



Pollutants and diet influence carotenoid levels and integument coloration in nestlings of an endangered raptor

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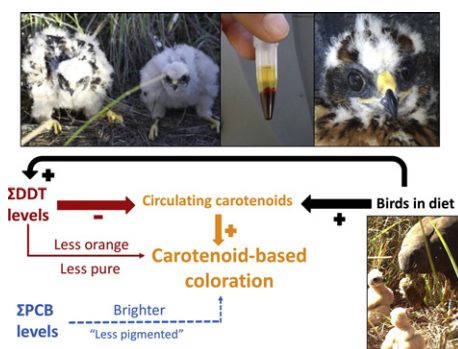
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HIGHLIGHTS

- PCBs and DDTs may affect the expression of bird carotenoid-based signals.
- Carotenoid-based coloration and plasma carotenoids increased with bird consumption.
- Carotenoids and coloration decreased in harrier nestlings with increasing DDT levels.
- Nestlings harriers with higher PCB levels had whiter, less pigmented integuments.
- Organochlorines may affect social communication based on carotenoid signals.

GRAPHICAL ABSTRACT



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ABSTRACT

Carotenoid-based traits or ornaments, such as yellow-red integuments (feathers, beaks, legs or eye-rings) displayed by birds, play key roles in social communication by reliably advertising an individual's quality or health. In some species, these traits are displayed not only by adults but also by nestlings, and function in parent-offspring communication or sibling competition by advertising an individual's physical or physiological condition. Pollutants such as organochlorine compounds (OCs) could have disruptive effects on the coloration of these traits, thereby interfering with communication processes. Such effects have been reported in adult birds, but are still largely unknown for nestlings. Here we investigated associations between polychlorinated biphenyl (PCB) and dichlorodiphenyltrichloroethane (DDT) blood-levels, circulating carotenoid levels and the yellow-orange coloration of the cere and tarsi of wild Black Harrier *Circus maurus* nestlings, a scarce raptor endemic to southern Africa. As carotenoid pigments must be acquired through the diet, we also tested for an effect of dietary composition. The orangeness-purity of cere and tarsi coloration positively correlated with circulating carotenoid levels, and increased with both nestling age and the proportion of birds consumed in the diet. Circulating carotenoid levels and the orangeness-purity of colored integuments were unrelated to blood PCB levels, although the brightness of integuments (i.e. lack of pigmentation) increased with PCB levels. Nestlings with more DDT had lower levels of circulating carotenoids and reduced carotenoid-based coloration (i.e. higher hue and lower saturation, reflecting a yellow rather than orange and less intense color, respectively). Together, our results are consistent with the hypothesis that OC contaminants, in particular DDT, may disrupt carotenoid-based signaling in exposed nestlings.

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1. Introduction

Carotenoids are pigments responsible for the red, orange and yellow coloration of integuments such as exposed skin, eye, cere, tarsus and plumages displayed by many avian species (Brush, 1990; Badyaev and Hill, 2000; Hill and McGraw, 2006). These carotenoid-based traits or ornaments play key roles in bird communication and in social interactions (Bortolotti et al., 2000; Costantini et al., 2007; Sternalsky et al., 2009) and are displayed by both adults and nestlings, sometimes from a very young age (Bortolotti et al., 2003; Sternalsky et al., 2009, 2011, 2012). In adults, colored traits are commonly used as an honest signal of individual quality such as a superior physical condition, foraging efficiency or ability to resist parasites (e.g. Hill and McGraw, 2006; Mougeot et al., 2007a). In the context of competition or mate choice, more colorful individuals are often dominant or more attractive (e.g. Goodwin, 1984; Webster et al., 2008; Lindsay et al., 2011). In nestlings, however, these colored displays have other functions, and likely play a role in parent-offspring communication, i.e. for parents to assess nestling condition and needs and so adjust their feeding and caring efforts (Lyon et al., 1994), or sibling competition, i.e. for sibs to assess each-other's competitive abilities and dominance (Biard et al., 2006; Sternalski et al., 2011, 2012). Understanding the factors that influence variation in the coloration of carotenoid-based traits is particularly relevant for understanding the functions and biological importance of these traits. In that respect, little is known about the potential effects that contaminants may have on the expression of carotenoid-based traits, and the broader implications for social communication.

Vertebrates cannot synthesize carotenoid pigments *de novo* but must ingest them; thus carotenoids may be a diet-limited resource (Goodwin, 1984). The total amount of carotenoid pigments available for an individual will depend on the quantity and the quality, in terms of carotenoid content, of the ingested food (Negro et al., 2002; Eeva et al., 2009). For predators, small mammal prey, for instance, are energy-rich but carotenoid-poor, while other prey such as birds, reptiles or insects have a greater carotenoid content (Goodwin, 1984). Dietary composition may, therefore, be important when studying variation in circulating carotenoid levels and the expression of carotenoid-based traits (e.g. Sternalsky et al., 2009), as well as interpreting the effect of persistent organic pollutants in birds (Mañosa et al., 2003). Carotenoids serve important health-related physiological functions: they can act as antioxidants and be used to limit oxidative damage (Pérez-Rodríguez, 2009). They can also boost the immune system through immuno-stimulation and immune-regulation functions (Faivre et al., 2003; Blount et al., 2003). Carotenoid-based traits have been proposed to reliably signal an individual's healthiness, or for instance the ability to deal with parasites (e.g. Mougeot et al., 2007a). In this context, only healthy individuals could afford to deposit carotenoids to increase their ornamental coloration, rather than using them to limit oxidative damage or boost their immune defenses (Lozano, 1994; Møller and Mousseau, 2001; Mougeot et al., 2009; Biard et al., 2010; Pérez-Rodríguez et al., 2013). The carotenoid-based coloration may also vary with individual characteristics, including nestling age, sex (Sternalski et al., 2009, 2011, 2012), hatching order (i.e. rank within the brood) or condition (Senar et al., 2003; Sternalsky et al., 2009, 2012). It seems, therefore, important to consider these variables, as they may help explain variations in physiological aspects that may ultimately influence future reproductive outcomes of breeding birds (Eeva et al., 2012; Sassani et al., 2016).

Carotenoid-based coloration and circulating carotenoids have been shown to be affected by both non-organic contaminants such as heavy metals (Camplani et al., 1999; Møller and Mousseau, 2001; Eeva et al., 2008, 2009; Giraudeau et al., 2015; Vallverdú-Coll et al., 2015, 2016a) and organic contaminants such as persistent organic pollutants (POPs) (McCarty and Secord, 2000; Bortolotti et al., 2003; López-Antia et al., 2013; Blévin et al., 2014; López-Antia et al., 2015a, 2015b) or fuel oil pollution (Pérez et al., 2010). Pollutants could thereby potentially interfere with communication processes that rely on carotenoid-colored traits

(see Marasco and Costantini, 2016). For example, the carotenoid-based coloration of eye-rings, gapes and tongues of adult female Kittiwakes *Rissa tridactyla* decreased with increasing levels of POPs in the blood (Blévin et al., 2014). By contrast, no association was found between POPs contamination and the carotenoid-based coloration of integuments of breeding Great Black-Backed Gulls *Larus marinus* (Bustnes et al., 2007). Several studies have related exposure to pollutants to both circulating carotenoids and carotenoid-based coloration (López-Antia et al., 2013, 2015a, 2015b; Vallverdú-Coll et al., 2015, 2016a; García-de Blas et al., 2016), but few of them have been performed with birds in the wild (Bortolotti et al., 2003; Eeva et al., 2008; Vallverdú-Coll et al., 2016b). In addition, most studies have been conducted on adult birds, but relatively few have been undertaken with nestlings and the knowledge and understanding of how these contaminants affect them is particularly lacking.

The main goal of the present study was to investigate whether OCs affect the circulating carotenoid levels and the carotenoid-based coloration of integuments (yellow-orange cere and tarsi) developed by Black Harrier *Circus maurus* nestlings. Unlike feathers, the carotenoid-based coloration of fleshy integuments is dynamic, with the potential for rapid change (Velando et al., 2006; Pérez-Rodríguez, 2008). Therefore, changes in coloration of integuments such as cere and tarsi and in circulating carotenoid levels may reflect a more recent intake of carotenoids and recent changes in individual condition (Faivre et al., 2003; López et al., 2011).

The Black Harrier is a scarce medium-sized raptor, endemic to southern Africa, and classed as Endangered in South Africa, Namibia and Lesotho (Simmons et al., 2015; Taylor, 2015). This ground-nesting bird of prey breeds in indigenous vegetation of south western South Africa, preferentially along the coast within the Fynbos biome, and also inland within the Karoo biome (Curtis et al., 2004; Curtis, 2005; García-Heras et al., 2016). Like most raptors, Black Harriers display carotenoid-based coloration from a young age, i.e. a few days after birth. Black Harrier broods include generally 2 to 4 nestlings (Simmons, 2000; Simmons et al., 2005) and competition for food can be intense in harsh conditions, sometimes leading to chick mortality (Simmons, 2000; MSGH, *personal observations*). A recent study revealed the presence of OCs in both adults and nestlings (García-Heras et al., *under review*). PCBs (PCBs 52, 101, 138, 153, 180) and DDTs (p,p'-DDT and its metabolite p,p'-DDE) were detected in the blood plasma of 82% and 81% of nestlings, respectively. Detected concentrations were high enough to induce physiological effects, such as increased heterophil to lymphocyte ratio in PCB-exposed individuals and increased white blood cell counts in DDT-exposed individuals, but had no effect on nestling's body condition (García-Heras et al., *under review*). Black Harriers prey mostly on small mammals, but also on birds such as Common Quails (*Coturnix coturnix*) as alternative prey (García-Heras et al., 2017a, b). Birds are generally known to bio-accumulate more OCs than small mammals (Fossi et al., 1995; Mañosa et al. 2003; van Drooge et al. 2008), and García-Heras et al. (*under review*) found a positive association between blood-p,p'-DDE levels and the proportion of bird biomass in Black Harriers diet. In this context, diet may affect carotenoid availability directly because small mammals are carotenoid-poor prey compared with birds, as well as indirectly because, in this region, individuals eating more birds are also more exposed to DDTs (García-Heras et al., *under review*).

We first tested whether carotenoid levels and carotenoid-based coloration varied with nestling age, sex, rank, physical condition and dietary composition. We expected well-fed nestlings in better condition (i.e. mass corrected for age) to be more colored, and individuals eating more birds to be less carotenoid-limited and more colored. Second, we tested whether circulating carotenoid and coloration varied with DDT and PCB levels, and expected exposed nestlings to display less colored integuments, because they may allocate available carotenoids towards detoxification to counter adverse physiological effects of OCs.

2. Material and methods

2.1. Study sites and data collection

Fieldwork was conducted in South Africa during the 2012, 2013 and 2014 Black Harrier breeding seasons (July to December). Study nests were located along the coast of the Western Cape Province (33.700°S, 18.450°E; 33.133°S, 18.083°E) and inland in the Northern Cape Province in the Nieuwoudtville area (31.316°S, 19.083°E). Breeding sites were in National Parks (South African National Parks – SANParks properties), Provincial Protected Reserves (Cape Nature), or on private land (see García-Heras et al., 2016).

Nestlings were measured and sampled during nest visits when 15–39 days old ($n = 118$, 63 females and 55 males). Each was individually marked with a metal and a color ring, with a unique alpha-numeric code. The rank of each nestling within the brood (i.e. hatching order, rank 1 being the first hatched chick) was also determined from nestling age, itself determined from biometrics. We measured tarsus length to the nearest 0.1 mm using an electronic caliper, and body mass to the nearest 5–10 g using a Pesola balance, and subsequently calculated a body condition index (i.e. physical condition) using the residuals from the relation between the body mass and the age of nestlings, calculated for each sex separately.

We collected a blood sample from the brachial vein (0.7–1 ml) using a heparinized syringe. The blood was stored in a heparinized Eppendorf vial and kept in a polystyrene cool box previously filled with ice blocks. Within 30–40 min after collection, the samples were centrifuged for 15 min with a mini portable centrifuge (VWR, Radnor, Pennsylvania) to separate the plasma from the red cells. Both set of samples were immediately placed in a portable freezer, and frozen at -80°C on arrival at the lab from the field (< 3 h after collection) until analysis. Plasma samples were used to identify and quantify the concentrations of OCs and circulating carotenoids. Red blood cell samples were used to determine the sex of nestlings by molecular procedures using the primers 0057F and 002R (Round et al., 2007) and protocols described in García-Heras et al. (under review). We obtained blood samples from a total of 101 nestlings from 44 nests. Twenty-three nestlings were blood sampled twice: at 15–20 days old, and again at 30–39 days old. Fieldwork protocols were approved by the University of Cape Town's science Faculty Animal Ethics Committee (Permit number: A1/2014/2013/V21/GC).

2.2. Dietary composition

Diet information was available for a total of 29 nests and 63 nestlings. We assessed dietary composition at each nest either by analyzing bones, scales, feathers or hairs within pellets collected at the nest ($n = 17$ nests), by analyzing video and picture footage from cameras set at nests ($n = 2$ nests), or by using a combination of both techniques ($n = 10$ nests). Estimates of diet from pellets and cameras correlated with each other when the proportion of unidentified prey in cameras was low (see supplementary material in García-Heras et al., 2017b). Identified prey were categorized as small mammal, bird or reptile (García-Heras et al., 2017a), and the percentage biomass contributed by each prey category was estimated by allocating an average weight to each category (García-Heras et al., 2017a). The contribution of reptiles to consumed biomass was $< 5\%$, and the proportion of bird biomass was thus strongly negatively related to the proportion of small mammal biomass (Pearson correlation: $r = -0.98$, $p < 0.0001$, $n = 29$). Therefore, variations in diet composition are reflected by variations in the proportion of bird biomass, and we only used this variable in analyses, as an indicator of dietary composition. An average (\pm SE) of 55.4 ± 0.21 prey items (min-max: 10–164) were identified for each nest. Ten prey was determined to be sufficient for an accurate estimation of the proportion of birds or small mammals in the diet using bootstrapping analyses (supplementary material in García-Heras et al., under review).

2.3. Plasma concentrations of organochlorine compounds and carotenoids

OCs were identified and quantified using the protocol described in García-Heras et al. (under review). In short, we looked for PCB and DDT components present in the Pesticide-Mix 13 (Dr. Ehrenstorfer standard). Plasma samples were extracted and cleaned up following the methods described and validated by Mateo et al. (2012). These are based on the sulfuric acid clean-ups using the n-hexane extraction procedure, and organochlorine concentrations were measured by gas chromatography coupled to an electron capture detector (GC-ECD) equipped with a column HP-5 30 m, 0.32 mm, 0.25 μm , both from Agilent Technologies. OC levels (expressed in ng/ml) were determined for 90 nestlings comprising 50 females and 40 males, each nestling sampled only once.

Carotenoids were extracted from the plasma following a procedure validated by García-de Blas et al. (2013), and identified and quantified using high performance liquid chromatography (HPLC) coupled with a photodiode detector. Briefly, 50 μl of plasma were put into a polypropylene microtube, in which 200 μl of distilled water and 150 μl of ethanol were added. The headspace tube was filled with nitrogen to avoid carotenoid oxidation, vortexed for 5 min and vibrated with ultra-sound (sonicated) for 1 min. The mixture was then extracted three times with 1 ml of hexane using vortex mixing for 15 min each time. Hexane phases were recovered after centrifuging for 5 min at 2500 g and at 4°C . The supernatant (i.e. hexane phase) was extracted, combined and put into a long tube kept in the dark. The supernatant was then evaporated to dryness under a stream of nitrogen. Residues were immediately re-dissolved with 100 μl of methanol, transferred to a glass vial for HPLC analysis with an Agilent Technologies 1200 Series. The HPLC method used to separate, identify and quantify the carotenoid pigments present in the samples is described in Rodríguez-Estival et al. (2010). We identified two main carotenoids in the plasma of nestling Black Harriers, lutein and zeaxanthin (see Supplementary Material), as found in most raptor species (Hill and McGraw, 2006). Based on a sub-sample of 67 analyzed plasma, we found that lutein and zeaxanthin levels were strongly positively correlated ($r = 0.88$, $p < 0.0001$, $n = 67$). To simplify the statistical analyses, and because in some chromatograms both substances co-eluted, we summed the lutein and zeaxanthin levels and used a total carotenoid concentration in all analyses. Circulating carotenoid levels (expressed in nmol/ml) were thus determined for a total 101 nestlings, 58 females and 43 males.

2.4. Carotenoid-based coloration

To measure the carotenoid-based coloration, we took digital photographs of the cere and tarsi of each nestling using a Panasonic DMC-FZ38 digital camera. All pictures were taken by the same person (MSGH) with the same camera, at a standard distance of 40 cm between the objective lens and the body parts, avoiding direct sun light. The same yellow board card and color chart were placed next to each photographed body part (i.e. cere or tarsus) to standardize color measurements. Each integument was photographed three times, and the best quality picture (one for each integument part) was selected for analysis. Photographs were analyzed using Adobe Photoshop (CS5), a method used previously to study coloration in raptors (Martínez-Padilla et al., 2013) or other birds (Mougeot et al., 2009; Vallverdú-Coll et al., 2016a). For each body part, we selected a homogeneous area with the magic wand tool, and calculated its three-color characteristics in the HSB color space: hue (H), saturation (S) and brightness (B). These measurements were repeated four times and we used average values. We did the same with the yellow standard reference to standardize the body part measurements among all photographs.

The hue reflects the perceived color of the integument (e.g. yellow, orange, red), while the saturation indicates the intensity and the purity of a color. The brightness reflects the “quantity of white” present in each color (Hill and McGraw, 2006). The digital photography method was preferred to other methods, such as spectrophotometry, because

of fieldwork constrains and because it is better suited to quantify the coloration of irregular surfaces such as cere and tarsi (Montgomerie, 2006). We obtained color measurements of both for a total of 115 nestlings (54 males and 61 females), with 37 of these being measured up to three times during their growth.

The values of hue, saturation and brightness of the cere and the tarsi were positively correlated (Pearson correlations: Hue: $r = 0.32$, $p < 0.0001$, $n = 115$; Saturation: $r = 0.64$, $p < 0.0001$, $n = 115$; Brightness: $r = 0.43$, $p < 0.0001$, $n = 115$). Therefore, to reduce the number of coloration variables and to account for collinearity among them, we conducted a Principal Component Analysis (PCA) on the 6 coloration variables, i.e. hue, saturation and brightness of both cere and tarsi. The first Principal Component (hereafter coloration-PC1) explained 44% of the variance, and the second Principal Component (hereafter coloration-PC2) explained a further 23% of the variance (Table 1). High values of coloration-PC1 were indicative of a lower hue value and a greater saturation, i.e. cere and tarsi that were more orange than yellow, and with a purer and more intense coloration. This was indicative of a greater carotenoid-based coloration of these integuments. High values of coloration-PC2 were indicative of a greater brightness (whiteness), thus indicating integuments that were less pigmented overall, with more reflectance from the unpigmented (i.e. white) skin (Mougeot et al., 2007b).

2.5. Statistical analyses

All statistical analyses were conducted using R 3.2.3 (The R Foundation for statistical computing, 2015).

Because sample sizes varied depending on the variables measured (i.e. not all data could be measured for all nestlings), we conducted two sets of General Linear Mixed Models (GLMMs) to investigate how coloration and circulating carotenoids varied with 1) age, sex, nestling rank, body condition index, and dietary composition ($n = 93$ samples from 63 nestlings from 29 nests,) and 2) age, diet, and contaminants (Σ PCB and Σ DDT, which includes p,p'-DDE and p,p'-DDT) ($n = 56$). We initially also considered lay date as an explanatory variable in our models, but it was correlated with dietary composition (the occurrence of birds in the diet of nestling increases as the breeding season progresses; García-Heras et al. 2017b). To avoid collinearity issues, we thus only considered the % of bird biomass in our statistical models, considering this variable more biologically meaningful to explain carotenoid intake and availability. Circulating carotenoid levels were log-transformed to adjust for normality. Dependent variables were fitted to models using a normal distribution (package lme4, log function, Bates et al., 2012). Nest identity and nestling identity were included as random effects in all models to account for the non-independence of samples coming from the same nest and the repeated measures on some nestlings.

In the first set of models, we tested whether the coloration PCs and circulating carotenoid levels varied with age (from 15 to 39 days old, continuous variable; reflecting changes during nestling growth), sex, brood rank (from 1 to 4, continuous variable; reflecting effects of

hatching asynchrony and nestling competition), body condition index (continuous variable) and dietary composition (proportion of ingested bird biomass, square root transformed to adjust better to a linear model). We also included the interaction age \times sex. Non-significant interactions and variables were removed from the initial models using a stepwise backward procedure and we present the results of the final models (Type III results).

In the second set, we investigated additional effects of OC levels (Σ PCB as the sum of all detected PCBs, and Σ DDT as the sum of all detected DDTs) on coloration and circulating carotenoids, adding these variables as explanatory variables to those that were retained in previous models. We checked for correlations and collinearity among these explanatory variables and calculated their Variance Inflation Factors (VIF). All VIF values were below 1.4, indicating that collinearity was not an issue (Zuur et al., 2010). For a better adjustment to a linear model, Σ PCB was log-transformed. These models were performed using data from 56 individuals (each sampled once).

3. Results

Plasma concentrations of carotenoids averaged 10.7 ± 6.5 nmol/ml (minimum-maximum: 0.7–36.7). Coloration-PC1 (i.e. hue-saturation) was positively correlated with circulating carotenoids ($\chi^2 = 38.37$, d.f. = 1, $p < 0.0001$; slope: $+1.270 \pm 0.205$; $n = 93$), indicating that higher circulating carotenoid levels were associated with a more orange and purer coloration of integuments (Fig. 1). No significant correlation was found between coloration-PC2 (i.e. brightness) and circulating carotenoids ($\chi^2 = 0.029$, d.f. = 1, $p = 0.865$; $n = 93$).

3.1. Carotenoid-based coloration, body condition and dietary composition

Circulating carotenoids and coloration-PC1 increased with nestling age: older nestlings had more circulating carotenoids and developed a more orange and purer coloration than younger ones (Table 2, Fig. 2a,d). The coloration-PC2, however, did not vary with age (Table 2). Circulating carotenoids, coloration-PC1 and PC2 did not differ between sexes, and were not associated with brood rank or body condition index (Table 2). The latter indicated that relatively heavier nestlings were not more colorful than lighter ones.

Circulating carotenoids and coloration-PC1 both increased with the percentage of bird biomass in nestlings' diet (Table 2, Fig. 2b,e). Nestlings consuming more birds had more circulating carotenoids and integuments of a more orange and purer color than that of nestlings consuming more small mammals. No association was found between diet and coloration-PC2 (Table 2).

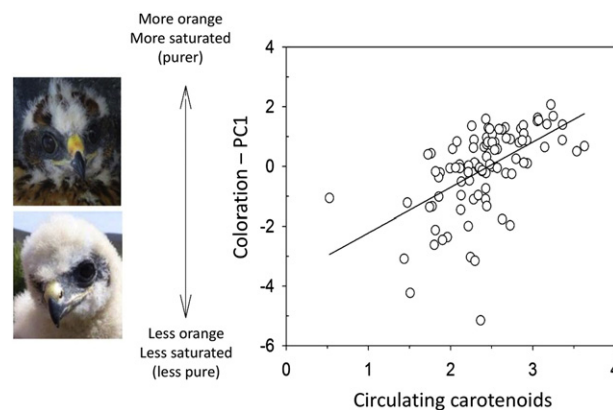


Fig. 1. Relationship between coloration-PC1 (i.e. hue-saturation) and circulating carotenoids levels (in nmol/ml, log transformed) in wild Black Harrier nestlings.

Table 1

Results of the Principal Component Analysis conducted on the six coloration measurements (i.e. hue, saturation and brightness of the cere and tarsi) for Black Harrier nestlings ($n = 124$). Variable loadings >0.4 or lower than -0.4 are highlighted in bold.

	PC1	PC2
Cere Brightness	-0.18	0.68
Tarsus Brightness	-0.01	0.71
Cere Hue	-0.48	-0.18
Tarsus Hue	-0.42	0.04
Cere Saturation	0.53	0.08
Tarsus-Saturation	0.53	0.04
Variance explained		
Proportion	0.44	0.23
Cumulative		0.67

Table 2

Results of GLMMs testing for the effects of nestling age, sex, body condition index, rank and dietary composition (% of bird biomass in diet) on circulating carotenoid levels and carotenoid-based coloration (coloration-PC1: hue-saturation; coloration-PC2: brightness; see Table 1). Significant variables are highlighted in bold, and parameter estimates for those variables are also included. Models were performed using 93 data points from 63 nestlings. Nest and nestling identity were both included as random effects in models. d.f. = degrees of freedom.

Independent variables	Explanatory variables	Chi-square	d.f.	P	Estimate \pm SE
Circulating carotenoids*					Intercept:
	Age*	4.123	1	0.042	0.821 ± 0.528
	%Bird in diet[§]	23.953	1	<0.0001	0.306 ± 0.151
	Sex	0.156	1	0.693	1.511 ± 0.309
	Body condition	0.805	1	0.369	
Coloration-PC1	Rank	0.003	1	0.959	
					Intercept: -10.547 ± 1.548
	Age*	40.831	1	<0.0001	2.846 ± 0.445
	%Bird in diet[§]	10.643	1	0.001	2.535 ± 0.777
	Sex	1.628	1	0.202	
Coloration-PC2	Body condition	1.137	1	0.286	
	Rank	1.539	1	0.215	
	Age*	0.909	1	0.340	
	%Bird in diet[§]	0.961	1	0.327	
	Sex	0.425	1	0.515	
	Body condition	1.174	1	0.259	
	Rank	0.026	1	0.872	

[§]Square-root transformed.

*Log transformed.

3.2. Carotenoid-based coloration and organochlorine compounds

In sampled nestlings, Σ PCB levels averaged 3.5 ± 3.1 ng/ml (minimum-maximum: 0–13.7 ng/ml), while Σ DDTs (p,p'-DDE + p,p'-DDT) levels averaged 1.9 ± 2.0 ng/ml [minimum-maximum: 0–9.8 ng/ml]. We found a significant negative association between DDT levels and carotenoid levels, coloration-PC1 and coloration-PC2 (Table 3). Nestlings with more DDTs circulated fewer carotenoids (Fig. 2f) and had yellower rather than orange, less saturated and less bright colored traits. Coloration-PC2 was positively associated with PCB levels: nestlings exposed to PCBs displayed brighter (i.e. whiter) cere and tarsi. No significant

associations were found between PCB levels and circulating carotenoids or coloration-PC1 (Table 3).

4. Discussion

We investigated the associations between diet, OCs, carotenoid-based coloration, and circulating carotenoid levels in nestlings of a wild raptor species, and contribute novel knowledge by revealing that both diet and OCs contamination influence the expression of carotenoid-based traits displayed by nestlings. Detected OC levels in our study were within the range or slightly higher than those found in recent studies conducted on

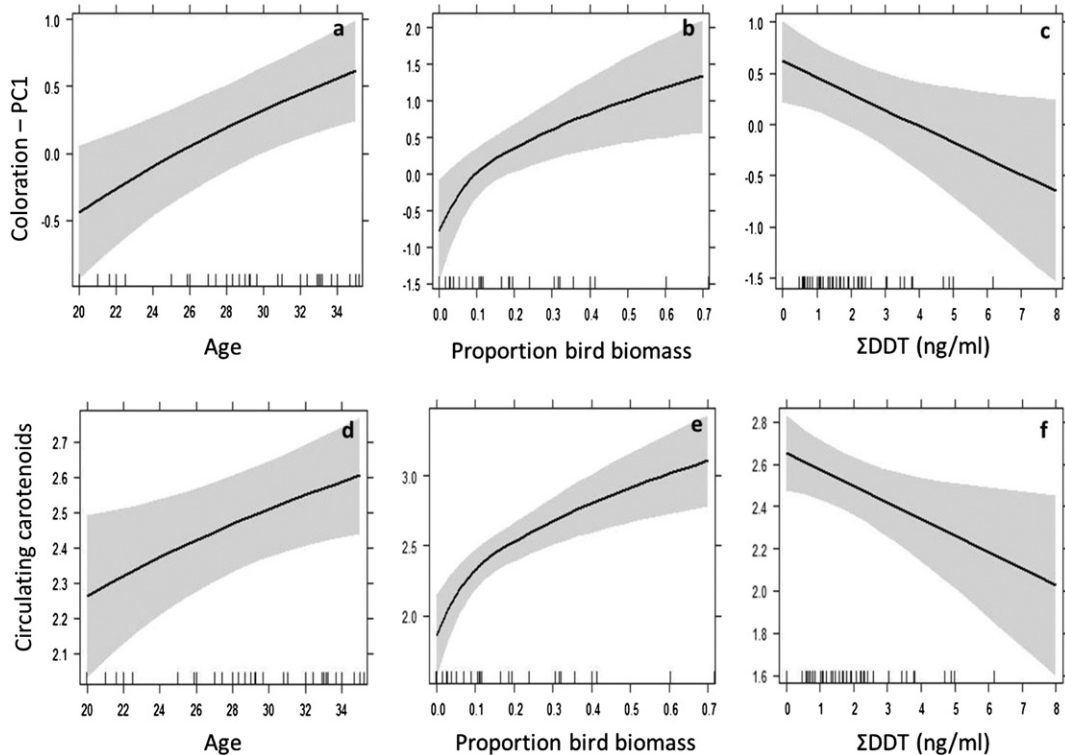


Fig. 2. Results of the models (fitted curve: black lines; 95% confidence intervals in grey) testing for associations between coloration-PC1 or circulating carotenoid levels and: (a,d) nestling age, (b,e) dietary composition (% of bird biomass in diet), (c,f) Σ DDT levels. Model outputs are given in Table 3. Plots with the raw data are shown in Fig. S3 (Supplementary Material).

Table 3
Results of GLMMs testing for the effects of age, dietary composition (% of bird biomass in diet), and Organic Compound (Σ PCB and Σ DDT) levels on circulating carotenoids and carotenoid-based coloration (Coloration-PC1: hue-saturation; Coloration-PC2: brightness; see Table 1). Significant variables are highlighted in bold, and parameter estimates for those variables are also included. Models were performed with 56 nestlings. Nest identity was included as a random effect in models. d.f. = degrees of freedom.

Independent variables	Explanatory variables	Chi-square	d.f.	P	Estimate \pm SE
Circulating carotenoids*	Age*	5.966	1	0.015	Intercept: -0.184 ± 0.919
	%Bird in diet[§]	20.001	1	< 0.0001	0.636 ± 0.260
	ΣDDT	5.782	1	0.016	1.494 ± 0.334
	ΣPCB*	1.306	1	0.253	-0.079 ± 0.033
Coloration-PC1	Age*	12.976	1	0.0003	Intercept: -7.262 ± 1.928
	%Bird in diet[§]	10.227	1	0.001	1.971 ± 0.547
	ΣDDT	5.684	1	0.017	2.527 ± 0.790
	ΣPCB*	1.839	1	0.175	-0.159 ± 0.066
Coloration-PC2	ΣDDT	5.150	1	0.023	Intercept: 0.211 ± 0.278
	ΣPCB*	4.385	1	0.036	-0.180 ± 0.079
	Age*	0.339	1	0.560	0.394 ± 0.188
	% Bird in diet[§]	1.128	1	0.288	

*Log transformed.

[§]Square-root transformed.

other raptors (e.g. Sonne et al., 2012; Bustnes et al., 2013; Eulaers et al., 2014; Gómez-Ramírez et al., 2014), and for which adverse physiological effects have been reported (e.g. Ortiz-Santaliestra et al., 2015).

4.1. Variation in carotenoid levels and coloration according to age and body condition

Black Harrier nestlings circulating more carotenoids had a more orange and purer coloration of integuments. The orangeness of cere and tarsi and the circulating carotenoid levels increased with nestling age. This may reflect an increasing absorption, deposition and accumulation of circulating carotenoids throughout nestling growth (distinguishing it from carotenoids received from the egg yolk, or stored in fat or in the liver; Hill and McGraw, 2006). Such accumulation of carotenoids can be allocated towards nestling coloration or for self-maintenance needs (Casagrande et al., 2007; see also below). As carotenoid pigments in animals must be acquired through the diet, their accessibility will depend on the type of food, but could also depend on the quantity of the ingested food. When prey type does not vary, nestlings that receive more food would have also acquired more carotenoids and should be more colored as well as in better physical condition (relatively heavier). Under such a scenario, carotenoid-based coloration is expected to be condition-dependent (Hill, 1990; Senar et al., 2003; Tschirren et al., 2003; Biard et al., 2006; McGraw and Hill, 2006). In our study, however, no significant associations were found between carotenoid-based coloration or circulating carotenoids and a nestlings' body condition index: heavier nestlings were not more colorful, nor did they have more circulating carotenoids than lighter ones. This may arise when carotenoid intake primarily depends on the quality (carotenoid content) of the ingested food rather than its quantity. This concurs with results found in wild Montagu's Harrier *Circus pygargus* and Western Marsh Harrier *Circus aeruginosus* nestlings (Sternalsky et al., 2009, 2012), and in Great Tits *Parus major* nestlings (Eeva et al., 2009). It may also explain why we did not find differences in carotenoid levels or coloration between male and female nestlings, or according to nestling rank (hatching order), as reported elsewhere. These differences are usually associated with differences in food quantity rather than quality among nestlings of different sex or rank (Magrath, 1990; MacWhirter, 1994; Bortolotti et al., 2003; Sternalsky et al., 2009, 2012).

4.2. Variation in carotenoid levels and coloration according to diet and OC levels

While the coloration of feathers reflects an earlier availability and accumulation of carotenoid pigments (during moult), the coloration of

integuments such as cere and tarsi usually reflects a more recent intake or mobilization of carotenoids (López et al., 2011). As expected, we found that nestlings feeding on a diet rich in birds had higher levels of circulating carotenoids and displayed a greater carotenoid-based coloration than those feeding regularly on small mammals. Our results are in accordance with other studies showing the tight link between the expression of the carotenoid-based coloration and the type and amount of carotenoid pigments ingested by wild vertebrates (Hill et al., 2002; Negro et al., 2002; Eeva et al., 2008, 2009; Sternalski et al., 2009). Indeed, small mammals are known to be carotenoid-poor, unlike other birds, reptiles and insects (Goodwin, 1984). In this context, feeding on small mammals, the Black Harrier's primary prey (García-Heras et al., 2017a, b), may be profitable for nestlings in terms of energy gain, but consuming more birds seems also to be beneficial as it increases carotenoid availability; this in turn has health-related physiological benefits (Lozano, 1994). On the other hand, we also demonstrated that the greater the proportion of ingested bird biomass in the diet, the higher the levels of blood Σ DDT in nestlings and in adults (García-Heras et al., under review). This is consistent with other studies that reported a positive link between the consumption of bird prey and OC exposure (Fossi et al., 1995; Mañosa et al., 2003; van Drooge et al., 2008). Toxic substances in diet, such as Σ DDT, can impose stress that alters nutrient requirements or use (Phillips and Hidirolglou, 1965). For instance, rats fed over 10 mg/kg of DDT in the diet decreased utilization of orally administered carotene and vitamin A (Phillips, 1963). Similarly, cows fed with forage containing 40 to 60 mg/kg of o,p'-, p,p'-DDT (dry weight) exhibited lower levels of carotenoids and vitamin A in both serum and liver (Phillips and Hidirolglou, 1965). It may be reasonable to expect a reduction in the levels of circulating carotenoids in Black Harrier nestlings with higher levels of DDTs, which in turn may lead to a reduced carotenoid-based coloration of integuments. Furthermore, several organic contaminants (e.g. PCBs and perfluoroalkoxy alkanes -PFAs) have been shown to alter various physiological processes, causing changes in endocrine (Rattner et al., 1984) and neuroendocrine pathways (Frye et al., 2011) and in oxidative balance (Whysner and Wang, 2001). These changes may induce a disruption of the coloration of integuments and/or plumages (Surai, 2002; Bortolotti et al., 2003; Marasco and Costantini, 2016). Our results indeed show that DDT-contaminated Black Harrier nestlings had significantly lower levels of circulating carotenoids and reduced carotenoid-based coloration (yellow rather than orange and less saturated integuments). In this context, the beneficial "extra-amount" of circulating carotenoids obtained by harrier nestlings eating birds (rather than small mammals) seems to be compromised when consuming birds that simultaneously expose harriers to DDT-contamination. Future experimental studies will, however, be necessary to confirm this. Additionally, we found a

negative association between DDT levels and coloration-PC2 indicating a reduced brightness of integument color. This is rather counter-intuitive, because a reduced brightness (lower PC2 values) would be indicative of a less white, more pigmented integument. Since PC2 is unrelated to circulating carotenoid levels and unrelated to PC1 by definition, this result suggests that the two characteristics of integument coloration (hue and saturation versus brightness) are affected by contaminants via different pathways (the former being more clearly linked with carotenoid availability than the latter).

To the best of our knowledge, our study is the first to report an association between blood-DDT levels, integument coloration and circulating carotenoids in a bird species. Similar results were, however, found in amphibians where DDT-congeners have been shown to negatively affect the endocrine system in the Asian Common Toad *Bufo melanostictus* and in the Reed Frog *Hyperolius argus*, resulting in changes in the skin-coloration of individuals (Noriega and Hayes, 2000).

We also found that the brightness of cere and tarsi color significantly increased with blood-PCB levels. As emphasized above, it is unclear to what extent the brightness of cere and tarsi is dependent upon carotenoid availability and allocation priorities. Higher PC2 values could be indicative of whiter, less pigmented integuments. Alternatively, variation in integument brightness could be unrelated to carotenoid availability. PCBs are known to activate the cytochrome P450 enzyme system (which acts on the cycles of oxidation-reduction and generates free radicals; Schelzinger et al., 1999), and may thereby increase the production of pro-oxidant molecules, which may in turn cause oxidative stress. PCBs may also cause a de-regulation of specific antioxidants, which in turn would also amplify the effects of oxidative stress (Marasco and Constantini, 2016), and concomitantly affect the brightness of integument coloration of Black Harrier nestlings. By increasing oxidative stress, PCBs could affect carotenoid allocation priorities: carotenoid pigments have antioxidant properties and their allocation to colored integuments is usually reduced under conditions of increased oxidative stress (e.g. Mougeot et al., 2010; Perez-Rodríguez et al., 2010; Vallverdu-Coll et al., 2015, 2016a).

In a previous work, we found no association between the levels of OCs (DDTs or PCBs) in nestlings' blood and their body condition (García-Heras et al., under review). Here, we found no relationships between carotenoid-based coloration or circulating carotenoid levels and the body condition of nestlings, indicating that the reported effects of OCs, and in particular of DDT, on carotenoid-based coloration were unlikely to be mediated via effects on body condition, but would rather be mediated by physiological effects (e.g. detoxification need, increased oxidative stress or reduced immunity).

PCB- (and DDT-) exposure may also have important health implications for nestlings, which may be more vulnerable to other threats, for example more prone to parasite infection (López et al., 2011). Only a few studies have related OC exposure to indicators of health, but with inconsistent results (Bustnes et al., 2004, 2007; Rivera-Rodríguez and Rodríguez-Estrella, 2011; Ortiz-Santaliestra et al., 2015). While Bustnes et al. (2004) showed an increase of the numbers of heterophils with higher OC levels in adult breeding Glaucous Gulls *Larus hyperboreus*, no association between white blood cell counts and OC levels were found in the Great Black-Backed Gulls *Larus marinus* (Bustnes et al. 2007). In adult and nestling Bonelli's Eagles *Aquila fasciata* contaminated with PCBs at similar levels to the Black Harriers in our study, Ortiz-Santaliestra et al. (2015) found a reduction of dietary antioxidants (i.e. circulating carotenoids) and of alkaline phosphatase (indicators of osteoblastic activity). Similarly, levels of PCBs and DDTs equivalent to Black Harriers' were found to affect several blood clinical-chemical parameters in Golden Eagles *Aquila chrysaetos*, Northern Goshawks and White-Tailed Eagles *Haliaeetus albicilla* (Sonne et al., 2012; Bustnes et al., 2013; Eulaers et al., 2014). In this context, further studies should be undertaken to investigate how these OCs may physiologically compromise Black Harrier nestlings as appears to be the case in other raptors (Rivera-Rodríguez and Rodríguez-Estrella, 2011; Ortiz-Santaliestra et al., 2015).

5. Conclusions

Our results are consistent with the hypothesis that organic contaminants disrupt carotenoid-based coloration, and may thereby affect communication processes in birds and other vertebrates that rely on these colored traits (McCarty and Secord, 2000; Bortolotti et al., 2003). Black Harrier nestlings feeding on more birds had higher levels of circulating carotenoids (which have important health-related physiological functions), but were also more exposed to DDT-contamination. In turn, and paradoxically, DDT-exposed nestlings appeared more carotenoid-limited as they circulated relatively lower carotenoid levels, and developed less orange carotenoid-based integuments. Therefore, the potential benefits of eating carotenoid-rich prey such as birds seemed to be compromised when a nestling was simultaneously exposed to DDTs. An influence of OCs on the expression of carotenoid-based traits may have further implications given their communication roles. Further research is needed to better understand the mechanisms linking OC exposure and carotenoid-based coloration, as well as the broader implications for social communication.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.scitotenv.2017.06.048>.

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