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Oil pollution increases plasma antioxidants but reduces coloration in a seabird

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Abstract It has been suggested that condition-dependent signals may be a useful measure of environmental quality. In this study, we tested the hypothesis that oil pollution enhances oxidative stress and impairs expression of a carotenoid-based signal in a wild population of the yellow-legged gull (*Larus michahellis*). During the courtship period, a group of gulls were fed a supplementary diet containing heavy fuel oil from the *Prestige* oil spill and were compared with control gulls fed a similar supplementary diet without fuel oil. Blood levels of polycyclic aromatic hydrocarbons, the most toxic components of crude oils, were higher (30%) in the *Prestige* oil-fed gulls than in the control gulls. Plasma concentrations of vitamin E and carotenoids were also significantly higher in the *Prestige* oil-fed gulls (31 and 27%, respectively). Although, the plasma levels of lipid peroxidation markers were higher (13%) in gulls fed with *Prestige* oil than in the control gulls, these differences were not significant, possibly because of the small number of gulls analyzed. The red bill spot was significantly smaller (16%) in the oil-fed gulls than in the control individuals. This study provides the first experimental evidence that a carotenoid-based signal in a free-living seabird is affected by exposure to oil pollution and is hence indicative of environmental quality. Since the yellow-legged gull belongs to a complex of species widely distributed throughout the northern hemisphere, the method described may constitute a useful tool for evaluating sub-lethal effects of oil spills in seabirds.

Keywords Bioindicator · *Larus michahellis* · Oil pollution · Oxidative stress · Sexual selection

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Introduction

Many animals exhibit elaborate ornamental traits that have evolved as signals of the bearer's quality and can be evaluated by prospective mates or opponents (Andersson 1994). The honesty of these traits is often based on the fact that their maintenance entails an associated cost that only can be afforded by higher quality animals (see Zahavi and Zahavi 1997). The expression of such traits thus often signals reliable information about the physiological condition of the bearer (Hamilton and Zuk 1982; Grafen 1990). Sexual signals display high phenotypic plasticity (Cotton et al. 2004), and their expression relative to other traits (Peters et al. 2008) is particularly sensitive to the cascade of physiological mechanisms produced by stressful events (Hill 1995; Buchanan 2000). Accordingly, it has been suggested that condition-dependent signals may be a useful measure of environmental quality as they represent the sum of environmental pressures on the animal (Hill 1995). In this context, carotenoid-based coloration may be especially valuable for monitoring and detecting sub-lethal effects of toxic chemicals in the environment, because in many cases the mechanisms underlying coloration and the response to pollutants (and any subsequent damage) are interconnected (Dauwe and Eens 2008).

The carotenoid-based colorations displayed by fishes and birds are considered as good examples of honest sexual signals (e.g., Olson and Owens 1998; Badyaev and Hill 2000; Pike et al. 2007). Carotenoids are lipid-soluble pigments and can only be synthesized by algae, bacteria, fungi and plants (Goodwin 1984), and thus vertebrates must obtain them from food. As well as being involved in the expression of color signals, carotenoids also have important physiological functions, acting as immuno-enhancers in the immune system and as oxygen radical scavengers in antioxidant activity (Lozano 1994; von Schantz et al. 1999).

Recent studies have shown that the availability of colorless antioxidants affects the expression of carotenoid-based signals (Bertrand et al. 2006; Pike et al. 2007; Pérez et al. 2008a), indicating that oxidative stress plays a key role in regulating such coloration. Since many pollutants induce oxidative stress (Kappus 1987), it is expected that they will cause an increase in demand for antioxidants and lead to reduced expression of carotenoid-based coloration, which should therefore be useful in ecotoxicological monitoring (Camplani et al. 1999; Dauwe and Eens 2008). Indeed, other biomarkers of oxidative stress are commonly used in environmental pollution monitoring (McCarthy and Shugart 1990). Two recent experimental studies in captive birds and fishes showed that carotenoid coloration was impaired when the animals were exposed to pollutants (Bortolotti et al. 2003; Arellano-Aguilar and Garcia 2008). Moreover, another recent study showed that carotenoid coloration can be used as a biomarker of pollutant-derived oxidative stress (Geens et al. 2009).

Here, we experimentally evaluated the effect of naturally occurring oil pollution (from the *Prestige* oil spill) on oxidative stress and coloration in a free-living seabird. Seabirds are particularly vulnerable to oil pollution (Peterson et al. 2003; Velando et al. 2005) and, since they occupy high trophic positions, important toxic effects (due to persistent exposure to oil) are expected in these organisms (Alonso-Alvarez et al. 2007a, b). One of the most recent examples of a large marine oil spill occurred in November 2002 when the supertanker *Prestige* sank off the coast of Galicia (NW Spain). The tanker spilled between 40,000 and 63,000 tonnes of heavy fuel oil into the Atlantic Ocean, polluting coastal areas as far apart as Portugal and France. Polycyclic aromatic hydrocarbons (PAHs), the most toxic components of crude oils (Ramachandran et al. 2006), are still being detected in the marine food chain in the affected area (e.g., Laffon et al. 2006; Ordas et al. 2007; Pérez et al. 2008b).

The acute toxicity of PAHs is mainly attributed to oxidative stress and cellular damage associated with the metabolic response, such as cytocrome P450 catalytic activity, by which PAHs are eliminated from tissues (Gonzalez 2005; Shimada 2006; Ramos and García 2007). The cytochrome P450 cycle produces different reactive oxygen species (Lewis 2002), which organisms counteract by activating antioxidant systems (Matés 2000; Nordberg and Arnér 2001). Thus, antioxidant systems are induced as an adaptive response, allowing an organism to partially or totally overcome oxidative stress in a polluted environment (Di Giulio et al. 1989; Winston and Di Giulio 1991). Induction of antioxidant defence components is used as biomarker of acute oil exposure (Cossu et al. 1997; Cheung et al. 2001).

The damaging effects on seabirds of exposure to petroleum products following oil spills are well documented (e.g., Seiser et al. 2000; Balseiro et al. 2005; Alonso-Alvarez et al. 2007a, b). However, as far as we know, the effect of ingestion of oil on sexual ornaments has not previously been explored. In this study, we tested the hypothesis that oil pollution enhances oxidative stress and impairs expression of a carotenoid-based signal in a wild population of the yellow-legged gull (*Larus michahellis*), a seabird species in which both sexes show intense integumentary carotenoid-based coloration of legs, eye rings, gape, and bill spot (Cramp and Simmons 1983). Expression of the red spot in gulls is known to vary throughout the breeding cycle, and is enhanced during courtship (Cramp and Simmons 1983). Moreover, as a signal, the red spot affects the partner's reproductive investment (i.e., differential allocation); thus, a larger red spot promotes higher partner investment in both sexes (Morales et al. 2009). Recent studies have revealed that this trait is carotenoid-based, reliably reflects the bearer's antioxidant status (Pérez et al. 2008a) and reflects sub-lethal effects following the *Prestige* oil spill (Pérez et al. 2009). Thus, we predict that the oxidative stress produced by ingestion of oil may affect carotenoid mobilization and hence bird coloration. To test this idea, we measured the effect of pollution on the plasma levels of different non-enzymatic antioxidants by analysis of carotenoid and vitamin E levels and plasma antioxidant activity. In addition, we determined plasma levels of lipid peroxidation products as a measure of oxidative cellular damage. Finally, we predicted that oil ingestion would be reflected by the carotenoid-based coloration of gulls, as a consequence of oxidative stress.

Materials and methods

The fieldwork was carried out on a breeding colony of yellow-legged gulls (*Larus michahellis*) on the Illas Cíes (Ría de Vigo, Galicia, NW Iberian Peninsula). At the end of April 2005, during the gulls' courtship period, we randomly allocated 36 breeding pairs for the experiment: 16 pairs were fed a supplementary diet containing *Prestige* oil

(oil-supplemented group) and 20 pairs were treated as controls (control group). Ethical considerations were taken into account in the experimental design to avoid unnecessary harm to animals while still eliciting a measurable response. Thus, the number of experimental subjects was kept as low as possible (Dawkins and Gosling 1996) and the amount of crude oil administered was well below the dosage used in previous experiments (e.g., Butler and Lukasiewicz 1979; Leighton 1991). Blood concentrations of PAHs in gulls fed with *Prestige* oil (see "Results") were much lower (50%) than in gulls sampled in oiled colonies 17 months after the *Prestige* oil spill (Pérez et al. 2008b). Thus, the dosage used probably resembles a low–medium pollution event.

The oil-supplemented group was restricted to 16 pairs, which were fed daily with 0.04 ml of *Prestige* oil (kindly provided by the Instituto Español de Oceanografía; daily individual dose of PAHs: 59.15 μg), mixed with sunflower oil and spread on a slice of bread (0.3 ml in total per pair). The pairs in the control group were fed in a similar manner but without fuel oil and only with sunflower oil (for more details about food supplementation, see Pérez et al. 2006, 2008b).

All gulls were given the corresponding supplementary diets between 26 April (just before the egg-laying period in the population) and 5 days after the first egg was laid (to ensure that the egg laying was complete). The *Prestige* oil diet was only administered on seven consecutive days (26 April–3 May). From the end of the 7-day period until the end of the egg-laying period, birds in both groups received bread with only sunflower oil (mean ± SE 9.2 ± 0.98 days, range 1–21 days). Food supplementation started 10.41 ± 1.06 days (range 2–22 days) before egg laying. The period of supplementation prior to laying did not differ significantly between treatment groups ($t_{27} = 0.08$, $P = 0.94$). Egg length (L) and width (W) were measured (to the nearest 0.01 mm), and egg volume (V) was calculated with the following formula: $V = 0.51 \times L \times W^2$ (Hoyt 1979).

After the clutch was complete, 20 control (9 females and 11 males) and 12 oil-supplemented gulls (7 females and 5 males) were trapped in their nests (one gull per pair; 1–21 days after egg laying). The proportion of trapped gulls did not differ between experimental groups (chi-square test, $\chi^2 = 1.125$, $df = 1$, $P > 0.289$). The time between the end of supplementary feeding and capture of the gulls did not differ between experimental groups ($t_{27} = 0.05$, $P = 0.96$). Head, bill width (measured at the widest part of the lower mandible), and tarsus length were measured (to the nearest 1 mm). Body mass was also determined (to the nearest 10 g). The tarsus length allowed confirmation of the sex of the birds by means of discriminant analysis (Bosch 1996). The bill was photographed against a white standard, together with a red standard and a millimetric scale, inside a black box, with a digital camera (Nikon Coolpix 5200). The distance from the lens to the bill (15 cm) was maintained constant. The red spot area was measured by the same person (C.P.) by use of image analysis software (analySIS FIVE) and blindly with respect to treatment. The repeatability of the method (intra-class correlation coefficient *r*; Lessells and Boag 1987), performed three times on six randomly selected photographs, was very high ($r = 0.98$, $F_{5,12} = 161.01$, $P < 0.001$). The intensity of the color of the red spot (i.e., redness) was calculated as the mean value of each component: red (r), green (g) and blue (b) in RGB color-space at three pixel positions from the central part of the spot. A single redness intensity value (*R* value) was calculated according to Pike et al. (2007). To calculate the luminosity of the photograph, three pixel values were obtained from the white standard beside the bill spot. The luminosity was defined as the mean value of the sum of all three components (r, g, b). Bill color analysis was restricted to human-visible wavelengths, although the ultraviolet proportion represents <0.05 of total reflectance spectrum of bill color in yellow-legged gulls (unpublished data).

A blood sample (about 1.5 ml) was taken from the brachial vein, with a 25G heparinized needle. The blood was immediately transferred to plastic tubes and maintained on ice in cool boxes (4°C), then centrifuged in the laboratory at the end of the day. Plasma and blood cells (pellet) were frozen separately at −80°C until analysis.

Biochemical assays

Blood cells were analyzed to determine and quantify haematological levels of PAHs derived from oil spilled during the *Prestige* disaster. The PAH levels were determined in a high performance liquid chromatography (HPLC) system coupled to a wavelength programmable fluorescence detector. Samples (100 μl) were injected into a HPLC system equipped with a Waters PAH analytical column (250 mm × 4.6 mm × 5 μm). The mobile phase was acetonitrile:water in gradient elution, run at a flow rate of 1.2 ml/min (see Pérez et al. 2008a, b). The PAHs analyzed were acenaphthene, anthracene, benz(a)anthracene, benzo(a)pyrene, benzo(b + j) fluoranthene, benzo(g,h,i)perylene, benzo(k)fluoranthene, chrysene, dibenz(a,h)anthracene, fluorene, fluoranthene, indeno (1,2,3-c-d)pyrene, naphthalene, phenanthrene and pyrene. To estimate the individual degree of oil contamination, the sum of concentrations from all these hydrocarbons was used as a variable (hereinafter PAH levels).

The carotenoids and vitamin E contained in the plasma samples were also measured by high-performance liquid chromatography (HPLC). Plasma samples (50 μl) were diluted in 250 μl of absolute ethanol (Alonso-Alvarez et al.

2004) in tubes protected from high temperatures and direct light. The solution was mixed on a vortex mixer and subsequently centrifuged at 10,000 rpm for 10 min. The supernatant was collected in a new tube, dried under a nitrogen atmosphere and diluted again in 200 µl of methanol. Samples (20 µl) were injected into an HPLC system (JASCO Comparison Proven, model 1500), fitted with a SecurityGuard column and a C18 reverse-phase analytical column (15 cm × 4.6 mm × 3 µm) (SphereClone type ODS (2); Phenomenex). The mobile phase was methanol-milliQ water (90:10 v/v) in gradient elution (gradient: 0–21 min 90:10 v/v, 21–25 min 100:0 v/v, 25–35 min 90:10 v/v) and the flow rate, 1.5 ml/min. Carotenoids were determined at 445 nm with a UV detector (JASCO Comparison Proven, model UV-1570) and quantified by use of external standards (canthaxanthin, astaxanthin and β-carotene: Dr. Ehrenstorfe; Lutein: Sigma–Aldrich; zeaxanthin, echinenone and β-cryptoxanthin: LGC Promochem). The calibration curves for the carotenoids present in the samples revealed high correlation coefficients (in all cases $R^2 > 0.99$). The concentration of the unknown carotenoids was calculated in relation to a lutein standard (see Alonso-Alvarez et al. 2004; Costantini and Dell'Omo 2006; Eraud et al. 2007). Vitamin E (α-tocopherol) was determined simultaneously in the same extract with the same column, mobile phase, gradient, and flow rate, but with a fluorescence detector (JASCO Comparison Proven, model FP-1520). The excitation and emission wavelengths used were 295 and 330 nm, respectively. Concentrations were calculated in relation to the vitamin E standard (α-tocopherol; Sigma–Aldrich; calibration curve, $R^2 = 0.99$). Concentrations of carotenoids and vitamin E were expressed in µg/ml.

The plasma antioxidant activity was measured by the method described by Erel (2004). Basically, the method consists of the use of the molecule 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonate) (ABTS*+), which is decolorized by antioxidants according to their concentration and antioxidant capacity. The change in color is measured as the change in absorbance at 415 nm (BIO-RAD 550 microplate reader). The concentration of plasma antioxidant activity is expressed as millimoles of Trolox equivalent/L. We used "plasma antioxidant activity" rather than the common term "total antioxidant capacity" because the method used only quantifies the reaction of antioxidants present in the aqueous phase of the plasma and not the reaction of total antioxidants (Miller et al. 1995; Young 2001; Prior et al. 2003). Lipid peroxidation was quantified with a lipid peroxidation assay kit (Calbiochem, cat no. 437634). This method measures malondialdehyde (MDA) and 4-hydroxyalkenals (HAE), which are end products derived from peroxidation of polyunsaturated fatty acids and related esters.

Data analyses

The effects of the *Prestige* oil supplementation on red spot size, intensity of red spot, blood levels of PAHs, and plasma concentrations of vitamin E, carotenoids, plasma antioxidant activity, and end products derived from lipid peroxidation were analyzed by use of General Linear Models (GLMs). Experimental treatment and sex were included in the models as factors, and body mass, bill width (in the red bill spot model), luminosity, and R value of red standard (in the bill spot redness model), as covariates. Non-significant terms (P value > 0.05) were backward dropped by a stepwise elimination procedure. Moreover, the full models are reported as recommended (Whittingham et al. 2006). We also explored the effect of PAHs, carotenoids, the time from feeding (the number of days elapsed from the start of feeding to first egg-laying), and the time to capture (days elapsed from the end of egg-laying to the capture of gulls) by including these as covariates in the models. In order to avoid type II errors due to small sample size (see ethical considerations above), in variables with expected directional effects (PAHs, lipid peroxidation, and red bill spot), the effect of ingestion of *Prestige* oil was analyzed by use of one-tailed tests with significance levels set at 0.05, as recommended in studies involving manipulations that are potentially detrimental to animals (Still 1982). In GLMs, the observed power (the power to detect a population effect equal to the obtained sample effect; Onwuegbuzie and Leech 2004) was calculated. Antioxidant activity was square transformed in order to meet model requirements (normality and homoscedasticity variance structure). Data are expressed as means ± standard errors. Sample sizes varied somewhat among statistical analyses as plasma volumes were not always sufficient for all biochemical analysis and because spot size was not measured in three birds sampled during harsh weather conditions.

Results

Prestige oil supplementation did not affect body mass ($F_{1,26} = 0.06$, $P = 0.81$; sex $F_{1,26} = 28.29$, $P < 0.001$), laying date ($t_{27} = 0.08$, $P = 0.94$), clutch size ($Z = 0.38$, $P = 0.70$), or egg volume ($t_{27} = 0.97$, $P = 0.34$), suggesting similar breeding characteristics in both groups of gulls studied. Blood levels of PAHs were higher in oil-supplemented gulls (30% higher) than in control gulls (Table 1; see Pérez et al. 2008b).

Antioxidants, carotenoids and oxidative damage

The plasma concentration of vitamin E was significantly higher (31%) in gulls administered the diet containing

Table 1 General linear models showing treatment effects on yellow-legged gulls (*Larus michahellis*) with all covariates (full models) and the model that retained only variables that caused a significant increase in deviance (minimal adequate models)

Dependent variable	Variables	Full model					Minimal model			
		Degrees of freedom	Parameter estimate	Observed power	*F*	*P*	Parameter estimate	Observed power	*F*	*P*
Polycyclic aromatic hydrocarbons	Intercept	1.25	94.88				75.28			
	Treatment (control)		−9.64	0.37	2.90	0.10	−17.58	0.44	3.48	**0.036**
	Sex (female)		4.07	0.05	0.04	0.83				
	Weight		−0.02	0.08	0.25	0.62				
	Sex × treatment		−14.43	0.11	0.53	0.47				
Vitamin E	Intercept	1.23	19.79							
	Treatment (control)		−4.71	0.66	6.17	**0.02**	−3.96	0.64	5.85	**0.023**
	Sex (female)		−3.26	0.20	1.38	0.25				
	Weight		−0.01	0.05	0.03	0.85				
	Sex × treatment		1.07	0.06	0.10	0.75				
Antioxidant activity	Intercept	1.23	−5.73				−6.48			
	Treatment (control)		−1.20	0.07	0.15	0.70				
	Sex (female)		1.68	0.40	3.22	0.09	2.74	0.51	4.23	**0.050**
	Weight		0.01	0.49	4.11	**0.05**	0.01	0.51	4.25	**0.050**
	Sex × treatment		1.62	0.12	0.65	0.43				
Total carotenoids	Intercept	1.23	12.18				11.20			
	Treatment (control)		−2.83	0.44	3.60	0.07	−2.37	0.49	4.04	**0.055**
	Sex (female)		4.00	0.72	7.05	**0.01**	4.77	0.98	17.49	**<0.01**
	Weight		−0.01	0.05	0.01	0.91				
	Sex × treatment		1.00	0.07	0.16	0.69				
Lipid peroxidation	Intercept	1.23	145.19							
	Treatment (control)		−30.64	0.22	1.52	0.11				
	Sex (female)		1.55	0.06	0.09	0.77				
	Weight		0.07	0.12	0.60	0.44				
	Sex × treatment		13.07	0.06	0.11	0.74				
Bill spot redness	Intercept	1.23	0.59				0.74			
	Treatment (control)		−0.06	0.23	1.59	0.11				
	Sex (female)		0.03	0.11	0.56	0.46				
	Bill width		0.01	0.07	0.20	0.66				
	R-red standard		0.40	0.96	15.31	**<0.01**	0.47	1.00	36.62	**<0.01**
	Luminosity		−0.01	0.85	9.82	**<0.01**	−0.01	0.94	13.20	**0.001**
	Sex × treatment		0.03	0.08	0.29	0.60				
Bill spot size	Intercept	1.23	−153.72				−6.07			
	Treatment (control)		8.11	0.36	2.83	**0.055**	20.95	0.59	5.21	**0.015**
	Sex (female)		11.20	0.17	1.09	0.31				
	Bill width		16.58	0.54	4.59	**0.04**	8.51	0.64	5.83	**0.023**
	Sex × treatment		17.27	0.16	0.96	0.34				

Significant values are shown in *bold*. The observed powers of the analyses are provided

Prestige oil than in the control gulls (Table 1; Fig. 1a). Plasma antioxidant activity was higher (43%; Fig. 1c) in gulls fed with *Prestige* oil than in control gulls, but the difference was not significant (Table 1). Moreover, plasma levels of vitamin E were negatively correlated with carotenoids levels (parameter estimate −0.44, $F_{1,25} = 4.95$, $P = 0.03$), but plasma antioxidant activity was not correlated with carotenoids ($F_{1,21} = 2.64$, $P = 0.12$).

The total levels of plasma carotenoids were higher (27%) in *Prestige* oil-supplemented gulls than in controls, but this difference was close to significance (Table 1; Fig. 1b). In addition, plasma carotenoids were negatively related to blood levels of PAHs ($F_{1,21} = 8.82$, $P < 0.01$; Fig. 2).

Finally, the plasma levels of lipid peroxidation markers were higher in gulls fed with *Prestige* oil than in control

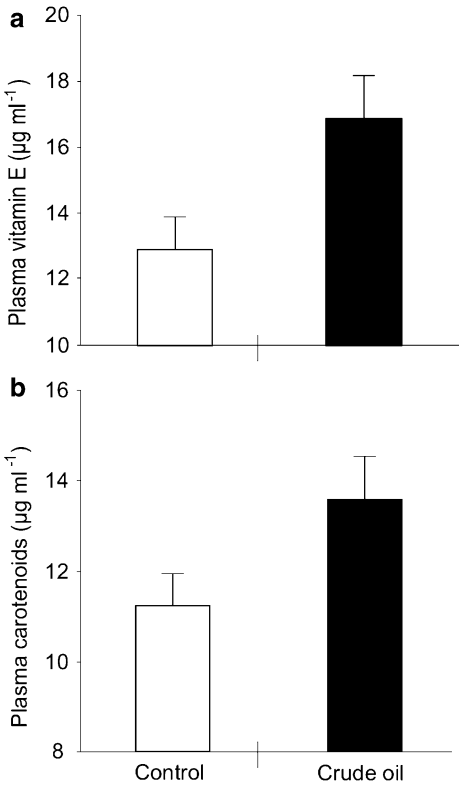

Fig. 1 Effect of *Prestige* oil-supplementation on plasma levels of yellow-legged gulls (*Larus michahellis*): **a** vitamin E, **b** total carotenoids. Control group ($n = 18$) and *Prestige* oil-fed group ($n = 10$). *Values* are expressed as least square means ± standard errors

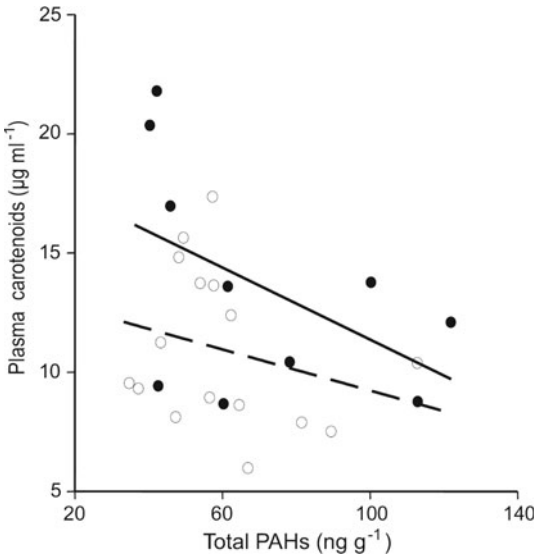

Fig. 2 Relationship between the blood levels of total polycyclic aromatic hydrocarbons (sum of 15 PAH compounds; see "Materials and methods") and the plasma levels of carotenoids in control gulls (*open dots* and *dashed line*, $n = 16$; $R^2 = 0.07$) and *Prestige* oil-fed gulls (*filled dot* and *solid line*, $n = 10$; $R^2 = 0.24$)

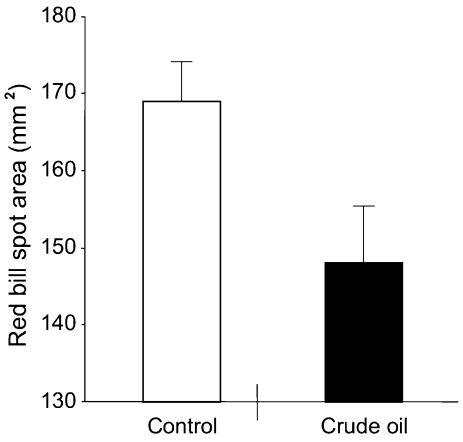

Fig. 3 Effect of *Prestige* oil supplementation on size of red bill spot in yellow-legged gulls. Control group ($n = 19$) and *Prestige* oil-fed group ($n = 10$). *Values* are expressed as least square means ± standard errors

gulls (13%; Fig. 1d), although the difference was not significant (minimal model, $F_{1,26} = 1.75$, $P = 0.098$).

Carotenoid-based signal

The redness of the bill spot did not differ between groups (minimal model, $F_{1,25} = 0.92$, $P = 0.17$). As expected, *Prestige* oil supplementation affected the size of the red bill spot (Table 1); the red spot of oil-supplemented gulls was 16% smaller than that of controls gulls (Fig. 3). Moreover, there were no differences between males and females in relation to size of bill spot or redness of the spot ($P > 0.2$ in all cases).

Interestingly, the blood levels of PAHs did not affect the size and the redness of the bill spot ($P > 0.5$ in both cases). Finally, redness or size of the bill spot were not correlated with plasma levels of carotenoids ($P > 0.2$ in both cases).

Discussion

In this study, we showed that plasma levels of vitamin E and carotenoids were higher in gulls experimentally exposed to oil pollution (ingestion of *Prestige* oil) than in control gulls. In addition, as expected, the oil pollution caused a reduction in expression of a carotenoid-based signal. Similar breeding characteristics in randomized experimental groups suggest that these effects are not attributable to differences existing prior to the experiment.

The feeding ecology of yellow-legged gulls makes them susceptible to continued exposure to remnant crude oil (Alonso-Alvarez et al. 2007a). We previously found that yellow-legged gulls breeding in colonies affected by the oil spill were exposed to residual *Prestige* oil (Pérez et al. 2008b), with important long-term sub-lethal effects

(Alonso-Alvarez et al. 2007a, b). At that time, a negative correlation between hepatic damage (oil-induced sub-lethal effects) and the size of the red bill spot was also observed in gulls captured in oiled areas (Pérez et al. 2009). The present findings are consistent with these results and provide the first experimental evidence in a free-living seabird that a carotenoid-based signal is depressed by oil pollution.

After an oil pollution event, animals typically mobilize antioxidant defences to counteract petroleum-hydrocarbon-induced free radical toxicity (Matés 2000; Nordberg and Arnér 2001; Cheung et al. 2001). We did not find any enhancement of plasma antioxidant activity, but levels of vitamin E and carotenoids were higher in gulls supplemented with *Prestige* oil than in control gulls. Gulls exposed to *Prestige* oil may have mobilized antioxidants from body stores, such as the liver, to blood (Cohn et al. 1988; Costantini and Dell'Omo 2006). Nevertheless, since the experimental gulls were free-living, we cannot completely rule out the possibility of a dietary shift to a vitamin E- and carotenoid-rich diet. However, body condition and hematocrit values (Alonso-Alvarez et al. 2007b) did not suggest differences in nutritional condition between experimental groups.

Lipid peroxidation products did not differ between experimental groups, suggesting that the availability of antioxidants allowed gulls to overcome—to some extent—oxidative stress promoted by ingestion of *Prestige* oil. Vitamin E is a major lipid-soluble antioxidant (Halliwell and Gutteridge 1999; Surai 2002) involved in detoxification processes (see Murvoll et al. 2005) and is the most important antioxidant agent that protects polyunsaturated fatty acids against lipid peroxidation (e.g., İnal et al. 1999; Mateo et al. 2003). The increase in antioxidant defences after acute exposure to crude oil pollution has been well documented in animals (e.g., Achuba and Osakwe 2003; Reid and MacFarlane 2003). Interestingly, although plasma antioxidant activity also increased in gulls fed with *Prestige* oil, the experimental effect was not significant. Sample size limitations may have compromised the power to detect the expected differences between experimental groups in relation to lipid peroxidation products and the plasma antioxidant activity. Thus, although the effects of sample size on these variables were similar to the observed effects on vitamin E and carotenoids, the levels of lipid peroxidation products and plasma antioxidants were more variable, perhaps as a result of measurement error caused by limitations of the analytical techniques (Monaghan et al. 2009).

In birds, carotenoids are mobilized under stressful conditions (Eraud et al. 2007). Accordingly, we found that plasma carotenoids were enhanced by *Prestige* oil ingestion, which suggests a response to overcome the oxidative stress promoted by crude oil exposure. Indeed, in *Prestige* oil-supplemented gulls, those birds with higher levels of plasma carotenoids showed reduced blood levels of PAHs, suggesting that carotenoids improved PAH degradation. The ROS produced by PAH degradation may be counteracted by the mobilization of carotenoids to balance oxidative stress (see Krinsky and Yeum 2003; Rao and Rao 2007). For instance, oxidative damage to DNA is reduced when the total concentration of carotenoids in plasma is high (e.g., Zhao et al. 2006; Thomson et al. 2008). Nevertheless, in birds, the role of carotenoids as free radical scavengers remains controversial (Hartley and Kennedy 2004; Costantini and Møller 2008; Isaksson and Andersson 2008), and it has been suggested that antioxidant activity is not the main biological role for carotenoids in birds (Hartley and Kennedy 2004; but see Catoni et al. 2008; Cohen and McGraw 2009; Pérez-Rodríguez 2009). However, in gulls, experimental carotenoid supplementation reduces the susceptibility of eggs to lipid peroxidation and increases the antioxidant capacity of adult birds (Blount et al. 2002a, b). Alternatively (or additionally), carotenoids are also modulators of the immune response (see Blount et al. 2003; McGraw and Ardia 2003; Velando et al. 2006). Thus, the higher plasma levels of carotenoids in *Prestige* oil-supplemented gulls may also reflect a mechanism used to counteract the well-documented immunodepressive effects of PAHs (White et al. 1994). In any case, the present results suggest that the carotenoids were mobilized to overcome the harmful effects of PAHs ingestion, highlighting the fact that carotenoids may play an important role in the detoxification process of the oil pollution. Otherwise, although *Prestige* oil ingestion appeared to increase the levels of both vitamin E and carotenoids, there was a negative relationship between plasma levels of the two compounds. It is therefore possible that they are differentially mobilized by birds in response to oxidative stress, or that carotenoids and vitamin E compete for plasma lipoproteins (plasma carriers).

Plasma carotenoid levels were higher in *Prestige* oil-supplemented gulls than in the control gulls, but the size of the red spot was smaller. These findings suggest that carotenoids are probably a limited resource and that their use for oil detoxification is prioritized at the expense of carotenoid-based coloration. Similarly, previous studies found that metal pollution and thermal stress increase circulating levels of carotenoids and antioxidants but depress bird coloration (Eraud et al. 2007; Geens et al. 2009). In the present study, plasma levels of carotenoids were not correlated with red spot size, indicating that carotenoids are not exclusively used for pigmentary function. In a previous study, we found that red coloration was affected by the availability of colorless antioxidant (Pérez et al. 2008a). Overall, these findings are consistent with the hypothesis that there is a trade-off between allocation of carotenoids to the sexual signal and to physiological functions (von Schantz et al. 1999;

Eraud et al. 2007). Importantly, plasma levels of carotenoids increased under scenarios of low (Pérez et al. 2008a) and high (present study) oxidative stress. Thus, high levels of carotenoids (or antioxidants) in plasma should not be interpreted as indicative of low levels of oxidative stress.

Because of the role played by carotenoid-based traits on social interactions (Hill and McGraw 2006), disruption of this signal by alteration after exposure to oil pollution could have effects on the reproductive output. In yellow-legged gulls, red spot size is a mutually selected sexual signal. Thus, in a previous study, we found that food provisioning by both parents depends on the size of red bill spot of their partner (Morales et al. 2009). Moreover, the red spot on the bill elicits chick begging behavior (Tinbergen and Perdeck 1950), smaller spots could also affect chick growth. Integration of biochemical, physiological, ecological, and behavioral approaches may be advisable when quantifying the real impact of pollution on wildlife (Zala and Penn 2004).

The present study provides experimental evidence regarding the plasticity of carotenoid-based color integuments in response to toxic chemicals in the environment, a long-standing hypothesis (Hill 1995). The present results are consistent with those of previous studies in captive and wild vertebrates in which exposure to pollutants was reflected in carotenoid-based signals (Bortolotti et al. 2003; Arellano-Aguilar and Garcia 2008; Geens et al. 2009). Since the mechanisms underlying coloration and the response to pollutants (and any subsequent damage) are probably interconnected (Dauwe and Eens 2008), carotenoid-based coloration may be useful in monitoring and detecting sublethal effects of pollutants at the population level (Geens et al. 2009; Pérez et al. 2009). Since the yellow-legged gull belongs to a complex of species widely distributed throughout the northern hemisphere (e.g., Liebers et al. 2001, 2004), the present method may be a useful tool for evaluating short- and long-term effects of oil pollution in seabirds.

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