concentrations were registered over the last decade, which coincides with the research period for this study.

Using agricultural production data available from the Murcia Regional Autonomous Government from 1999 to 2003 (CARM, 2005) (southern Spain) on crops mostly associated with dry farming areas (grains, vineyards, almonds and olives) and fruit trees from market garden areas and matching them against concentrations of the different compounds, an increase in endosulfan concentrations in the blood of booted eagle chicks was observed over the years which follows a progression coinciding with olive, grape and plum tree production, whilst displaying tendencies to the contrary of those for grain production (Fig. 3).

Logic dictates that shifts in cultivation trends in the area would have produced a change in the use of certain insecticides focusing on a greater usage of endosulfan as a result of restrictions imposed by the European Union, which was substantiated by the large increase in the registration of products containing this substance.

5. Conclusion

According to our results we can conclude that the blood of booted eagle nestlings is an excellent sampling unit for monitoring the use of pesticides. The variable "year" has proven to be the most influential in organochlorine-insecticide blood concentrations in forest raptor nestlings from the Region of Murcia. Although endosulfan has recently been banned, during the studied period it was the sole organochloride compound legally traded and used in agriculture and recommended for those plagues which affect the chief crops in the area, had experienced a five-fold increase in blood concentrations in booted eagle nestlings since the year 2000. This progressive rise observed for endosulfan makes ongoing monitoring necessary in order to determine how this trend is evolving.

On the other hand, the sudden drop in lindane levels in the blood of raptor chicks and its chronological concurrence with the European Union's legislative measures regarding its prohibition, allows the blood of raptor nestlings to serve as a unit for monitoring the practical application of these measures and that the reduction in levels could suppose a guarantee for minimizing health risks.

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