Available online at www.sciencedirect.com
ScienceDirect
Environment International 33 (2007) 773–781
ENVIRONMENT INTERNATIONAL
www.elsevier.com/locate/envint

Sublethal toxicity of the *Prestige* oil spill on yellow-legged gulls

Carlos Alonso-Alvarez [a,*], Ignacio Munilla [b], Marta López-Alonso [c], Alberto Velando [b]

[a] *Unidad de Ecología, Instituto de Investigación en Recursos Cinegéticos, IREC (CSIC, UCM, JCCM), Ronda de Toledo s/n, 13005, Ciudad Real, Spain*
[b] *Departamento de Ecoloxía e Bioloxía Animal, Facultade de Ciencias, Universidade de Vigo, Pontevedra, Spain*
[c] *Departamento de Patoloxía Animal, Facultade de Veterinaria, Universidade de Santiago de Compostela. Lugo, Spain*

Received 7 January 2007; accepted 15 February 2007
Available online 26 March 2007

Abstract

The *Prestige* oil spill in November 2002 is considered the biggest large-scale catastrophe of its type in Europe, thousands of seabirds dying in the subsequent months. Here, the total concentration of 16 polycyclic aromatic hydrocarbons (TPAH) was measured in the blood cell fraction of adult and chick yellow-legged gulls (*Larus michahellis*) from unoiled and oiled coastal areas in North Western Spain. In addition, hematocrit, plasma metabolites, electrolytes and enzymes, as well as body mass were determined in the same individuals. Our results strongly suggest the presence of health damages of sublethal nature in adult gulls breeding in oiled colonies 17 months after the *Prestige* oil spill. This is supported by the following evidences: (1) gulls sampled in unoiled and oiled colonies differed in blood TPAH levels, (2) gulls sampled in unoiled and oiled colonies differed in several blood parameters indicative of physiological disorders, and (3) TPAH in blood was significantly related to several of these parameters. Differences in the level of asparatate aminotransferase (AST), gamma-glutamyl transferase (GGT), total protein, glucose and inorganic phosphorus suggest damages on some vital organs (i.e. liver and kidney) in adult birds from oiled areas. Meanwhile, chicks presented weaker effects than adults, showing only between-area differences in hematocrit. Since TPAH levels in blood did not differ between both age-groups, the stronger effects on adults should be due to their longer exposure to these pollutants and/or to severe exposure in the months following the spill. The presence of PAHs in chicks indicates that these pollutants were incorporated into the food chain because nestlings would have been only exposed to contaminated organisms in the diet (e.g. fishes and crustaceans). Our findings support the view that PAHs may deeply alter the physiology of seabirds, and emphasize the necessity of quantifying the circulating levels of these compounds in order to evaluate the sublethal effects associated to large oil spills.

Keywords: AST; Before-after-control-impact sampling; Body mass; Hematocrit; Liver damage; Plasma biochemistry; Polycyclic aromatic hydrocarbons; *Prestige* oil spill; Seabirds

1. Introduction

Large oil spills at sea are likely to have dramatic effects on marine ecosystems and result in an increased exposure to oil by marine organisms, including seabirds (e.g. Peterson, 2001). Lethal short-term effects of oil spills often involve large seabird losses and draw much public attention when oiled seabirds and carcasses are washed ashore (e.g. Piatt et al., 1990). These birds are especially sensitive to marine oil spills, which can have dramatic effects on their populations (e.g. Velando et al., 2005a, b; Votier et al., 2005). However, oil pollution, whether chronic or acute, at levels that do not lead to immediate death, has the potential to seriously impair the ability of seabirds to survive and reproduce (Jessup and Leighton, 1996; Esler et al., 2000; Golet et al., 2002).

Whereas the acute effects of crude oil ingestion on avian physiology have been well described (e.g. Leighton, 1995; Stubblefield et al., 1995; Yamato et al., 1996; Prichard et al., 1997; Newman et al., 1999, 2000; Balseiro et al., 2005), the effects of long-term sub-lethal exposures to these contaminants have been rarely explored (some exceptions: Seiser et al., 2000;

Abbreviations: AST, asparatate aminotransferase; Ca, calcium; CK, creatine kinase; CV, coefficient of variability; df, degrees of freedom; GGT, gamma-glutamyl transferase; HPLC, high performance liquid chromatography; iP, inorganic phosphorus; TPAH, total polycyclic aromatic hydrocarbons; SE, standard error.

* Corresponding author. Tel.: +34 926295450; fax: +34 926295451.
E-mail address: carlos.alonso@uclm.es (C. Alonso-Alvarez).

0160-4120/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.envint.2007.02.012

Trust et al., 2000; Golet et al., 2002). Sub-lethal effects from direct oil ingestion or indirect oil incorporation (across the marine trophic chain) would include a large array of inflammatory and toxic effects, immune-suppression, and finally, an impairment of the general health status (Leighton, 1995; Briggs et al., 1996; Golet et al., 2002).

One of the last examples of a large marine oil spill took place in November 2002 when the supertanker *Prestige* sank in front of Galicia (NW Spain). The wreck spilled about 60,000 t of crude oil into the Atlantic Ocean, spreading pollution from Northern Portugal to France (Fig. 1). The *Prestige* oil spill is considered the biggest large-scale catastrophe of its type in Europe and thousands of seabirds died in the subsequent months (Camphuysen et al., 2002). Since seabirds are long-lived animals and upper trophic level consumers and the oil incorporation of the *Prestige* pollution into the marine food chain is currently being detected (e.g. Fernandez et al., 2006; Laffon et al., 2006; Morales-Caselles et al., 2006), sublethal effects on seabirds due to this chronic exposure are expected.

Plasma biochemistry of yellow-legged gulls (*Larus michahellis*) from the Galician coast (formerly *Larus cachinnans*) has been deeply studied before the *Prestige* oil spill. Such knowledge would constitute a useful tool to understand the effects of this kind of catastrophe on the physiology of seabirds. Reference plasma values of several metabolites have been determined in wild gulls from different ages and sexes (Alonso-Alvarez et al., 2002a; Alonso-Alvarez, 2005). Moreover, the dynamics of many physiological parameters in relation to food availability has been experimentally studied. Thus, we know their patterns of variation from an optimal level of physical condition (birds with *ad libitum* food) to the limit before death (after a long food-deprivation period; Alonso-Alvarez and Ferrer, 2001). We have also determined the level of correlation between the studied parameters and the individual range of variation in body mass. Thus, relatively high body mass (with regard to body mass range) positively correlated to total cholesterol level, which was proposed as a good index of condition for this species (Alonso-Alvarez et al., 2002b; Alonso-Alvarez and Velando, 2003).

In the present study, the total concentration of 16 polycyclic aromatic hydrocarbons (TPAH) found in the *Prestige* crude oil (Bosch, 2003; CSIC, 2003) was measured in the blood of yellow-legged gulls. Adult and chick yellow-legged gulls captured in unoiled and oiled colonies 17 months after the *Prestige* oil spill were sampled to establish spatial differences in TPAH concentration, as well as in several plasma biochemical parameters, hematocrit and body mass. The analyzed plasma metabolites provide information on the level of energetic reserves (e.g. Le Maho et al., 1981; Boismenu et al., 1992; Alonso-Alvarez and Ferrer, 2001). Moreover, the levels of four plasma chemicals and body mass obtained before the *Prestige* wreck were used as reference in a attempt to complete the classic before-after-control-impact (BACI) approach (e.g. Stewart-Oaten et al., 1986; Osenberg et al., 1994). In addition, two enzymes (transaminases) were assessed for the first time in this species. High levels of these parameters are indicatives of hepatic damages (Campbell, 1994; Harr, 2002). To conclude, TPAH levels were correlated to the above cited parameters. This allowed us to infer the level of damage associated to the intensity of intoxication. As far as we know, the relationship between the analysed parameters and circulating levels of polycyclic aromatic hydrocarbons has not been previously explored for any avian species.

2. Materials and methods

2.1. Bird sampling

Bird sampling was performed in seven breeding colonies located in islands distributed along the coastline of the North-western Spain (Fig. 1). Three of them were located in an area that did not suffer the impact of the *Prestige* spill (unoiled area), whereas the other four were heavily oiled (oiled area). In total, 95 adults (40 males and 55 females) and 135 chicks were captured. Adult birds

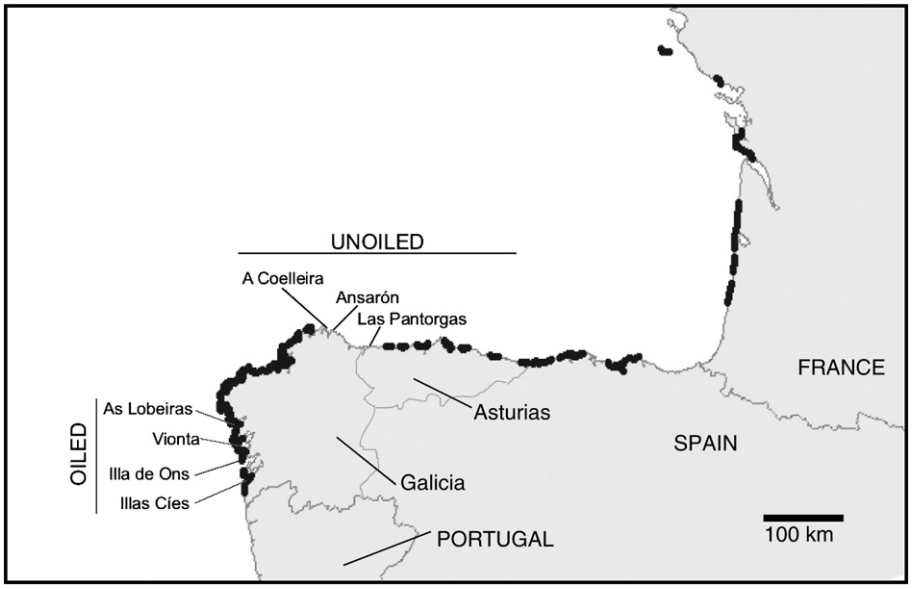

Fig. 1. Coastal areas affected by the *Prestige* oil spill. Unoiled and oiled sampling areas are shown. Source Coordination Technical Bureau against Accidental Marine Spills, Spanish Ministerio de Educación y Ciencia. (http://otvm.uvigo.es/accidentprestige/litoralafectado.html).

were nest-trapped from May 19 to June 5, 2004. Chicks were captured between June 20 and 28, 2004. Several morphometries including body mass (±1 g), wing length (±1 mm) and tarsus length (±1 mm) were determined. Tarsus length allowed sexing birds by means of a discriminant function (Bosch, 1996). To avoid the confounding effects of early development variability on plasma chemicals (see e.g. Szabo et al., 2005; Juráni et al., 2006), chicks were sampled after their asymptotic-growth period, that is, when most part of body size has been acquired (estimated age: 3–5 weeks).

A blood sample (1–2 mL, depending on body mass) was taken from the ulnar vein with a heparinized 25 G needle. Blood was immediately transferred to plastic tubes and a microcapillar was taken from them. Both tubes and microcapillars were maintained cooled in ice boxes (4 °C), and centrifuged at the end of the day. In all sampled birds, hematocrit values were determined from centrifuged microcapillars. Plasma was removed from tubes and both plasma and blood cell fraction (pellets) were frozen with liquid Nitrogen (−196 °C). Plasma chemicals were randomly assessed in 92 adults and 76 chicks. TPAH concentration was determined in a random sub-sample of 62 adults and 54 chicks. Only 39 chicks were simultaneously evaluated for both TPAH and plasma chemical levels.

2.2. TPAH levels

The most toxic polycyclic aromatic hydrocarbons (PAHs), according to U.S. Environmental Protection Agency (EPA) recommendations (Keith and Telliard, 1979), and present in the *Prestige* crude oil (CSIC, 2003) were measured on blood cell fraction. The PAHs analysed were naphthalene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benz(a)anthracene, chrysene, benzo(b+j)fluoranthene, benzo(k)fluoranthene, Indeno(1,2,3-c-d)pyrene, benzo(a)pyrene, benzo(e)pyrene, dibenz(a,h)anthracene, benzo(g, h, i) perylene. To estimate the individual degree of oil contamination, the sum of concentrations from all these hydrocarbons was used as a variable (TPAH level).

The concentrations of PAHs were determined by high performance liquid chromatography (HPLC). After microwave extraction with a mixture of acetone: hexane 1:1, the extract was cleaned-up using a deactivated alumina column with hexane as eluant. The PAHs were determined by HPLC coupled to a wavelength programmable fluorescence detector (Viñas-Diéguez 2002). The method used certified quality controls from the National Institute of Standards and Technology (Gaithersburg, USA; references NIST SRM 1647d and 2977). Moreover, the method was involved in the "Prestige 2004 Intercalibration Trial" organized by the Instituto Español de Oceanografía (www.ieo.es).

2.3. Plasma biochemicals

Plasma chemicals were measured in a Lambda PerkinElmer spectrophotometer (Wellesley, USA) using commercial kits and certified controls from Spinreact labs (Girona, Spain; http://www.spinreact.com). The analyzed biochemical parameters were (method in parenthesis): glucose (glucose oxidase), cholesterol (cholesterol esterase), total protein (biuret reaction), uric acid (uricase method), creatinine (kinetic Jaffee reaction), inorganic phosphorus ("iP"; molybdenum blue reaction), calcium ("Ca"; arsenaze III), asparatate aminotransferase ("AST"; NADH-method) and gamma-glutamyl transferase ("GGT"; carboxy substrate). The coefficient of variation (CV) for all these parameters ranged between 3.79–9.39%. Analyses for each age-category (adults or chicks) were performed during the same time series, randomizing the origin throughout the session to avoid time-related bias.

2.4. Birds sampled before the Prestige oil spill

Adult birds from Cíes and Ons islands (oiled area; Fig. 1) were sampled in the 1997 and 1998 breeding seasons, respectively (Alonso-Alvarez, 2000; Alonso-Alvarez et al., 2002a,b). Morphometric measures, blood sampling and plasma extraction were performed as described above. Samples were stored at −20 °C. Plasma biochemistry was measured in a Hitachi-U2000 spectrophotometer (Tokyo, Japan) in the same year of sampling. Commercial kits from SIGMA (Missouri, USA) were used to determine: cholesterol (cholesterol esterase), total protein (Bradford method), uric acid (uricase method) and iP (molybdenum blue reaction). In order to avoid differences in phenology or seasonal cycles (Alonso-Alvarez et al., 2002a) only those birds sampled from May 19 to June 5 (23 males and 25 females) were used in the analyses.

2.5. Statistical analyses

Comparisons of TPAH values between sites and age-categories (adults and chicks) were carried out by means of a generalized mixed model (PROC MIXED in SAS software; SAS Institute, 2001), including the coastal area of sampling (oiled vs unoiled areas) and the age (adults vs chicks) as fixed factors, and the identity of each population (colony) as a random factor. The interactions between both fixed factors were also tested.

The general mixed models assessing the influence of the coastal area on plasma biochemicals, hematocrit and body mass were performed separately for each age group and including the sampling site (oiled vs unoiled areas) as fixed factor. In adults, the sex was also included as fixed factor and sampling date as a covariate. Tarsus and wing length of adults were also added as covariates when analysing body condition (i.e. size-corrected body mass; e.g. García-Berthou, 2000; Stevenson and Woods, 2006). Since adult body mass follows a bimodal distribution (i.e. yellow-legged gulls are sexual-dimorphic in size; Bosch, 1996), the analyses on this variable were performed separately for each sex. In chicks, wing length was included as a covariate to control for individual variability in age (e.g. Genovart et al., 2005) and/or body size (i.e. age- and size-independent body mass). In the sub-sample of birds where TPAH was measured, the relationship between this parameter and plasma biochemicals, hematocrit or body mass was investigated by similar models as above described but including TPAH as covariate.

Comparisons between birds sampled before and after the *Prestige* oil spill were performed by including time as fixed factor (before vs after) and the identity of the colony as a random factor. Analyses on body condition were again separately performed for each sex.

In order to selected the best fitted model, we always started from the saturated model including factors, covariates and their interactions, removing all non-significant terms ($P>0.05$) by a backward procedure. The random factor (colony) showed low significance (P-value range = 0.061–0.370). Nonetheless, it was conservatively maintained in all models order to maintain the independence of data, adjusting properly the degrees of freedom (e.g. Crawley, 1993).

Most dependent variables met normality and heteroscedasticity assumptions. In analyses on adult birds, TPAH covariate was however log-transformed in order to meet the normal distribution. Means, SE and dots in Figures come from the real data, that is, uncorrected for the random effect. Slopes were obtained from mixed models. *Post hoc* analyses were performed with Tukey tests. Results are given as mean ± SE.

3. Results

3.1. Oiled versus unoiled areas

3.1.1. TPAH levels

Birds sampled in oiled areas showed higher TPAH concentration (190.63 ± 24.5 ng/g) than birds from unoiled areas (101 ± 11.1 ng/g) and this difference was significant (Table 1). TPAH concentration did not differ between chicks and adults (167.5 ± 21.9 ng/g and 156.8 ± 27.3 ng/g, respectively) and the age × area interaction was not significant (Table 1).

3.1.2. Plasma biochemistry, hematocrit and body mass variability

Adult gulls from oiled colonies showed significant lower levels of creatinine, iP, glucose and total protein, and higher levels of AST (Table 2; Fig. 2). In the case of GGT, the interaction between sex and area was significant ($F_{1,83}=13.08$, $P=0.0005$; Fig. 2). Thus, whereas

Table 1
Generalized mixed linear model testing the effect the sampled area and the age of the bird on the level of TPAH in blood cell fraction

	F	df	P
Area (unoiled/oiled)	8.91	1,106	0.004
Age (chick/adult)	1.15	1,106	0.286
Area × age interaction	0.07	1,106	0.791

The identity of each colony was included as a random factor ($Z=0.70$, $P=0.242$).

Table 2
Circulating levels of several biochemical parameters, hematocrit and size-corrected body mass from adult yellow-legged gulls breeding in colonies located in *Prestige* oiled and unoiled areas

Parameter	Unoiled area		Oiled area		Area factor		
	Mean	SE	Mean	SE	*F*	df	*P*
AST (U/L)	89.8	3.35	107.2	2.02	4.06	1,85	0.047
GGT (U/L)	4.00	0.12	4.27	0.09	2.05	1,85	0.156
Creatinine (mg/dL)	0.81	0.02	0.76	0.01	4.38	1,85	0.039
Ca (mg/dL)	10.0	0.02	9.96	0.01	1.07	1,82	0.304
iP (mg/dL)	3.88	0.05	3.44	0.05	5.55	1,82	0.021
Glucose (mg/dL)	267.5	5.92	242	4.58	7.04	1,82	0.009
Cholesterol (mg/dL)	228	5.23	222.5	3.70	0.11	1,82	0.745
Total protein (g/dL)	5.29	0.13	4.93	0.07	4.64	1,82	0.034
Uric Acid (mg/dL)	9.83	0.90	12.31	0.70	2.03	1,82	0.159
Hematocrit (%)	41.53	0.71	42.53	0.85	0.18	1,76	0.673
Male size-corrected body mass(g) [a]	1031	16.8	1012	14.7	0.68	1,32	0.416
Female size-corrected body mass (g) [a]	858	15.9	870	12.0	4.04	1,24	0.067

The difference between both areas is tested as a fixed factor in generalized linear mixed models.
F and *P* values from the final model or just before to be removed as non-significant term during the cited backward process. The identity of each colony as random factor in every model (see text).
[a] Size-corrected body masses were least squared values from the mixed model including tarsus length as a covariate (males: $F_{1,32}=8.10$, $P=0.0077$; females: $F_{1,46}=20.96$, $P<0.0001$).

males failed to show significant differences (Tukey test: $P=0.26$), females sampled in colonies from the oiled area clearly showed higher GGT values than females from the unoiled area (Tukey test: $P=0.003$; Fig. 2). Differences in body condition were not significant (Table 2). Sex, sampling date and their interactions never showed significant effects, being all removed (all $P>0.05$).

In chicks, only hematocrit showed a significant difference between areas (lower values in chicks from oiled sites; Table 3). Sampling date showed a positive relationship with hematocrit ($F_{1,125}=5.79$, $P=0.018$; estimated slope: $+1.136\pm0.472$), and was retained in this model.

3.2. Before–after comparisons

Birds sampled before the *Prestige* oil spill showed higher plasma cholesterol and lower total protein concentrations (Table 4). Plasma iP levels were higher before the spill, and the difference was close to significance. Finally, both males and females sampled in 1997–1998 showed weaker body condition than birds sampled after the catastrophe. Sex, sampling date and interactions were also tested but removed as non-significant terms (all $P>.05$).

3.3. TPAH in relation to plasma chemistry, hematocrit and body mass variability

3.3.1. Adult gulls

TPAH showed a significant positive relationship with AST (Fig. 3). Nevertheless, TPAH did not show a significant influence in the rest of plasma biochemicals or hematocrit. Sex, sampling date and their interactions did not show a significant effect on these models (all $P>0.05$).

TPAH was negatively related to body condition in both males ($F_{1,20}=7.29$, $P=0.014$; estimated slope±SE: -69.95 ± 25.9) and females ($F_{1,24}=5.75$, $P=0.025$; estimated slope±SE: -38.61 ± 20.33). Wing length remained in both models (males: $F_{1,20}=11.11$, $P=0.0033$; females: $F_{1,24}=18.43$, $P=0.0003$). The area factor, the sampling date, tarsus length and their interactions were removed from these models as non-significant terms (all $P>0.06$).

3.3.2. Chick gulls

TPAH showed a positive relation with GGT (Fig. 4). TPAH was also positively related to calcium values (Fig. 4), wing length remaining in the same model ($F_{1,32}=6.38$, $P=0.017$; estimated slope ±SE: $+0.0023\pm0.0009$). Finally, TPAH was positively related to the hematocrit (Fig. 4).

4. Discussion

Our study suggests that a delayed impact of a sublethal nature on seabirds is operating 17 months after the catastrophe. This is supported by several lines of evidence. First, gulls sampled in unoiled and oiled colonies differed in blood TPAH levels. Second, gulls sampled in unoiled and oiled colonies differed in several blood parameters indicative of physiological disorders. Third, TPAH in blood cell fraction was significantly related to several of these parameters. The last suggests that PAHs were indeed able to induce the cited disorders. Furthermore, the presence of TPAH in chicks and the fact that they could be not directly exposed to the crude indicates that pollution was in fact incorporated into the food chain.

Both adult males and females from oiled colonies showed a higher AST level, which suggests the presence of hepatic damages. Studies on the effect of the *Exxon-Valdez* oil spill on pigeon guillemots (*Cepphus columba*) reported higher AST levels in adults, but not chicks, captured in oiled areas more than 7 years after the spill (Seiser et al., 2000; Golet et al., 2002). AST enzyme is used as an index of hepatocellular disease in birds (Brugère-Picoux et al., 1987; Harr, 2002). Alternatively, high AST values would be also indicators of muscle damage when high creatine kinase (CK) levels are present (e.g. Dabber and Powell, 1993). CK catabolizes phosphocreatine to creatinine in order to produce energy for the muscle activity (Wyss and Kaddurah-Daouk, 2000). Here, the lower creatinine levels of gulls from the oiled area suggest that high AST levels were mostly related to hepatic damage (see also Seiser et al. 2000). Supporting the relation between AST and liver damage in a context of PAH pollution, Golet et al. (2002) found a positive correlation between AST levels and a hepatic citochrome (P4501A), which is specifically sensitive to PAH exposure (Collier and Varanasi 1991; Trust et al., 2000).

Spatial differences in GGT values were only significant in adult females (Fig. 2). GGT would again be an index of liver disease and damages of biliar ducts and renal epithelium among avian species (Lewandowski et al., 1986; Hochleithner, 1994; Harr, 2002). A stronger impact on breeding females might be explained by the additive effect of oil toxicity and laying effort (the sampled females were, in fact, incubating). We know that female liver can increase in size during the laying period to face the effort of egg yolk production (e.g. Christians and Williams, 1999; see also Barboza and Jorde 2002). In the same line, we know that in female mallards (*Anas platyrhynchos*) serum GGT activity increased 20-fold during the egg laying period (Fairbrother et al., 1990). Alternatively, the result could be also explained by sex-related differences in feeding habits (described

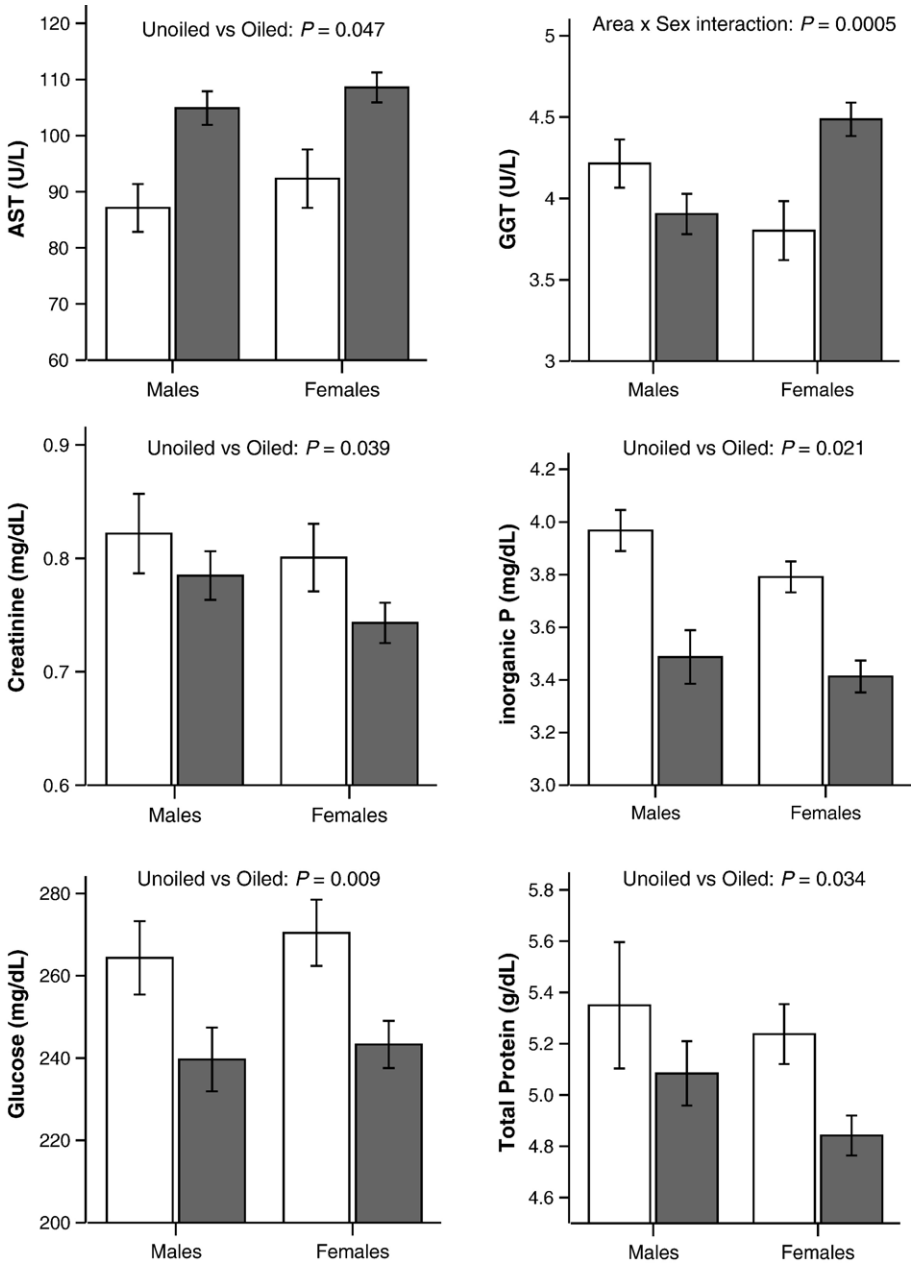

Fig. 2. Plasma levels of several biochemical parameters from male and female adult yellow-legged gulls sampled in unoiled and oiled areas (white and dark bars, respectively). *P*-values for significant factors and interactions in Table 2 models and text are again shown. Means±SE.

in gulls: e.g. Pons, 1994). Such differences could have resulted in increased exposure of females to oil pollutants (see also Martínez-Abraín et al., 2006).

The lower glucose and total protein values in birds from oiled areas indirectly suggests, in a first glance, the impact of the *Prestige* pollution on food availability. Circulating glucose is strongly regulated because it is used as energy resource for most tissues and cells (e.g. the central nervous system; Castellini and Rea, 1992). Here, the glucose values of gulls from both areas were around the lowest mean concentration found in yellow-legged gulls at the end of a starvation period (267 and 282 ng/mL for absolute fasting and food-restricted gulls, respectively; i.e. Alonso-Alvarez and Ferrer, 2001). Lower glucose levels (around 200 mg/dL) have been reported in American coots (*Fulica americana*) fed with crude-oil from the Unocal–Metrolink oil spill (Newman et al., 2000). These authors also found increased total protein levels, explaining both findings as consequence of concomitant bacterial infections. Here, the total protein decrease might instead support the idea of undernutrition. Total protein levels diminish during food-shortage periods in yellow-legged gulls (Alonso-Alvarez and Ferrer, 2001). Surprisingly, total protein levels of gulls from both sites were considerably higher than those found in starved gulls (i.e. around 3 g/dL; Totzke et al., 1999; Alonso-Alvarez and Ferrer, 2001) and in those sampled before the oil spill (Table 4). Nonetheless, plasma cholesterol level, an index of individual condition in this species (Alonso-Alvarez et al., 2002b), showed higher values before the *Prestige* event, though this could be

Table 3
Circulating levels of several biochemical parameters, hematocrit, body mass and size-corrected body mass from yellow-legged gull chicks captured in colonies located in *Prestige* oiled and unoiled coastal areas

Parameter	Unoiled Area		Oiled Area		Area factor		
	Mean	SE	Mean	SE	*F*	df	*P*
AST (U/L)	111.6	1.81	112	1.98	0.001	1,71	0.951
GGT (U/L)	4.40	0.10	4.47	0.12	0.22	1,71	0.644
Creatinine (mg/dL)	0.74	0.02	0.81	0.02	3.28	1,71	0.075
Ca (mg/dL)	9.87	0.05	9.88	0.08	0.01	1,71	0.910
iP (mg/dL)	3.68	0.07	3.88	0.10	1.94	1,71	0.168
Glucose (mg/dL)	221.9	2.45	217.3	2.94	0.78	1,71	0.381
Cholesterol (mg/dL)	227	3.25	222.7	3.42	0.19	1,71	0.670
Total protein (g/dL)	4.41	0.04	4.42	0.07	0.02	1,71	0.887
Uric Acid (mg/dL)	7.84	0.26	7.61	0.25	0.38	1,71	0.537
Hematocrit (%)	33.1	1.74	26.6	1.26	5.44	1,125	0.021
Body mass (g)	634.7	23.2	678.4	14.68	0.42	1,127	0.519
Size-corrected body mass (g) [a]	674.5	23.22	652.4	19.65	0.52	1,125	0.472

The difference between both areas is tested as a fixed factor in generalized linear mixed models.
F and *P* values from the final model or just before to be removed as non-significant term during the cited backward process. The identity of each colony as random factor in every model (see text).
[a] Size-corrected body masses were least squared values from the mixed model including wing length as a covariate ($F_{1,125}=173.3$, $P<0.0001$).

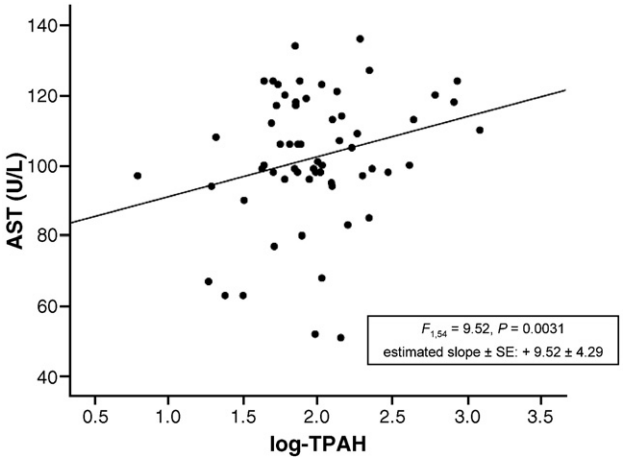

Fig. 3. Relationship between AST plasma level and total polycyclic aromatic hydrocarbons (TPAH) in blood cell fraction of adult yellow-legged gulls.

also related to differences in freezing conditions between studies (see Bustamante and Travaini, 1994). Alternatively to the effects of the oil spill on food availability and nutritional condition, differences in glucose and total protein levels best fit with a TPAH-induced liver damage. The hepatic tissue has a pivotal role in both glycogenesis and protein synthesis (e.g. Whittow, 2000). Moreover, both glucose and total protein values were negatively correlated to AST (Pearson correlation coefficients: $r=-0.257$, $P=0.015$ and $r=-0.209$, $P=0.049$, respectively), which is involved in both gluconeogenesis and amino acid metabolism (e.g. Stevens, 1996).

Our analyses did not detect a clear effect of the *Prestige* oil spill on the nutritional condition of yellow-legged gulls. First, body condition (i.e. size-corrected body mass) did not differ between areas. Second, body condition after the spill was in fact better than before. Third, Galician fisheries showed a significant recovery in 2004 (Sanchez et al., 2006; Serrano et al., 2006), which would not agree with a scenario of reduced food availability as consequence of the spill. Nonetheless, TPAH levels and adult body condition were negatively correlated. Thus, although these pollutants could be able to impact on nutritional status of gulls, the effect of the spill was mostly expressed in terms of organ dysfunctions.

In the same line, liver damage could be also responsible of differences in inorganic phosphorus concentrations. Avian embryos treated with fluoranthene and benz(k)fluoranthene, that is, two of the PAHs present in our gulls, induced liver necrosis and a decrease of alkaline phosphatase activity (Kertesz and Hlubik, 2002), which is involved in phosphorus metabolism. Since this enzyme is produced in liver, kidney, intestines and bones (Lewandowski et al., 1986; Hochleithner, 1994), any toxic acting on any of these tissues could potentially affect its activity, and consequently, iP levels. As in the case of glucose and total protein, the negative correlation between iP and AST levels ($r=-0.223$, $P=0.036$) supports the hypothesis of liver damage. In addition, birds sampled before the *Prestige* oil spill tended to have significantly higher iP levels than birds sampled in 2004 (Table 4). Reduced iP levels have been

Table 4
Circulating levels of several biochemical parameters and size-corrected body mass from yellow-legged gulls captured before (1997–1998) and after (2004) the *Prestige* oil spill

Parameter	Before		After		Area factor		
	Mean	SE	Mean	SE	*F*	df	*P*
iP (mg/dL)	3.93	0.35	3.17	0.06	3.40	1,68	0.070
Cholesterol (mg/dL)	295.9	9.32	228.9	6.96	24.69	1,68	<0.0001
Total protein (g/dL)	3.18	0.80	5.07	0.11	192.8	1,69	<0.0001
Uric Acid (mg/dL)	15.24	1.33	12.61	0.97	1.84	1,68	0.180
Male size-correctedbody mass (g) [a]	957.7	11.15	1021	15.45	11.07	1,31	0.002
Female size-correctedbody mass (g) [a]	827.8	14.85	882.5	17.17	6.77	1,37	0.013

The identity of each colony as random factor in every model (see text).
The difference between both sampling events is tested as a fixed factor in generalized linear mixed models.
[a] Size-corrected body masses were least squared values from the mixed model including tarsus length as a covariate (males: $F_{1,31}=7.49$, $P=0.010$; females: $F_{1,37}=14.3$, $P=0.0006$).

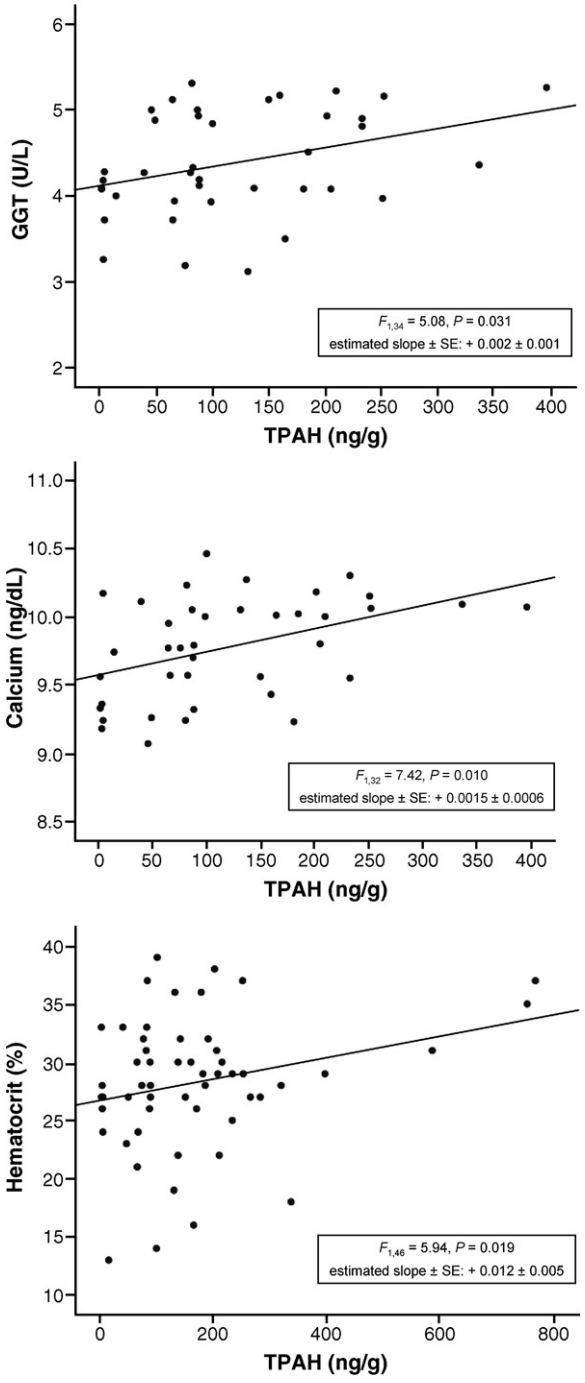

Fig. 4. Concentration of total polycyclic aromatic hydrocarbons (TPAH) in blood cell fraction of gull chicks related to GGT and calcium plasma levels as well as to hematocrit values.

reported in mallards fed with crude-oil (Stubblefield et al., 1995) and on pigeon guillemots sampled in the *Exxon-Valdez* oiled sites (Golet et al., 2002). Nonetheless, our levels were within the range of variation of captive (Alonso-Alvarez and Ferrer, 2001) and free-ranging yellow-legged gulls (Alonso-Alvarez, 2005), suggesting that the effect was in fact subtle.

In the case of chicks, only hematocrit showed differences between sampled sites, presenting lower values in oiled areas. In contrast, this parameter was positively related to TPAH-values, which could be explained by dehydration of some individuals (Dawson and Bortolotti, 1997; Kertesz and Hlubik, 2002). In fact, when the two most extreme TPAH values were excluded (Fig. 4, upper right corner), the correlation disappeared ($P=0.290$), but the difference between areas remained ($P=0.021$). The reduced hematocrit value of chicks from oiled sites could be associated to starvation (Boismenu et al., 1992; Brown, 1996; but see Dawson and Bortolotti, 1997), but also to haemolytic anaemia. However, chick body condition did not differ between areas and haemolytic anaemia has been only described in acute exposures (e.g. Leighton, 1995; Yamato et al., 1996; Balseiro et al., 2005). The fact that TPAH values were positively correlated to GGT and Ca concentration suggests that PAH's were indeed able to affect chicks' health, but not according to the sampling area. Increased GGT levels would indicate liver and/or kidney damages (above), whereas elevated Ca values could be associated to tumours and/or dehydration (Lewandowski et al., 1986; Hochleithner, 1994).

The presence of PAHs in chick blood is very relevant, indicating that these pollutants have been incorporated into the food chain. Seiser et al. (2000) and Golet et al. (2002) emphasized that adult pigeon guillemots are a better model for the study of oil spill contamination than chicks because adult birds would be exposed to higher amounts of pollutants as a consequence of age-related differences in the diet. However, information from chick seabirds is also crucial to know the level of contamination in the marine ecosystem because they are not directly exposed to the crude-oil, but to contaminated organisms in the diet (e.g. fishes and crustaceans).

Chicks showed similar TPAH levels than adult gulls, which suggests that the difference in the degree of health damage was not related to differences in diet (see Seiser et al., 2000; Golet et al., 2002), but to differences in the time of exposure to pollutants, longer in adults. Additionally, adults were likely directly exposed to oil in the few months following the *Prestige* wreck. Adult yellow-legged gulls in NW Spain are sedentary, with overlapping wintering and breeding areas (Munilla, 1997). Oiled seabirds ingest oil when preening and data on the proportion of birds that were observed with oil stained plumage suggests that between November 2002 and Febuary 2003 a noticeable proportion of the yellow-legged gull population in Galicia was oiled to some (Munilla, unpublished data).

In summary, our results not only suggest that the *Prestige* oil spill was responsible of an increase in contamination levels of marine organisms, but also that a delayed impact of a sublethal nature is operating 17 months after the catastrophe. There is a risk of underestimating the impact of oil pollution on seabirds by overlooking the sublethal effects of chronic exposures. In fact, sublethal effects could even have a stronger impact on population dynamics than direct mortality (see Peterson et al., 2003). Therefore, the evaluation of long-term sub-lethal effects will be imperative when quantifying the real impact of oil pollution on wildlife.

Acknowledgments

We acknowledge Xunta de Galicia staff (Spain) for providing us with logistic support during the sampling campaigns.

C. A.-A. and A.V. were supported by a Ramon y Cajal Fellowship (Ministerio de Educación y Ciencia, Spain). The present study was founded by the program Plan Nacional I+D+I 2004–2007 (Ministerio de Educación y Ciencia, Spain).

References

Alonso-Alvarez C. Ecofisiología del comportamiento y de la reproducción en la gaviota patiamarilla (*Larus cachinnans*). PhD Thesis dissertation (in Spanish). Santiago de Compostela, Spain: Universidade de Santiago de Compostela; 2000.

Alonso-Alvarez C. Age dependent changes in plasma biochemistry of yellow-legged gulls (*Larus cachinnans*). Comp Biochem Physiol A 2005;140: 512–8.

Alonso-Alvarez C, Ferrer M. A biochemical study about fasting, subfeeding and recovery processes in yellow-legged gulls. Physiol Biochem Zool 2001;74: 703–13.

Alonso-Alvarez C, Ferrer M, Velando A, Veira JAR. Changes in plasma biochemistry and body mass during incubating in the yellow-legged gull. Waterbirds 2002a;25:253–8.

Alonso-Alvarez C, Ferrer M, Velando A. The plasmatic index of body condition in yellow-legged gulls *Larus cachinnans*: a food-controlled experiment. Ibis 2002b;144:147–9.

Alonso-Alvarez C, Velando A. Female body condition and brood sex ratio in yellow-legged gull *Larus cachinnans*. Ibis 2003;145:220–6.

Balseiro A, Espi A, Marquez I, Perez V, Ferreras MC, Marin JFG, et al. Pathological features in marine birds affected by the *Prestige*'s oil spill in the north of Spain. J Wildl Dis 2005;41:371–8.

Barboza PS, Jorde DG. Intermittent fasting during winter and spring affects body composition and reproduction of a migratory duck. J Comp Physiol B 2002;172:419–34.

Boismenu C, Gauthier G, Larochelle J. Physiology of prolonged fasting in greater snow geese *Chen caerulescens atlantica*. Auk 1992;109:511–21.

Bosch M. Sexual size dimorphism and determination of sex in yellow-legged gulls. J Field Ornithol 1996;67:534–41.

Bosch X. Exposure to oil spill has detrimental effect on cleanup workers' health. Lancet 2003;361:147.

Briggs KT, Yoshida SH, Gershwin ME. The influence of petrochemicals and stress on the immune system of seabirds. Regul Toxicol Pharmacol 1996;23: 145–55.

Brown ME. Assessing body condition in birds. In: Nolan V, Ketterson ED, editors. Current ornithology, vol. 13. New York: Plenum Press; 1996. p. 67–135.

Brugère-Picoux J, Brugère H, Basset I, Sayad N, Vaast J, Michaux JM. Biochemie clinique en pathologie aviaire. Introit et limites des dosages enzymatiques chez la poule. Rec Med Vet 1987;163:1091–9.

Bustamante J, Travaini A. Effect of keeping plasma frozen at −20 °C on the concentration of blood metabolites. Comp Biochem Physiol A 1994;107: 661–4.

Campbell TW. Hematology. In: Ritchie BW, Harrison GH, Harrison LR, editors. Avian medicine: principles and application. Lake Worth: Wingers Publishing; 1994. p. 176–98.

Camphuysen CJ, Heubeck M, Cox SL, Bao R, Humple D, Abraham C, et al. The *Prestige* oil spill in Spain. Atlant Seabirds 2002;4: 131–40.

Castellini MA, Rea LD. The biochemistry of natural fasting at its limits. Experientia 1992;48:575–82.

Christians JK, Williams TD. Organ mass dynamics in relation to yolk precursor production and egg formation in European starlings *Sturnus vulgaris*. Physiol Biochem Zool 1999;72:455–61.

Collier TK, Varanasi U. Hepatic activities of xenobiotics in English sole (*Parophrys vetulus*) exposed to environmental contaminants. Arch Environ Contam Toxicol 1991;20:462–73.

Crawley MJ. GLIM for ecologists. Oxford: Blackwell Scientific Publications; 1993.

CSIC (Consejo Superior de Investigaciones Científicas). CSIC technical reports No. 01 and 02; 2003 (in Spanish). http://csicprestige.iim.csic.es/Informes.htm.

Dabbert CB, Powell KC. Serum enzymes as indicators of capture myopathy in mallards (*Anas platyrhynchos*). J Wildl Dis 1993;29:304–9.

Dawson RD, Bortolotti GR. Are avian hematocrits indicative of condition? American kestrels as a model. J Wildl Manage 1997;61:1297–306.

Esler D, Schmutz JA, Jarvis RL, Mulcahy DM. Winter survival of adult female harlequin ducks in relation to history of contamination by the Exxon valdez oil spill. J Wildl Manage 2000;64:839–47.

Fairbrother A, Craig MA, Walker K, O'Loughlin D. Changes in mallard (*Anas platyrhynchos*) serum chemistry due to age, sex and reproductive condition. J Wildl Dis 1990;26:67–77.

Fernandez N, Cesar A, Gonzalez M, DelValls TA. Level of contamination in sediments affected by the Prestige oil spill and impact on the embryo development of the sea urchin. Cienc Mar 2006;32:421–7.

García-Berthou E. On the misuse of residuals in ecology: testing regression residual vs. the analysis of covariance. J Anim Ecol 2001;70:708–11.

Genovart M, Oro D, Forero MG, Igual JM, Gonzalez-Solis J, Ruiz X. Parental body condition does not correlate with offspring sex ratio in Cory's shearwater. Condor 2005;107:161–7.

Golet GH, Seiser PE, McGuire AD, Roby DD, Fischer JB, Kuletz KJ, et al. Long-term direct and indirect effects of the Exxon Valdez oil spill on pigeon guillemots in Prince William Sound, Alaska. Mar Ecol Prog Ser 2002;241:287–304.

Harr KE. Clinical chemistry of companion avian species: a review. Vet Clin Pathol 2002;31:140–51.

Hochleithner M. Biochemistries. In: Ritchie BW, Harrison GH, Harrison LR, editors. Avian medicine: principles and application. Lake Worth: Wingers Publishing; 1994. p. 223–45.

Jessup DA, Leighton TE. Oil pollution and petroleum toxicity to wildlife. In: Hoff G, Fairbrother A, Locke L, editors. Non infectious diseases in wildlife. Iowa: Iowa State University Press; 1996. p. 141–56.

Juráni M, Výboh P, Zeman M, Lamosová D, Kost'ál L, Blazýcek P. Post-hatching dynamics of plasma biochemistry in free-living European starlings (*Sturnus vulgaris*). Comp Biochem Physiol A 2004;138:89–95.

Keith LH, Telliard WA. Priority pollutants. I. A perspective view. Environ Sci Technol 1979;13:416–23.

Kertesz V, Hlubik I. Plasma ALP activity and blood PCV value changes in chick fetuses due to exposure of the egg to different xenobiotics. Environ Pollut 2002;117:323–7.

Laffon B, Rabade T, Pasaro E, Mendez J. Monitoring of the impact of *Prestige* oil spill on *Mytilus galloprovincialis* from Galician coast. Environ Int 2006;32:342–8.

Le Maho Y, Vu Van Kha H, Koubi H, Dewasmes G, Girard J, Ferré P, et al. Body composition, energy expenditure, and plasma metabolites in long-term fasting geese. Am J Physiol 1981;241:E342–54.

Leighton FA. The toxicity of petroleum oils to birds: an overview. In: Frink L, Ball-Weir K, Smith C, editors. Wildlife and oil spills. Newark, Delaware: Tri-State Bird Rescue and Research Inc; 1995. p. 10–22.

Lewandowski AH, Campbell TW, Harrison GJ. Clinical Chemistries. In: Harrison GJ, Harrison LR, editors. Clinical avian medicine and surgery. Philadelphia: Saunders; 1986. p. 192–200.

Martínez-Abraín A, Velando A, Oro D, Genovart M, Gerique C, Bartolome MA, et al. Sex-specific mortality of European shags during an oil spill: demographic implications for the recovery of colonies. Mar Ecol Prog Ser 2006;318:271–6.

Morales-Caselles C, Jiménez-Tenorio N, Gonzalez de Canales ML, Sarasquete C, DelValls TA. Ecotoxicity of sediments contaminated by the oil spill associated with the tanker Prestige using juveniles of the fish *Sparus aurata*. Arch Environ Contam Toxicol 2006;51:652–60.

Munilla I. Desplazamientos de la Gaviota Patiamarilla *Larus cachinnans* en poblaciones del norte de la Península Ibérica. Ardeola 1997;44:19–26.

Newman SH, Mazet JK, Ziccardi MH, Lieske CL, Fauquier DA, Gardner IA, et al. Haematological changes and anaemia associated with captivity and petroleum exposure in seabirds. Comp Hematol Int 1999;9: 60–7.

Newman SH, Anderson DW, Ziccardi MH, Trupkiewicz JG, Tseng FS, Christopher MM, et al. An experimental soft-release of oil-spill rehabilitated American coots (*Fulica americana*): II. Effects on health and blood parameters. Environ Pollut 2000;107:295–304.

Osenberg GW, Schmitt RJ, Holbrook SJ, Abu-Saba KE, Flegal AR. Detection of environmental impacts: natural variability, effect size, and power analysis. Ecol Appl 1994;4:16–30.

Peterson CH. The Exxon Valdez oil spill in Alaska: acute, indirect and chronic effects on the ecosystem. Adv Mar Biol 2001;39:1–103.

Peterson CH, Rice SD, Short JW, Esler D, Bodkin JL, Ballachey BE, et al. Long-term ecosystem response to the Exxon Valdez oil spill. Science 2003;302:2082–6.

Piatt JF, Lensink CJ, Butler W, Kendziorik M, Nysewander DR. Immediate impact of the Exxon Valdez oil spill on marine birds. Auk 1990;107:387–97.

Pons JM. Feeding strategies of male and female Herring gulls during the breeding season under various feeding conditions. Ethol Ecol Evol 1994;6: 1–12.

Prichard AK, Roby DD, Bowyer RT, Duffy LK. Pigeon guillemots as a sentinel species: A dose-response experiment with weathered oil in the field. Chemosphere 1997;35:1531–48.

Sanchez F, Velasco F, Cartes JE, Olaso I, Preciado I, Fanelli E, et al. Monitoring the *Prestige* oil spill impacts on some key species of the Northern Iberian shelf. Mar Pollut Bull 2006;53:332–49.

SAS Institute. SAS/STAT Software: changes and enhancements, version 8.2. North Carolina: SAS Publishing; 2001.

Seiser PE, Duffy LK, McGuire AD, Roby DD, Golet GH, Litzow MA. Comparison of pigeon guillemot, *Cepphus columba*, blood parameters from oiled and unoiled areas of Alaska eight years after the Exxon Valdez oil spill. Mar Pollut Bull 2000;40:152–64.

Serrano A, Sanchez F, Preciado I, Parra S, Frutos I. Spatial and temporal changes in benthic communities of the Galician continental shelf after the *Prestige* oil spill. Mar Pollut Bull 2006;53:315–31.

Stevens L. Avian biochemistry and molecular biology. Cambridge: Cambridge University Press; 1996.

Stevenson RD, Woods WA. Condition indices for conservation: new uses for evolving tools. Int Comp Biol 2006;46:1169–90.

Stewart-Oaten A, Murdoch WM, Parker KR. Environmental impact assessment: 'pseudoreplication' in time? Ecology 1986;67:929–40.

Stubblefield WA, Hancock GA, Prince HH, Ringer RK. Effects of naturally weathered Exxon-Valdez crude-oil on mallard reproduction. Environ Toxicol Chem 1995;14:1951–60.

Szabo A, Mezes M, Horn P, Suto Z, Bazar G, Romvari R. Developmental dynamics of some blood biochemical parameters inthe growing turkey (*Meleagris gallopavo*). Acta Vet Hung 2005;53:397–409.

Totzke U, Fenske M, Hüppop O, Raabe H, Schach N. The influence of fasting on blood and plasma composition of Herring Gulls (*Larus argentatus*). Physiol Biochem Zool 1999;72:426–37.

Trust KA, Esler D, Woodin BR, Stegeman JJ. Cytochrome P450 1A induction in sea ducks inhabiting nearshore areas of Prince William Sound, Alaska. Mar Pollut Bull 2000;40:397–403.

Velando A, Munilla I, Leyenda PM. Short-term indirect effects of the *Prestige* oil spill on European shags: changes in availability of prey. Mar Ecol Prog Ser 2005a;302:263–74.

Velando A, Álvarez D, Mouriño J, Arcos F, Barros A. Population trends and reproductive success of European Shag following the Prestige oil spill in the Iberian Peninsula. J Ornithol 2005b;146:116–20.

Viñas-Diéguez L. Evaluación de hidrocarburos aromáticos policíclicos (HAPs) por cromatografía líquida de alta eficacia (CLAE) en el entorno marino gallego. PhD Thesis dissertation (in Spanish). Vigo, Spain: Universidade de Vigo; 2002.

Votier SC, Hatchwell BJ, Beckerman A, McCleery RH, Hunter FM, Pellatt J, et al. Oil pollution and climate have wide-scale impacts on seabird demographics. Ecol Lett 2005;8:1157–64.

Whittow GC. Sturkie's Avian Physiology. London: Academic Press; 2000.

Wyss M, Kaddurah-Daouk R. Creatine and creatinine metabolism. Physiol Rev 2000;80:1107–213.

Yamato O, Goto I, Maeda Y. Hemolytic anemia in wild seaducks caused by marine oil pollution. J Wildl Dis 1996;32:381–4.