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Changes in eggshell thickness and ultrastructure in the Bearded Vulture (*Gypaetus barbatus*) Pyrenean population: A long-term analysis

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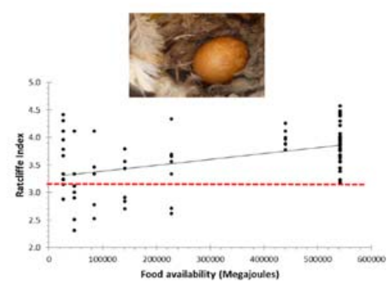
HIGHLIGHTS

- Shell thickness of Bearded Vulture eggs obtained from 1989 to 2012 was compared with eggs collected from 1858 to 1911.
- Bearded Vulture eggs exhibit significant variability in gross and ultrastructural biometry.
- The OC and PCB levels found in Bearded vulture eggs were lower than those considered critical for their survival.
- A decrease in the Ratcliffe Index and eggshell thickness was observed in eggs collected since 2001.
- Changes in egg and eggshell parameters seem to be the result of extrinsic factors as food availability and quality.

GRAPHICAL ABSTRACT



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ABSTRACT

The Spanish Bearded Vulture (*Gypaetus barbatus*) population has suffered from negative trends in a number of reproductive parameters that could jeopardize its long-term viability. From 1989 to 2012, 27 entire eggs and 63 eggshell fragments were collected from nests after breeding failure and/or fledging. Longer-term changes in eggshell thickness were made by examining 69 eggs collected in Spain from 1858 to 1911, and now held in European museums. Low levels of contamination with organochlorine pesticides and polychlorinated biphenyls were found in whole eggs and in conjunction with the high fertility rates observed in the field (66.7%) do not indicate a population suffering from the effects of organochlorine contamination. However, a decrease in the Ratcliffe Index and eggshell thickness were observed in eggs collected since 2001, increasingly so in samples post-2004, indicating an abrupt loss of egg quality. We found no significant relationship between organochlorine residues and eggshell in any of the variables measured. In contrast, we found a positive relationship between food availability and the Ratcliffe Index, eggshell thickness and eggshell surface area. A density-dependent explanation of reduced egg quality could arise from ecological constraints as the decrease of food resources. The impact of sudden changes in food availability due to sanitary regulations between 2006 and 2011 could be related with the loss of Bearded Vulture egg quality.

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1. Introduction

Contamination by OC pesticides has had serious consequences for bird populations, mainly in raptors through increased reproductive failure (Wiemeyer and Porter, 1970; Newton and Bogan, 1974), Reduction of eggshell thickness was one of the earliest recorded effects of dichlorodiphenyl dichloroethylene (DDE) on bird reproduction (Hickey and Anderson, 1968; Ratcliffe, 1970; Peakall and Kiff, 1979). Decreases in breeding success are related to DDE concentrations in the parent birds (Bowerman et al., 1995) leading to a potential relationship between DDE level and productivity (Wiemeyer et al., 1984: Helander et al., 2002; Hernández et al., 2008).

Measurements of eggshell thickness therefore provide a useful tool to assess the DDE contamination level of a threatened species as a precursor to improved management and conservation measures. A case in point is the Bearded Vulture (*Gypaetus barbatus*), an endangered species of conservation concern in Europe (Annex I, EU Wild Birds Directive 79/409/EEC, Appendix II of the Bern Convention, Bonn Convention and CITES), which has recovered since the 1990s to about 230 European breeding territories in 2016. Bearded Vultures are a long-lived species, with a low annual reproductive rate and a pronounced deferral of age of first breeding (Antor et al., 2007; Margalida et al., 2015). Factors which negatively impact on demographic parameters can have extremely detrimental effects on their population dynamics (Oro et al., 2008; Margalida et al., 2015).

In recent decades, the Pyrenean population of the Bearded Vulture increased progressively (from 57 territories in 1989 to 174 in 2016) but has suffered decreasing breeding success in a density-dependent productivity depression scenario (Carrete et al., 2006). Productivity fell from 0.6 young/territorial pair in the 1980s to 0.30 young/territorial pair currently (Carrete et al., 2006; Margalida et al., 2014) and its prospects of survival are reduced (Oro et al., 2008; Margalida et al., 2014). Recent studies have revealed high adult mortality as a result of the increased incidence of illegal poisoning (Margalida et al., 2008; Berny et al., 2015), a factor which seriously threatens the survival of the population (Margalida et al., 2015). For example, between 1990 and 2010 a total of 53 cases of Bearded Vultures illegally poisoned were reported in Spain (Margalida, 2012).

The number of studies on these parameters in Bearded Vultures contrasts with the lack of studies on pesticide contamination of the Pyrenean population (Hernández, 2005a, 2005b), although the OC levels found in eggs and carcasses seem to be, as yet, insufficient to cause breeding impairment or reduced reproductive rates (Elliot and Norstrom, 1998; Hernández et al., 2008; Odsjö and Sondell, 2014).

The recent outbreak of bovine spongiform encephalopathy (BSE) led to changes in sanitary regulations that have resulted in significant food shortages for scavenging animals (Donázar et al., 2009; Margalida et al., 2010). The reduction of available food resources (by c. 80%, see Cortés-Avizanda et al., 2010; Margalida et al., 2014) as a consequence of the retrieval and incineration of livestock carcasses, and the closure of several vulture feeding stations between 2006 and 2011, has been shown to have affected the breeding ecology and behaviour of some vulture species through damaging changes to their breeding and demographic parameters (Donázar et al., 2009; Margalida et al., 2014) and their behavioural characteristics (Zuberogoitia et al., 2010; Margalida et al., 2011). The impact of sudden changes in food availability, and therefore in habitat quality, could affect population trends through changes in life-history parameters (Margalida et al., 2014). Moreover, the effects of organochlorine compounds on egg quality have not been fully examined.

In this study, we used material from a long-term study of eggs and eggshells of the Pyrenean Bearded Vulture population to assess the influence of these reductions in food availability on eggshell parameters and egg quality. We evaluated several egg and eggshell parameters, including eggshell thickness, and their trends over the last 24 years (1989–2012) as well as the levels of OCs present in the eggs. We compared these results from the field with measurements taken from Bearded Vulture eggs added to museum collections between 1858 and 1911 to look at the longer-term changes in eggshell thickness.

2. Material and methods

2.1. Study area and population monitoring

Samples taken from Bearded Vulture populations in Spain and France (130 and 43 breeding territories in 2016, respectively) were studied. The study was carried out within the framework of the conservation plans for this species in France and the Autonomous Communities in Spain. Programs to monitor population trends, breeding parameters and survival rates (including a capture-mark-resighting program) have been performed (Oro et al., 2008; Margalida et al., 2014).

Between 1989 and 2012, all known territories were visited two or three times each month to search for signs of occupancy, territorial and/or courtship activity, and nest building, and to record reproductive parameters (Margalida and Bertran, 2000). Observations began in September–October each year, coinciding with the start of nest-building and sexual activity, and ended during the fledging period (June–August). Nests were observed using 20–60× telescopes and video cameras. During the egg-laying period, 14–20 pairs were selected each year for detailed daily monitoring to determine the date of egg-laying. Egg-laying was confirmed when an incubation shift between parents was observed, or once the eggs could be directly observed in the nest. Failing these methods, clutch size (a simple, one-egg clutch vs a two-egg clutch) was determined by visiting the nest after a confirmed breeding failure and/or after the breeding season, recovering the egg remains. In these cases, we only included the data when entire eggs were found or when the nest inspection was carried out as soon as possible after breeding failure, to avoid possible loss of eggs due to predation by Common Ravens (*Corvus corax*), or after fledging.

2.2. Sample collection

Addled or abandoned eggs or eggshell fragments were collected from the nests of Bearded Vultures breeding in the Pyrenees from 1989 to 2012, after confirmation of breeding failure and/or after fledging. Eggs and eggshells were removed from nests by authorized staff and official agents working under the framework rules of this species' recovery plans in the Autonomous Communities of Spain. Entire eggs were transported to rehabilitation centres in portable incubators and, upon arrival, were weighed, measured (length and breadth), and, when necessary, artificially incubated for some days before confirming breeding failure.

A total of 27 addled eggs, and eggshell fragments from a further 63 eggs were collected and analyzed (a total of 90 samples collected from 49 pairs). Fifty of the eggs were from one-egg clutches and 40 were from two-egg clutches. Although eggs may have been collected from the same territory or nest in different years the adults involved may have been different individuals because adult birds were not marked.

In cases where eggshell fragments were the only material retrieved from a nest after breeding failure, they were classified according to their size and external appearance before examination using a scanning electron microscope (SEM; Phillips Model KX-20 electron microscope, Philips Electronics Nederland, Eindhoven, The Netherlands). In 18 eggs from two-egg clutches (28.6% of the total) the fragments were large enough to distinguish between the pieces belonging to each egg. Only eggshell fragments from the equator of the egg were studied, to avoid bias due to ultrastructural changes occurring over the eggshell. In 68.3% (n = 43) of all eggs examined, large fragments were chosen to discriminate between those from the equator of the egg, rather than those from the blunt or pointed end. In 31.8% of all eggs (n = 20), the retrieved material consisted of eggshell fragments < 1 cm^2. In these cases, assorted fragments were examined using an SEM. Those fragments showing the morphological characteristics of the blunt end (Hernández, 2005a, 2015) or greater curvature indicating the pointed end (Solomon, 1997), were excluded from the study.

In addition, we included measurements taken from 69 Bearded Vulture eggs collected from Spanish nests between 1858 and 1911 and held in various European museums. The ultrastructural data from of eight of these eggs was included in the study. Samples were divided between those of Pyrenean origin (n = 24), those from territories in Southern Spain (n = 39), and eggs collected in Spain from unknown origins (n = 11).

2.3. Egg fertility

Fertility and embryo development for each of the 27 whole eggs was determined during pathological and gross pathological examination (Hernández et al., 2008). Fertility and embryo development in the 63 eggshell fragments were determined by SEM looking at the erosion of the basal cones on the inner surface of the shell after removal of the egg membranes (Bland, 1992) using the fact that during the latter stages of normal embryonic development, calcium is withdrawn from the shell (Figs. 1 and 2). The basal caps become eroded as calcite is absorbed by the embryo as a source of calcium for development and metabolism (Fox, 1976). Eroded basal caps therefore serve as a reliable indicator of an advanced stage of embryonic development (Solomon, 1997; Bland, 1992).

2.4. Analyses of organochlorine contaminants

Samples were kept frozen at −20 °C until their analysis in the Forensic Laboratory of Wildlife (Las Matas, Madrid, Spain). The egg contents were removed through a hole (the cut shell piece being kept with the rest of the shell). The whole shell was oven dried at 110 °C after rinsing with distilled water. Analysis of OCs was performed using gas chromatography with an electron-capture detector as described elsewhere (Hernández et al., 2008), a technique which has previously proved consistent. Briefly, the egg contents, including the embryo when present, were homogenized with anhydrous sodium sulfate, extracted with petroleum ether using a Soxhlet extractor (Afora, Fuenlabrada, Madrid, Spain) and purified using a Strata FL-PR Florisil solid-phase extraction cartridge (Phenomenex, Torrance, CA, USA) according to EPA Methods 3540C and 3620B. Levels of Alachlor; α, β, and γ-hexachlorocyclohexane (HCH); *o,p′*- and *p,p′*-isomers of dichlorodiphenyl dichloroethane (DDD); dichlorodiphenyl dichloroethylene (DDE); dichlorodiphenyl trichloroethane (DDT); aldrin; endrin; dieldrin; heptachlor; heptachlor epoxide; α and β-endosulfan; endosulfan sulfate; hexachlorobenzene; mirex; oxychlordane; and α and β-chlordane were quantified with a Gow-Mac Series 600 gas chromatograph equipped with an electron capture detector and a 1% dimethylpolysiloxane (ZEBRON-1, Phenomenex, Torrance, CA, USA) column according to EPA 608 Method. Organochlorine pesticides and polychlorinated biphenyl (PCB) congeners were determined by comparing the relative retention times of the unknown peaks with the peaks of a standard solution (Pesticides Mix and Aroclor 1254 Solution; Supelco, St. Louis, MO, USA) using dichloromethane as an internal standard. The total concentrations of PCBs were determined by summation of peaks areas in the sample that coincided with those of the Aroclor 1254 standard. Different standards of different OCs were employed to determine the response factors for each analyte. Quality controls were carried out using a content sample of hen egg fortified with each analyte after extraction and analysis in the same conditions as the samples. The recovery percentages of the different pesticides varied between 67% for hexachlorobenzene and 116% for heptachlor epoxide. The detection limits of the OCs were 0.001 μg/g except for endosulfan sulfate and α and β-chlordane (0.005 μg/g) and between 0.003 and 0.005 μg/g for all of the PCB congeners analyzed, except for PCBs 8, 18, and 28, which were 0.015 μg/g.

Following these analyses, the concentrations of OCs were not corrected according to their recovery percentage. The concentrations of pesticides are expressed in μg/g wet weight to avoid the influence of embryonic development on the concentrations of OCs (Newton and Bogan, 1978; Peakall and Gilman, 1979), the effect of putrefaction on the lipid content, and the effect of leakage of part of the egg content (Helander et al., 2002). Because the weight of the egg contents was not available for most of the eggs, it was not possible to adjust the concentration of pesticides for moisture loss as a result of dehydration after incubation (Hernández et al., 2008).

2.5. Egg and eggshell parameters

Eggs were measured and weighed to the nearest 0.1 mm with a caliper and to the nearest 0.001 g with an electronic scale (EK200G; AandD Instruments, Yokohama, Japan). Eggshell Weight was determined after oven drying the whole shell at 110 °C. We calculated Egg Volume (V) from the measurements of Egg Length and Breadth using the Stonehouse (1966) formula:

$$V\ (mm^3) = 0.512\ \text{Egg Length} \times \text{Egg Breadth}^2$$

We obtained Eggshell Volume (Vs) by subtracting Egg Contents Volume (Vc) from Egg Volume (V) using the equation Vs = V − Vc, where Vc = 0.512 (Egg Length − Shell Thickness) × (Egg Wirth − Shell thickness)^2. We also calculated an estimate of Egg Fresh Weight (W) from the egg's linear dimension using the equation:

$$W = K_w\ \text{Egg Length} \times \text{Egg Breadth}^2$$

For K_w we used the value 0.0005474 following Hoyt (1979) and Burnham (1983).

The Ratcliffe Index (RI) was calculated as the relationship between the length and breadth of the egg and the weight of the shell, including the membranes (Ratcliffe, 1970) according to the formula:

$$RI\ (mg * mm^{-2}) = \text{Shell weight}/(\text{Length} * \text{Breadth}).$$

The ultrastructure of the shell was studied for all eggs from a piece obtained at the equator of the egg. Fragments were sorted and only those from the equator of the egg were included in the study. The ultrastructure was determined using SEM in three fragments of 3 mm^2. A single fragment was examined from a cross-section obtained after mechanical fracture. All specimens were mounted vertically on aluminum stubs with the break facing upwards, and spur-coated with gold at 15 mA in an argon atmosphere. A second fragment was mounted on an aluminum stub with the outer surface of the egg facing upwards to analyze its composition. A third fragment was soaked in distilled water and boiled in 5% NaOH for 5 min. The fragments were then washed with distilled water to remove the egg membranes, dried, and mounted on aluminum stubs so that the inner surface faced upwards. A Phillips Model KX-20 electron microscope (Philips Electronics Nederland, Eindhoven, The Netherlands) was used, with an acceleration voltage of 10–15 kV, and 200–400× magnification microphotographs were produced. Eggshell Thickness was determined as the arithmetic mean of five measurements taken from the microphotographs, as well as the thickness of the three layers of the shell (reticular, palisade, mammillary) and the height and breadth of the basal cones (Board, 1982). The Cooke Index (CI) was defined as the relationship between the height of the basal cones and the thickness of the mammillary layer (Cooke, 1979).

Fig. 1. Inner surface of the eggshell of Bearded Vultures after removing membranes by NaOH digestion. Basal cones (CB) of the mammillary layer and pore openings (P) between them are shown. Basal caps are eroded from resorption of calcite by the embryo as the calcium source for development and metabolism. This decalcification exposes Basal Cap Cores (CO).

Using SEM, we measured the diameter of the inner pore channel of ten pores and measurements were averaged to obtain the Mean Pore Diameter for each egg. We also calculated the number of pores per cm^2 for each egg using 400× SEM microphotographs of the inner surface of the shell. To obtain the Total Number of Pores for each egg, we calculated the surface area (S) of each egg and multiplied it by the number of pores per cm^2, according to Hoyt (1979):

$$S\ (mm^2) = \left[4.393 + 0.394\ \text{Egg Length} \times \left(\text{Egg Breadth}^{-1}\right)\right]\left(V^{0.667}\right)$$

To obtain the Total Functional Pore Area (Ap) of each egg, we multiplied the Mean Pore Area by the Total Number of Pores. We calculated Water Vapor Conductance according to Ar et al. (1974):

$$GH2O = 23.42\ Ap/\text{Shell Thickness}.$$

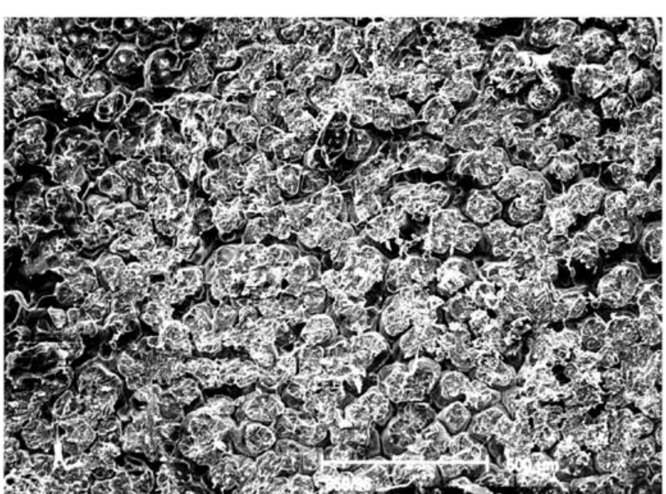

Fig. 2. Basal caps are not eroded in undeveloped Bearded Vulture eggs and Basal Cap Cores are not exposed and visible.

Piercing Strength (D), a measure of eggshell strength, was calculated for each egg according to Ar et al. (1979), using the formula:

$$D = (577 \times RI) - 362.$$

where RI is the Ratcliffe Index.

2.6. Assessing the effects of food shortages

In order to assess the effects of food availability on egg-shell parameters, we calculated the temporal changes in the availability of food resources (bone biomass) provided by wild and domestic ungulates, taking into account Bearded Vulture dietary habits and the presence and population trend of ungulate species in the study area (Colomer et al., 2011). Considering that 0.70% of the diet of the species is based on livestock (Margalida et al., 2009), we obtained the biomass available (bone remains expressed in megajoules) as a surrogate of food potentially available (see details in Margalida et al., 2014). Food shortages related to carcass disposal policies obtained from the agricultural insurance Spanish government agency Enesa (see Margalida et al., 2014) allowed to calculate the proportion of domestic ungulate carcasses that were destroyed. Afterwards, we inferred the proportion of such destroyed domestic carcasses collected from farms from those hypothetically available to scavenging birds at feeding sites obtained through censuses and mortality rates (for more details, see Margalida and Colomer, 2012; Margalida et al., 2014). Although the outbreak of bovine spongiform encephalopathy began in 2001, the restrictive legislation forced the closure of 80% of feeding stations (Donázar et al., 2009; Cortés-Avizanda et al., 2010; Margalida et al., 2014) and obliged the collection of domestic ungulates to be destroyed were not implemented effectively until 2005–2006 (Donázar et al., 2009; Margalida et al., 2010) when dead livestock were collected from farms and most feeding stations (80%) were closed. The changes in sanitary policies approved on November 2011 allowing farmers to provide cadavers to avian scavengers under sanitary controls were implemented progressively from the end 2012 until now (authors unpubl. data). Thus, we can consider 2012 inside the restrictive food shortage period (Margalida et al., 2012). The monitoring of several regions suggested that during the food shortage period, 80% of remains of Ovis/Capra were collected and nearly 100% for the remains of Bos/Equus.

2.7. Statistical analysis

Levels of OCs were expressed as the geometric mean ± standard deviation. Biometric values were expressed as the arithmetic mean ± standard deviation. We used general linear models (GLMs) with a Gaussian error distribution and identity link function to assess differences between the variables measured. Because clutches from the same pair may not be independent, we included the nest as a random term in the models. Similarly, analyses on egg fertility included clutch as a random variable to account for the lack of independence of eggs within a clutch. The correlations between the temporal variations in the trend observed in the Ratcliffe Index were assessed using the Spearman or Pearson rank coefficient depending on the distribution of the data. Differences between contemporary eggs and those kept in museums were tested using analyses of variance (ANOVA) and a posterior Tukey test. Statistical tests were performed using R2.15.2 (R Development Core Team 2014).

Temporal changes were analyzed by combining data obtained in four time periods of >5 years dating backwards from the period of food shortage (2006–2012): 2006–2012, coinciding with the application of restrictive sanitary regulations; 2001–2005, coinciding with the outbreak of bovine spongiform encephalopathy in 2001 (Tella, 2001; Donázar et al., 2009) but before the introduction of sanitary regulations (i.e. the period of food shortage); and 1996–2000 and 1989–1995, both without the effects of sanitary regulations.

3. Results

We analyzed the biometric parameters of 27 eggs collected in the Pyrenees. We found only one significant difference between values relating to the order of egg-laying or the time period of egg collection (Tables 1 and 2).

3.1. Differences according to egg-laying order and fertility

The only significant difference found was in Eggshell Thickness which was lower in one-egg clutches than in two-egg clutches (GLM t = −2.151, P = 0.034). We found no significant differences between fertile and undeveloped eggs in any of the variables measured.

3.2. Differences according to the time period of egg collection

The Eggshell Thicknesses of eggs collected during 2001–2005 and 2006–2012 were significantly less than those collected during the first study period (1989–1995) (GLM t = −3.44, P = 0.0009 and t = −3.48, P = 0.0008, respectively). The reticular layer was also significantly thinner during the periods 2001–2005 (t = −3.35, P = 0.001) and 2006–2012 (t = −2.20, P = 0.03). Similarly, the palisade layer was thinner during the periods 2001–2005 (t = −2.56, P = 0.007) and 2006–2012 (t = −3.92, P = 0.0002). Pore Density increased significantly from 1996–2000 (t = 3.5, P = 0.0007) to 2001–2005 (t = 4.0, P = 0.0001) to 2006–2012 (t = 2.3, P = 0.0024). The Piercing Strength also fell as time went by, eggshells being softer during the periods 2001–2005 (t = −2.11, P = 0.038) and 2006–2012 (t = −4.84, P < 0.0001). Water Vapor Conductance increased during the periods 2001–2005 (t = 3.66, P = 0.0004) and 2006–2012 (t = 4.43, P < 0.0001). The time trend of the Ratcliffe Index was statistically significant (r_s = −0.39, P = 0.0001, Fig. 3). In addition, the time trend of the Ratcliffe Index was also statistically significant with respect the Bearded Vulture population increase (r_s = −0.37, P = 0.0004), showing a negative density-dependent relationship.

3.3. Differences between eggs in museum collections (1880–1905) and contemporary eggs (1989–2012)

The differences in gross biometry and ultrastructural biometry between eggs examined from museum collections (Pyrenees and southern Spain) and contemporary eggs (Pyrenees) are shown in Table 3. There were significant differences in Egg Fresh Weight (F = 134.3, P < 0.0001) and Mammillary Layer Thickness (F = 4.41, P = 0.043), with lower values found in contemporary eggs compared with Pyrenean eggs from museums. Regarding Basal Cone Breadth, lower values were found in contemporary eggs (F = 19.91, P < 0.0001). In addition, marginal differences were found in Egg Length (F = 2.86, P = 0.063), Palisade Layer Thickness (F = 3.39, P = 0.074) and Total Number of Pores (F = 3.96, P = 0.056) (Table 3). Egg Length and Palisade Layer Thickness were less in contemporary eggs, whereas Total Number of Pores was greater in museum eggs.

When we compared samples from the Pyrenees (museum vs contemporary) differentiating three periods (1880–1905, 1989–2004 and 2005–2012), again there were significant differences in Egg Fresh Weight ($F_{2,36}$ = 91.43, P < 0.0001), Mammillary Layer Thickness ($F_{2,36}$ = 3.24, P = 0.051), Basal Cone Breadth ($F_{2,36}$ = 12.70, P < 0.0001) and marginal differences in Egg Length ($F_{2,36}$ = 3.03, P = 0.058) and Palisade Layer Thickness ($F_{2,36}$ = 3.06, P = 0.059). With respect Total Number of Pores, values were greater in museum eggs, with significant differences between 1989–2004 vs 2005–2012 period ($F_{2,36}$ = 7.75, P = 0.0017).

3.4. Fertility

Overall, fertility was 66.7% (n = 58) in the Bearded Vulture eggs studied. However, the method of determining fertility by SEM could present a bias in the sample, since the very early stages of embryonic development could be overlooked and an egg classified as infertile (Hemmings et al., 2012), so underestimating fertility in this study. Furthermore, among the fertile eggs (n = 58), a significant number (62.1%, n = 36) showed eroded basal caps indicative of advanced embryonic development. Fertility did not differ between the sample of whole eggs and the sample of fragments (χ^2_1 = 0.21, P = 0.65).

3.5. Organochlorine levels

Organochlorine pesticide residues were detected in all eggs analyzed (Table 4). The OC pesticide found at the greatest concentration was *p,p′*-DDE, with a geometric mean of 0.192 ± 0.525 μg/g (wet weight, ww hereafter), and was detected in all eggs analyzed, with a maximum level of 2.2 μg/g. The isomer *p,p′*-DDT was found in 90% of the eggs (geometric mean of 0.095 ± 0.174 μg/g (ww), and a maximum level of 0.590 μg/g (ww). The isomer γ-HCH (lindane) was detected in 80% of the eggs with a geometric mean of 0.007 ± 0.066 μg/g (ww) and a maximum level of 0.210 μg/g (ww). Among the cyclodienes, heptachlor and heptachlor epoxide were recorded in 80% and 60% of the eggs, respectively, with a geometric mean of 0.040 ± 0.182 μg/g (ww) and 0.029 ± 0.280 μg/g (ww), and maximum levels of 0.771 and 0.995 μg/g (ww), respectively. Other OC pesticides were recorded in <30% of the eggs and at levels below 0.025 μg/g (ww). PCBs were recorded in all eggs analyzed, with a geometric mean of 0.374 ± 0.665 μg/g (ww) and a maximum level of 1.258 μg/g (ww). Excluding sample 1 (collected near an industrial facility), PCBs values increased with the time (r_s = 0.607, P 0.002, n = 20, Table 4). We found no significant relationship between organochlorine pesticide residues and eggshell in any of the variables measured. In contrast, we found a positive relationship between food availability and the Ratcliffe Index (r = 0.461, P = 0.014, Fig. 4), Eggshell Thickness (r = 0.438, P = 0.019) and Eggshell Surface Area (r = 0.575, P = 0.0014). When food available decreased, the values of these parameters also diminished in a significant trend.

4. Discussion

The calcified layer of Bearded Vulture eggs generally corresponds to Tyler's (1966) and Becking's (1975) description, except that there is no surface crystalline layer. The Bearded Vulture eggshell is trilaminate and reticulate (Board, 1982), the calcified portion being composed of three

Table 1
Average biometric characteristics of eggs collected in the Pyrenees during the period 1989–2012, according to clutch size. Indexes are described in Material and methods.

	Single-egg clutch	First egg of two-egg clutch	Second egg of two-egg clutch
Egg Length mm	84.87 ± 3.31	84.81 ± 3.82	85.34 ± 3.54
Egg Breadth mm	66.17 ± 3.83	66.10 ± 2.47	65.83 ± 3.69
Egg Fresh Weight g	202.39 ± 28.04	204.32 ± 30.25	202.81 ± 16.44
Eggshell Surface Area mm^2	1877.34 ± 194.55	1898.89 ± 159.24	1863.76 ± 198.42
Total Number of Pores	156,292.7 ± 16,662.9	163,256.1 ± 33,423.27	160,183.5 ± 30,782.68
Egg Volume mm^3	189,291.6 ± 26,203.62	191,108.6 ± 28,297.47	185,578.5 ± 19,482.09
Eggshell Volume mm^2	4132.38 ± 504.07	4704.689 ± 704.77	4564.93 ± 822.42
Egg Volume Contents mm^2	185,159.2 ± 26,044.56	186,403.9 ± 28,064.41	181,576.9 ± 18,372.98

Table 2
Average values of ultrastructural morphometric characteristics of eggshells collected in the Pyrenees during the period 1989–2012, according to clutch size. Indexes described in Material and methods.

	Single	First egg of two-egg clutch	Second egg of two-egg clutch
Basal Cone Breadth μm	62.23 ± 22.47	73.61 ± 30.45	64.95 ± 27.20
Mammilary Layer Thickness μm	126.83 ± 27.57	134.41 ± 35.73	119.85 ± 29.63
Palisade Layer Thickness μm	292.56 ± 47.30	317.84 ± 49.95	298. 91 ± 53.57
Reticular Layer Thickness μm	119.80 ± 28.28	125.81 ± 31.05	124.89 ± 29.54
Pore Density mm^2	90.96 ± 13.24	85.05 ± 13.86	95.48 ± 15.49
Pore Diameter μm	17.85 ± 4.39	16.87 ± 6.21	16.01 ± 3.99
Eggshell Surface Area mm^2	1674. 49 ± 298.30	1834.16 ± 208.15	1806.76 ± 290.21
Eggshell Thickness μm	531.30 ± 66.99	572.24 ± 90.47	541.80 ± 69.74
Cooke Index	0.45 ± 0.13	0.49 ± 0.13	0.48 ± 0.09
Ratcliffe Index $mg*mm^C$	3.54 ± 0.53	3.80 ± 0.36	3.75 ± 0.50
Water Vapor Conductance $mm^2*\mu m^{-1}$	71.25 ± 20.47	60.81 ± 19.64	67.52 ± 21.63
Total Number of Functional Pores	1608.19 ± 421.96	1470.89 ± 449.22	1511.02 ± 407.46

layers, as described in other Accipitriformes (Tyler, 1966). It also has single, funnel-shaped pore channels and lacks an outer crystalline layer.

Distinguishing between infertility and early embryo mortality is valuable in studies of reproductive success and has important implications for conservation (Birkhead et al., 2008). However, determining fertility in random samples using SEM or necropsy introduces a bias, since very early stages of embryonic development could be overlooked and eggs erroneously recorded as infertile (Birkhead et al., 2008; Hemmings et al., 2012). Methods such as those used in the poultry industry are unacceptable in endangered species because they rely on the examination of fresh eggs (Birkhead et al., 2008). In any case, the high fertility rates (66.7%) found in the Pyrenean population does not indicate a population suffering from the effects of OC pesticide contamination (Bland, 1992; Newton et al., 1993; Bowerman et al., 1995; Helander et al., 2002; Hernández et al., 2008; Kurle et al., 2016). Furthermore, a significant number of failed but fertile eggs gathered in the field showed evidence of advanced embryonic development, and in field observations failure during breeding was found to occur mainly (51% of the cases) towards the end of the incubation period and/or during the first week after hatching (Margalida et al., 2003, 2004). Fertility rates were constant during the study period. Our findings therefore indicate that changes in egg and eggshell parameters could not be attributed to changes in fertility. Breeding failure due to DDE contamination may be a result of embryo mortality during early development (Bland, 1992; Hernández et al., 2008).

Bearded Vultures have been exposed to a wide variety of contaminants (Table 4) and a number of OCs of agricultural (DDT and its derivations, HCHs, cyclodienes, and chlordanes) and industrial origin (PCBs) were found in the eggs analyzed. However, none of the OC contaminants investigated reached levels considered critical in other studies (Risebrough and Peakall, 1988; Wiemeyer, 1996), neither were related with changes in eggshell variables measured. Although DDE levels in Bearded Vulture eggs were found to be far below critical levels throughout the entire study period, the OC pesticide levels in egg contents showed an increasing trend over time. This could be due to changes in the vulture's dietary habits during food shortage periods (2005–2011), wild prey having higher OC pesticide loads than livestock carcasses (Hernández and Margalida, 2009). The outliers found on HCH's, DDE and DDT' (samples 2 and 3, Table 4) can be explained because they belonged to a double clutch collected both, near an industrial facility, and after the spill of Lindane and other organochlorines from an industrial source (Hernández et al., 1991). Other cases of extreme values were documented in the Spanish imperial eagle (*Aquila adalberti*) population inhabiting Doñana, at which the average levels of DDE found in eggs were 1.64 μg/g and 1.19 μg/g in PCBs (Hernández et al., 2008), with one egg that showed a DDE value of 38.044 μg/g wet weight (Hernández et al., 1989).

Although, DDE-related effects on the reproduction of birds of prey have been well documented (Newton and Bogan, 1974, 1978; Bowerman et al., 1995; Helander et al., 2002), the levels of DDE detected in this study were well below those reported in the literature as responsible for breeding impairment and low productivity (Bowerman et al., 1995; Helander et al., 2002; Hernández et al., 2008).

Many new broad-spectrum pesticides have been used in developed countries in recent decades, so that all of the OC pesticide residues found in bird tissues have decreased significantly over time (Mora et al., 2016). However, even though these modern pesticides are available, OCs continue to be used to control agricultural pests and disease vectors in third world countries (Muralidharan et al., 2008). OC pesticide contamination therefore remains a concern for the recovery of many species in countries where they have been banned for many years (Helander et al., 2002; Kurle et al., 2016). The concentrations of OC hexachlorocyclohexanes, cyclodiene pesticides and PCB's found in this study were far below those reported to cause breeding impairment (Kiff et al., 1979; Wiemeyer et al., 1986; Helander et al., 2002; Muralidharan et al., 2008).

Our results showed that Bearded Vulture eggs exhibit significant variability in gross and ultrastructural biometry (Tables 1 to 3). This level of variation appears unique to the Bearded Vulture and has never been matched in other raptor species studied so far (Cooke, 1979; Kiff

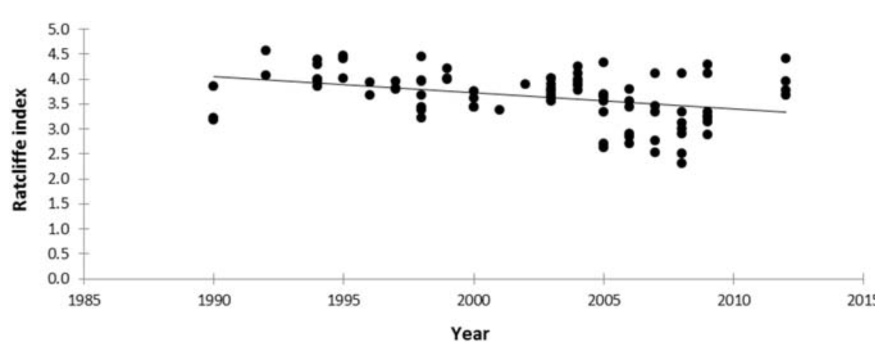

Fig. 3. Temporal variation in Ratcliffe Index values ($mg\times mm^{-2}$) found in the Bearded Vulture eggs in the period 1990–2012.

Table 3
Comparison of average biometric values of eggs collected in the Pyrenees during the period 1989–2012 compared with museum eggs collected during 1890–1906. Note that because the order of egg laying is not available in museum samples, date from one- and two- egg clutches are pooled to enable comparisons between time periods.

	Pyrenees 1989–2012 n = 90	Pyrenees Museum XIX century n = 24	Andalusia Museum XIX century n = 39
Egg Length mm	85.09 ± 3.48	82.67 ± 4.15	83.01 ± 4.25
Egg Breadth mm	65.91 ± 3.07	65.75 ± 2.09	65.40 ± 2.73
Egg Fresh Weight g	203.17 ± 24.64	297.85 ± 23.34	297.62 ± 16.44
Basal Cone Breadth μm	68.61 ± 22.59	106.99 ± 16.01	
Total Number of Pores	159.69 ± 27.83	138.23 ± 21.70	
Mammilary Layer Thickness μm	132.08 ± 33.66	105.09 ± 24.33	
Palisade Layer Thickness μm	304.93 ± 45.23	344.50 ± 76.28	
Reticular Layer Thickness μm	133.76 ± 27.99	129.69 ± 37.41	
Total Shell Thickness μm	564.69 ± 75.70	573.48 ± 121.80	
Cooke Index	0.47 ± 0.09	0.41 ± 0.07	

et al., 1979; Peakall and Kiff, 1979; Board, 1982; Helander et al., 2002; Burnett et al., 2013). Significant variability was even found in old specimens kept in museums (Table 3), and appears to be typical of this species, reflecting their special ability to adapt to different environmental conditions. In fact, in the Pyrenees we can find Bearded Vulture nests in an altitudinal range from 600 m to 2400 m. This wide variation in many biometric parameters accounts for the minimal differences observed between contemporary and old eggs (Table 3), which differed significantly between egg-laying order and fertility only in Egg Fresh Weight, and not in other biometric parameters.

Time trends were the only significant changes found in contemporary Bearded Vulture eggs and eggshells. They were statistically significant during two periods (2001–2005 and 2006–2012). Reduction in Eggshell Thickness and the Ratcliffe Index in these periods were due to thinning of the reticular and palisade layers. Increased Pore Density leading to lower Piercing Strength and increasing Water Vapor Conductance was also significant during these periods. The decrease in Ratcliffe Index and Eggshell Thickness, and the change in Pore Density observed since 2001 (and more importantly since 2006) might indicate that the Bearded Vulture is suffering from a progressive loss of egg quality (Solomon, 1997). The positive relationship between food availability and Ratcliffe Index, Eggshell Thickness and Eggshell surface area found in this study could support the hypothesis of nutritional stress affecting eggshell parameters. In fact, the 2006–2012 period coincides with the food shortage period. Since from a quantitative perspective food availability seems enough to cover the energetic requirements of Pyrenean Bearded Vulture population (Margalida and Colomer, 2012; Margalida et al., 2017), food quality could be affected by these changes, but also could be explained by the progressive increase in breeding density occurring in the study area, as was documented in the Wahlberg's Eagle (*Aquila wahlbergi*) (Simmons, 1993).

Reduction of the Ratcliffe Index seems to be one of the first signs of the effects of DDE on fertility in birds of prey (Helander et al., 2002). However, according to our results, none of the OC pesticide contaminants found in eggs reached levels considered critical by other studies (Ratcliffe, 1970; Risebrough and Peakall, 1988; Wiemeyer, 1996).

Changes in egg and eggshell parameters, and the subsequent loss of egg quality found in recent years, seem to be a result of extrinsic factors, such as food availability and their quality. For example, recent changes in EU sanitary regulations have led to significant food shortages for wildlife (Donázar et al., 2009; Margalida et al., 2010) over a very short period of time (beginning in 2001 but intensifying during 2006–2011), principally through the removal of dead livestock carcasses and an almost 80% closure of vulture feeding stations (Cortés-Avizanda et al., 2010; Margalida et al., 2014).

The nutritional quality of the pre-breeding diet has a significant influence on breeding performance in birds. Reynolds et al. (2003) showed that nutritional quality of the pre-breeding diet can influence laying date, clutch size, and egg size and composition. Smith et al. (1981) showed significant relationships between Black-tailed Jackrabbit (*Lepus californicus*) abundance and Ferruginous Hawk (*Buteo regalis*) reproduction. In this case, the population of nestling hawks, number of nesting pairs, total eggs laid and total young fledged varied in synchrony with jackrabbit abundance. Mundy et al. (1982) found a relationship

Table 4
Levels (μg/g [ww]) of organochlorine compounds found in Bearded Vulture eggs (n = 20) in the Pyrenees during the period 1989–2012. The first column indicates the period at what eggs were collected: 1) 1989–1995; 2) 1996–2000; 3) 2001–2005; 4) 2006–2012.

	aHCH	bHCH	Lindan	Heptaclor	H. epox	Aldrin	DDE	DDD	DDT	HCB	PCBs
1	0.001	0.001	0.001	0.007	0.012		0.013				0.232
1	0.140	0.940	0.150		0.270	0.510	0.120		0.590	0.031	0.112
1	0.011	0.62	0.210		0.065		2.200		0.081	0.93	0.958
2			0.005		0.007	0.005	0.065	0.007		0.001	0.568
2	0.005		0.005	0.005	0.005	0.005	0.112	0.005	0.012	0.001	0.123
2	0.005	0.005	0.0015	0.001	0.007	0.005	0.152	0.021	0.032	0.02	0.225
3			0.005	0.061			0.629		0.587		0.450
3							0.319	0.032	0.029		0.135
3				0.098			0.059	0.005	0.185		0.445
3			0.084	0.108	0.064		0.068		0.169		0.128
3			0.005	0.057			0.061		0.202		0.023
3			0.005	0.082		0.005	0.063		0.067		0.221
2				0.036			0.361		0.033		0.426
3	0.005	0.005	0.005	0.042		0.025	0.253	0.005	0.098	0.322	0.287
4	0.005		0.128	0.122	0.068		0.995		0.287		0.774
4		0.009	0.071	0.771	0.055		1.093		0.112		0.688
3			0.005	0.057	0.995		0.061		0.202		0.856
4			0.025	0.115			0.568		0.162		0.256
3			0.012	0.064	0.148		0.319	0.032	0.029		0.321
3				0.036	0.005		0.361		0.033		0.098

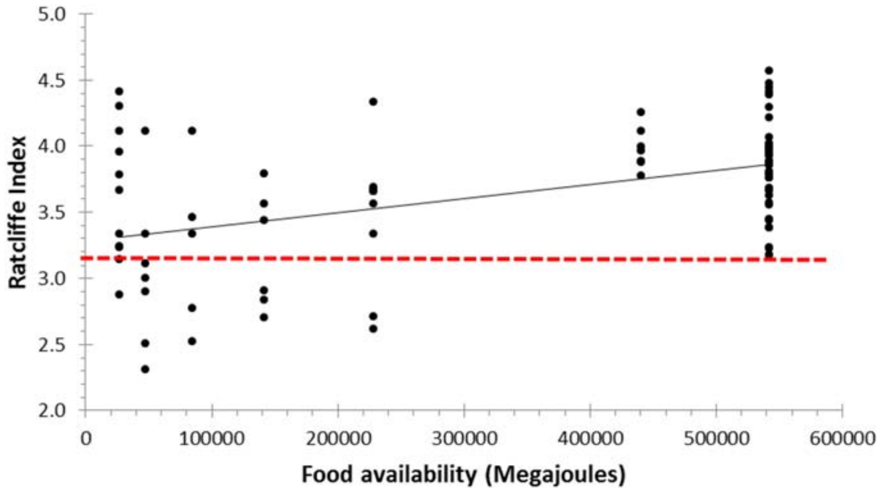

Fig. 4. Relationship between Ratcliffe Index of Bearded Vulture eggs and food availability between 1989 and 2012. Black line indicates the trend line whereas red dashed line indicates the minimum value of the Ratcliffe Index when the food available achieved their highest values (before 2004). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

between food quality and Ratcliffe Index in vulture eggs. A positive relationship between egg size and offspring fitness early in the chick-rearing period has been also been demonstrated (Krist, 2011). It has been suggested that variation in egg size might be the most important determinant of the probability of offspring survival during the first few days after hatching (Williams, 1994). Variation in egg composition is an adaptive mechanism; females may adjust egg composition facultatively to maximize reproductive investment in their offspring (Williams, 1994). The physiological characteristics of the female are also potential determinants of egg size and, therefore, of offspring fitness and probability of offspring survival (Christians, 2002).

Although the quantity of food available for Bearded Vultures in the Pyrenees could be enough to cover their energetic requirements (Margalida et al., 2017) there may be some effects on vulture demographics arising from food quality shortfalls (Margalida et al., 2014). The recent sudden changes in food availability seem to have had a significant impact on reproductive success through reduced egg quality (see Smith et al., 1981; Solomon, 1997). The progressive reduction in egg quality found in this study, mainly since 2006, could be due to the reduction in food availability affecting food quality. Application of EU sanitary regulations became increasingly intense from 2006, and coincides with significant changes in Bearded Vulture egg and eggshell parameters. Margalida et al. (2014) showed that this sudden reduction in food availability led to delays in laying dates and an increase in the proportion of single clutches, although it did not seem to affect the number of pairs that start breeding or the overall population trend (Margalida and Colomer, 2012; Margalida et al., 2014). Food shortages seem to have prompted Bearded Vultures to exploit less predictable food sources, possibly including wild prey species (Oro et al., 2008; Hernández and Margalida, 2008). Our findings show that PCBs values increased with the time suggesting a change in feeding habits that increased their exposure to lead poisoning since wild prey, including carcasses and offal from ungulates killed by hunters, may contain lead shot pellets or bullet fragments (Cade, 2007) and increases the risk of their consuming deliberately poisoned animals (Oro et al., 2008; Hernández and Margalida, 2009). However, the provision of specialized feeding stations obscures the effects of food shortages (Oro et al., 2008; Margalida et al., 2014). In this sense, most density-dependent explanations of reduced reproduction assume that smaller clutches, broods or breeding success arise from ecological constraints as the decrease of food resources (Lack, 1954; Newton, 1979; but see Simmons, 1993). These factors require further investigation, as do other issues related to the stresses associated with intraspecific interactions near to supplementary feeding stations and the density-dependent factors affecting fecundity (Carrete et al., 2006). On the other hand, the risk of exposure to topical antiparasitic pharmaceuticals present in livestock feet provided to feeding stations also needs further assessment (Mateo et al., 2015).

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