Exposure of black-necked grebes (*Podiceps nigricollis*) to metal pollution during the moulting period in the Odiel Marshes, Southwest Spain

Jaime Rodríguez-Estival[a]*, Marta I. Sánchez[b,c], Cristina Ramo[b], Nico Varo[b], Juan A. Amat[b], Juan Garrido-Fernández[d], Dámaso Hornero-Méndez[d], Manuel E. Ortiz-Santaliestra[a], Mark A. Taggart[e], Mónica Martinez-Haro[a], Andy J. Green[b], Rafael Mateo[a]

[a] *Instituto de Investigación en Recursos Cinegéticos (IREC – CSIC, UCLM, JCCM), Ronda de Toledo 12, 13005 Ciudad Real, Spain*. E-mail addresses: jaime.rodriguez.estival@gmail.com (J. Rodríguez-Estival), ManuelE.Ortiz@uclm.es (M. E. Ortiz-Santaliestra), monica.martinezharo@gmail.com (M. Martínez-Haro), Rafael.Mateo@uclm.es (R. Mateo).

[b] *Estación Biológica de Doñana (EBD – CSIC), Calle Américo Vespucio 26, 41092 Seville, Spain*. E-mail addresses: marta.sanchez@ebd.csic.es (M. I. Sánchez), cristina@ebd.csic.es (C. Ramo), nico@ebd.csic.es (N. Varo), amat@ebd.csic.es (J. A. Amat), ajgreen@ebd.csic.es (A. J. Green).

[c] *Instituto Universitario de Investigación Marina (INMAR), Campus de Excelencia Internacional/Global del Mar (CEI·MAR), Universidad de Cádiz, Puerto Real (Cádiz), Spain*

[d] *Instituto de la Grasa (IG – CSIC), Campus Universitario Pablo Olavide, Edificio 46, 41013 Seville, Spain*. E-mail addresses: jgarrido@ig.csic.es (J. Garrido-Fernández), hornero@ig.csic.es (D. Hornero-Méndez).

[e] *Environmental Research Institute, University of the Highlands and Islands, Castle St, Thurso, Scotland, KW14 7JD, UK*. E-mail address: Mark.Taggart@uhi.ac.uk (M. Taggart)

* Corresponding author (J. Rodríguez-Estival). IREC (CSIC, UCLM, JCCM); Phone: +34 926 295450; E-mail: jaime.rodriguez.estival@gmail.com;

Abstract

European populations of black-necked grebes (*Podiceps nigricollis*) congregate every year to moult at the salt ponds of the Odiel Marshes (SW Spain). However, the Odiel Marshes are part of one of the most metal-polluted coastal estuaries in the world, which may pose risks to wildlife. We assessed the exposure of grebes to metal pollution during the critical moulting period in the Odiel Marshes and its potential to cause adverse health effects. Levels of metals in red blood pellet (as a biomarker of exposure), plasma carotenoids, eye redness, and body condition (as biomarkers of effects) were studied. Metal content was also analyzed in the brine shrimp *Artemia parthenogenetica*, the most important food for grebes in this hypersaline ecosystem during the moulting period. Results showed that, in comparison to toxicity thresholds, grebes had relatively high blood levels of arsenic (As), mercury (Hg) and zinc (Zn). The high loads found in *Artemia* and the way blood levels vary during the moulting period indicate that shrimp consumption may be the main route of metal exposure for grebes. Plasma carotenoids and body condition showed a positive association with exposure to As, while the relationship of lutein-like carotenoids with Hg accumulation was negative at the beginning of the moulting period to become positive afterwards. Moreover, eye redness was negatively affected by As accumulation. Factors including food resource availability, seasonal fluctuations in physiological status, and interannual variations in the degree of environmental contamination should be considered in monitoring efforts when using moult migrant waterbirds as sentinel species.

Keywords: Waterbirds, *Artemia*, Body condition, Carotenoids, Eye redness, Metal pollution

1. Introduction

Moulting is a critical life-history event in the annual life cycle of birds since it requires a considerable amount of time and resources (energy and proteins), carrying important physiological costs (Barta et al., 2008). For many waterbirds, the most important moult occurs just after the breeding season and consists of the simultaneous replacement of their wing feathers, which renders them completely flightless for a period. In some species, this moulting period is associated with a migration to specific habitats, far from breeding areas, where animals can meet those high biological requirements. These moulting waterbirds typically select wetlands that provide the basic necessities for survival without the immediate need to fly, including sufficient food resources to meet their nutritional demands (moulting and accumulation of fat reserves before moving to wintering areas), shelter and safety (reduced risk of predation) (Gehrold, 2014). These bird species have also developed several physiological adaptations that increase their chances of survival (Fox and Kahlert, 2005; Portugal et al., 2007). However, flightless waterbirds may be vulnerable to anthropogenic disturbance occurring at wetlands during this sensitive period, such as environmental pollution. The release of contaminants into these fragile habitats may involve the impoverishment in water quality with the consequent impacts on their ecological processes and functions. Thus, these habitats can become ecological traps for their dependent biodiversity (Battin, 2004) because of the negative effects on the health and survival of exposed wildlife populations (Kingsford et al., 2016).

The black-necked grebe (*Podiceps nigricollis*) is probably the avian species that forms the largest aggregations during moulting throughout its worldwide range. In Europe, about 10,000 grebes from breeding areas across the continent may congregate from August to December at the salt ponds of the Odiel Marshes in Southwest Spain (province of Huelva, Andalusia), one of the most important moulting sites for European populations of the species. During this period, the

74 brine shrimp *Artemia parthenogenetica* is the most important food item for grebes (Varo et al.,
75 2011). Grebes adjust foraging effort during the moulting period in line with the progressive
76 decrease in brine shrimp biomass and water temperature, contributing to the natural decline in
77 *Artemia* abundance and causing grebes to move to wintering areas when they can no longer meet
78 their energy requirements (Varo et al., 2011). Grebes also adopt body mass accumulation
79 strategies according to the timing of the moult, i.e., delayed moulters acquire greater fat stores in
80 advance of moult to contribute to feather replacement and in anticipation of failing food supplies
81 (decline in *Artemia* population) late in the moulting season (Fox et al., 2013).

82 The Odiel Marshes, together with the estuary formed at the confluence of the rivers Odiel
83 and Tinto, are considered some of the most metal-polluted areas in the world (Vallés et al., 2017).
84 The main pollution source is past/present mining activity in the Iberian Pyrite Belt (located in
85 Northern Huelva), which is one of the largest polymetallic sulphide deposits in the world.
86 Additional pollution emerges from a large industrial chemical complex at the lower end of the
87 estuary, which discharges toxic waste into the Tinto river (Pérez-López et al., 2011; Nieto et al.,
88 2013). The oxidation of mining residues produces upstream acid mine drainage with high levels of
89 several metals and metalloids (hereafter referred as metals or elements) which reaches the Odiel
90 and Tinto river basin. This results in the accumulation of high levels of arsenic (As), mercury (Hg),
91 lead (Pb), cadmium (Cd), copper (Cu), zinc (Zn) and other metals in estuarine water and sediments
92 (Beltrán et al., 2010; Vallés et al., 2017). Previous work regarding exposure of wildlife to metal
93 pollution in the Odiel and Tinto estuary is scarce but has considered oysters, mussels (Funes et al.,
94 2006) and fish (Usero et al., 2004). However, few studies have examined native waterbirds
95 (Borghesi et al., 2011; Rubio et al., 2016), even though waterbird diversity is central to legal
96 protections place in the area (Vallés et al., 2017). Another reason to investigate metal pollution in
97 birds like the black-necked grebe is that high-level predators tend to bioaccumulate greater
98 amounts of toxic substances in their tissues than organisms lower in the food chain. Thus, these

types of waterbirds are considered especially useful, sensitive and accessible sentinels to evaluate ecological and health risks from pollutants in aquatic ecosystems (Zhang and Ma, 2011; Stankovic et al., 2014).

Here, the exposure of the grebes in the Odiel Marshes to metal pollution during the critical moulting period is evaluated alongside its potential to compromise their health. For this purpose, levels of metals in blood pellets were studied as biomarkers of exposure (Ortiz-Santaliestra et al., 2015), while levels of plasma carotenoids, eye redness (ornamental carotenoid-based coloration) and a mass/length body condition index were selected as biomarkers of potential subclinical health effects. Metal content was also studied in *Artemia* as a potential key source of metal exposure in the grebes. The main objectives were (1) to determine whether metal exposure changed during the moulting period, studying the role of moult status and *Artemia* in temporal variations; (2) to interpret the level of exposure in relation to certain toxicity thresholds; and (3) to analyse how selected biomarkers vary as a function of metal accumulation in order to evaluate the potential impact of metal exposure on grebes' health.

2. Materials and methods

2.1. Study area

The study was conducted in the Odiel Marshes (37°14'N, 6°57'W), which comprises of an estuarine complex (7185 ha) at the mouth of the Odiel and Tinto rivers in the province of Huelva (Southwest Spain). This estuarine complex includes 1174 ha of saltpans where seawater is pumped from primary to secondary evaporation ponds and then to crystallizers (Sánchez et al., 2006). The evaporation ponds are used as moulting sites by black-necked grebes every year between August and December (Varo et al., 2011; Fox et al., 2013; Amat et al., 2014). Here, fieldwork was conducted in four adjacent ponds with similar salinity, representative of the saltpan area. Water

level in these ponds is usually <1 m, and brine shrimp is the most abundant aquatic invertebrate (Sánchez et al., 2006).

2.2. Grebe trapping and sample collection

A total of 180 grebes caught for ringing from October to December 2008 (n=127) and in August and November 2009 (n=53) were used in the present study. Day of capture (based on the number of days from the 1st of January each year) ranged from 225 (August) to 341 (December). Some birds were recaptured (n=18: captured first in October and recaptured in November 2008), but only data from the initial capture were considered for analyses. However, metal exposure in recaptured birds was also compared with that from the initial capture. Captured grebes were aged according to iris coloration following Storer and Jehl (1985) and categorized as juveniles (≤1 year old; iris tan with an orange tint; captured in October: n=13; and November: n=4) and adults (>1 year old; iris orange to orange-red; captured in August: n=29; October: n=50; November: n=54; and December: n=30). Sex was also determined (for sexing procedures see Amat et al., 2014 and Sáez-Gómez et al., 2017). Depending on the state of the remiges, a total of 166 birds could be clearly assigned to one of three categories in terms of moult status: unmoulted (still with old feathers), moulting (actively moulting) and moulted (already with new feathers). Unmoulted grebes (n=9) were captured throughout the moulting period, from August to December; moulting grebes (n=41) were mostly detected in August (63.4%) and October (34.1%); and moulted grebes (n=116) were primarily identified in October (28.4%), November (44.8%) and December (25.0%).

Blood samples, taken by puncturing a tarsus vein, were stored in heparinized vials and kept refrigerated until processed in the laboratory the same day. Blood samples were centrifuged at 10000 *g* for 5 min at 4°C to separate plasma from the blood pellet, and both samples were immediately frozen at −80°C until analysis. The time elapsed between blood collection in the field and storage of the pellet and plasma sample at -80°C was 3-4 h.

149

150 *2.3. Elemental analysis*

151 Blood pellets (0.2-2 g; n=180) were used to quantify metal exposure. Samples were freeze-
152 dried (Christ Alpha 1-2, Braun Biotech), then acid-digested in Pyrex tubes using a heating block
153 (Micro, for 40 tubes, Selecta) following the methodology described by Rodríguez-Estival et al.
154 (2011), and analysed for As, Hg, Pb, Cd, Cu and Zn. Digest solutions were diluted to a final volume
155 of 15 ml with Milli-Q grade water. The analysis of Pb, Cd and As in digests was achieved using
156 graphite furnace atomic absorption spectroscopy (GF-AAS; AAnalyst 800, Perkin Elmer). The
157 analysis of Cu and Zn was undertaken using a flame-AAS system (AAnalyst 800, Perkin Elmer). Hg
158 analysis was achieved using the cold vapor technique and a hydride generation system (MHS-15,
159 Perkin-Elmer) coupled to the AAS. Calibration standards were prepared from certified commercial
160 solutions (Panreac).

161 Blanks (n=9) and certified reference material (lobster hepatopancreas: TORT-2, National
162 Research Council, Canada; n=9) were also processed and analyzed to identify potential laboratory
163 contamination and to evaluate method accuracy. Limits of detection (LOD, in µg/g dry weight,
164 back-calculated to in tissue concentrations) were 0.054 (As), 0.053 (Hg), 0.034 (Pb), 0.003 (Cd),
165 0.001 (Cu) and 0.001 (Zn). Recovery (mean±SD) of the certified reference material was 93.7±8.3%
166 (As), 90.7±13.0% (Hg), 100.5±21.3% (Pb), 95.8±4.0% (Cd), 93.7±1.7% (Cu), and 105.9±10.7% (Zn).
167 Dry residue (mean±SD) of digested samples was 36.06±1.92%. Final metal concentrations are
168 reported in µg/g dry weight (d.w.).

169

170 *2.4.* Artemia *sampling and elemental analysis*

171 *A. parthenogenetica* (adults) sampling was conducted in April 2014 in a 0.5 m deep channel
172 receiving water from secondary evaporation ponds where grebes moult every year at the Odiel
173 saltpans (Sánchez et al. 2006). Samples were collected from 4 different points within the water

body at a depth of ~30 cm using a 0.5 mm mesh net. Water salinity was 160 g/L. In the laboratory, *Artemia* samples were dried to constant weight and then digested using an Anton Paar Multiwave Pro microwave digestion system with a 24 Teflon vessel carousel. Dry samples (0.2-0.5 g) were added to vessels and high purity concentrated HNO_3 (2 ml) was then added for a pre-digestion step overnight at room temperature. The next day, after the addition of high purity hydrogen peroxide (0.5 ml), vessels were sealed and heated for 2 hours at 160°C. Finally, digests were diluted to 14 ml with H_2O (Milli-Q grade) and stored at 4°C until analysis.

Artemia digests were analyzed for a suite of 11 metals, including As, Pb, Cd, Cu, Zn, cobalt (Co), chromium (Cr), manganese (Mn), molybdenum (Mo), nickel (Ni), and selenium (Se), using inductively coupled plasma optical emission spectrometry (ICP-OES). Blanks (n=3) and certified reference materials (lobster hepatopancreas: TORT-2, National Research Council, Canada; n=2) were processed and analyzed with the *Artemia* samples. Limits of detection (LOD, in µg/g dry weight, back-calculated to in sample concentrations) were 0.036 (As), 0.017 (Pb), 0.005 (Cd), 0.005 (Cu), 0.009 (Zn), 0.007 (Co), 0.002 (Cr), 0.012 (Mn), 0.010 (Mo), 0.011 (Ni), and 0.044 (Se). Recovery (mean±SD) of the certified reference material was 91.3±10.5% (As), 104.7±8.4% (Pb), 95.9±2.2% (Cd), 93.6±15.3% (Cu), 94.7±8.4% (Zn), 90.5±6.5% (Co), 90.1±13.1% (Cr), 88.0±11.1% (Mn), 98.0±6.0% (Mo), 90.4±9.2% (Ni), and 102.8±6.4% (Se). Results are given in ng/g d.w.

2.5. Determination and quantification of plasma carotenoids

Carotenoids were extracted from a subset of plasma samples (n=139) as described by Blanco et al. (2013). Briefly, a plasma aliquot (100 µl) was lyophilised and the carotenoid pigments were extracted from the dry residue with 100 µl of N,N-dimethylformamide for 60 min, including sonication for 5 min every 20 min (three times). The resulting extract was analyzed by high-performance liquid chromatography (HPLC) following Mínguez-Mosquera and Hornero-Méndez (1993) with some modifications (Blanco et al., 2013). The chromatographic analysis was carried

199 out on the same day as the preparation of the extracts. All work was carried out under dimmed
200 light to prevent isomerization and photodegradation of carotenoids. Concentration of plasma
201 carotenoids is expressed in µg/ml.

202 The identification of the carotenoid pigments was carried out following standard
203 procedures, including co-chromatography with standards, acquisition of UV-visible spectra, as well
204 as chemical derivatization microscale tests for the examination of some functional groups
205 (hydroxyl and carbonyl) (Schiedt and Liaaen-Jensen, 1995; Eugster, 1995). The chromatographic,
206 spectroscopic and chemical properties of the pigments were compared with authentic carotenoid
207 samples and with data available in the literature (Britton, 1991, 1995; Britton et al., 2004).
208 Authentic pigment samples of carotenoids were isolated from natural sources: β-carotene, β-
209 cryptoxanthin and zeaxanthin were obtained from red pepper (*Capsicum annuum* L.) and lutein
210 from mint leaves (*Mentha arvensis* L.). Canthaxanthin was purchased from Sigma-Aldrich, and G.
211 Britton (School of Biological Sciences, University of Liverpool, UK) provided the echinenone
212 standard. The identification of *cis* isomers of lutein and zeaxanthin was based on the spectroscopic
213 and chromatographic data: the presence and relative intensity of the *cis* peak at about 330-340
214 nm in the UV-visible spectrum; the reduction in the fine structure and a small hypsochromic shift
215 in λ_{max} with respect to the *all-trans* isomer; and the chromatographic behavior in the C18 reversed
216 phase HPLC column (the *cis* isomers are slightly more retained than the *all-trans* isomer; Britton,
217 1995).

218 Quantitative analysis of carotenoids was carried out using HPLC according to the method
219 described by Mínguez-Mosquera and Hornero-Méndez (1993) with some modifications. The
220 system consisted of a Waters e2695 Alliance HPLC fitted with a Waters 2998 photodiode array
221 detector, all controlled with Empower2 software (Waters Cromatografía, S.A., Barcelona, Spain). A
222 C18 reversed phase analytical column (Mediterranea SEA18, 200 mm×4.6 mm i.d., 3 µm;
223 Teknokroma, Barcelona, Spain) was used. Separation was achieved using binary-gradient elution

and an initial composition of 75% acetone and 25% deionised water. This was increased linearly to 95% acetone over 10 min, then to 100% over 2 min, and maintained for 10 min (the return to initial conditions then took 5 min). An injection volume of 20 µl and a flow rate of 1 ml/min were used. Detection was performed at 450 nm, and the online spectra were acquired in the 350-700 nm wavelength range. Quantification was carried out using external standard calibration curves prepared with zeaxanthin, lutein, canthaxanthin, echinenone, β-cryptoxanthin and β-carotene standards. Calibration curves were prepared between 0.5 - 50.0 µg/ml, and constructed by plotting the peak area at 450 nm versus the pigment concentration. The calibration curves of *all-trans* isomers were also used to determine the concentration of the *cis* isomers.

2.6. Eye redness measurement

From the same subset of 139 individuals, we took a picture of the right eye in RAW format using a Canon EOS-400 digital camera equipped with a Canon EFS 18-55 mm macro lens. All pictures were taken against a neutral grey standard (Lastolite Ezybalance, 30 cm, 18% reflectance). For each picture we selected a region of interest in the iris: a square between the pupil and the border's eye free of glossiness (averaging around 1500 pixels). We used the SpotEgg tool (Gómez and Liñán-Cembrano, 2017) to linearize and normalize the images using the grey standard and to obtain the reflectance in the visible spectra (channels R, G and B). The eye redness was calculated as R/(R+G+B), producing a value between 0 and 1.

2.7. Determination of body condition index

Several body measurements, including the head-bill length and tarsus length (±0.01 mm; both measured with a digital caliper), wing length (±0.1 mm) and body mass (±0.1 g), were taken from 177 individuals. Body condition was calculated according to the Scaled Mass Index (SMI) proposed by Peig and Green (2009) using the body mass and head-bill length. The scaling

exponent b_{SMA} value (3.15) was calculated from the SMA regression of log-transformed body mass on head-bill length based on data collected during summer-autumn ringing campaigns carried out between 2005 and 2010 (n=3033) in the Odiel Marshes. A head-bill length of 56.91 mm, corresponding to the arithmetic mean value of the studied grebe population, was used as the L_o value to standardize the index. This species has a degree of sexual dimorphism, but not enough to sex birds reliably in the hand (for details, see Amat et al., 2014, Sáez-Gómez et al., 2017). For this reason, gender was not taken it into account when calculating the SMI.

2.8. Statistical analysis

For statistical purposes, metal concentrations <LOD were assigned a value of half of the respective LOD for each element. Statistical analyses were conducted after testing for normality (Shapiro-Wilk normality test) and homogeneity of variance (Levene test) for each variable, and log-transforming (natural logarithm) those variables that did not display a normal distribution. All tests were performed using IBM SPSS Statistics 23.0 software. Significance was set at the 0.05 level.

Temporal changes in metal exposure during the moulting period were studied through General Linear Models (GLMs), including the month of capture (August vs. October vs. November vs. December) as a predictor term. Gender (males vs. females), age (juveniles vs. adults) and moult status (unmoulted vs. moulting vs. moulted) were also tested in the models to consider potential biases. A backward stepwise procedure was used to find the best-fit model, sequentially removing non-significant interactions and terms and maintaining non-significant terms included in significant interactions (Vittinghoff et al., 2005). When a significant temporal variation was detected, specific differences between the months and moult status (when appropriate) were studied through post-hoc Tukey tests. These models were also analyzed after removing the smallest subgroups of individuals (1 unmoulted and 2 moulted birds in August, 2 unmoulted and 1 moulting birds in November, and 1 unmoulted bird in December) in order to avoid statistical

misinterpretation in the effect of moult status caused by such low number of birds. Differences in metal exposure between years (we only had data for November for both years) were also tested for each variable separately using one-way ANOVAs to consider potential interannual variations. When a difference between years was detected for any variable, models including the variable were explored both considering data collected in the two ringing campaigns and excluding those from 2009. Metal exposure in recaptured birds (November 2008) was explored and compared (one-way repeated measures analysis of variance) with exposure from the initial capture (October 2008) in order to support the interpretation of results. The effects of metal exposure on carotenoid status, eye coloration and body condition were evaluated with GLMs with each response as dependent variable, and initially including the same factors as reported above for metal statistical analyses. Metal concentrations in blood pellet were separately included in these models as covariates. Best-fit model selection was performed as described above.

3. Results

3.1. Metal residues in grebe blood pellet and Artemia

Blood As in grebes increased from October through to December ($F_{3,169}$=32.13, p<0.001; **Table 1**), such progression being explained by moulted birds ($F_{2,111}$=54.30, p<0.001; **Fig. 1**). Initially, he interaction "Month of capture × Moult status" was significant, revealing that in August, moulting grebes had lower As than those moulted, and in November, unmoulted grebes had higher As than those moulted ($F_{3,155}$=9.84, p<0.001). However, this effect disappeared from the model when the smallest subgroups of individuals were removed from the analysis. For Hg, blood levels were higher in August and November than in October and December ($F_{3,176}$=6.66, p<0.001; **Table 1**). For As, the interaction "Month of capture × Moult status" was significant in the model and revealed that, in August, moulting grebes had lower As than those moulted, and in November,

299 unmoulted grebes had higher As than those moulted. Levels of Pb increased from August through
300 to November and decreased again in December (to similar levels as in October; $F_{3,176}$=7.81,
301 p<0.001; **Table 1**). Levels of Cu increased in December with respect to the rest of the moulting
302 period ($F_{3,176}$=4.88, p=0.003; **Table 1**), whereas Zn levels, which were over 5% lower in males than
303 in females (p<0.001), decreased considerably in October, especially in females, in comparison with
304 the rest of the moulting period ($F_{3,175}$=36.67, p<0.001; **Fig. 2**).

305 Three individuals showed extremely high levels of Hg (23.71, 28.38 and 85.76 µg/g d.w.,
306 respectively), and one bird showed a particularly high level of Pb (1.09 µg/g d.w.); these were
307 excluded from the analysis above. Levels of Cd were above the analytical LOD in only six grebes
308 (mean±SE of 5.60±1.5 ng/g d.w.). Grebes captured in November 2009 had higher As, Hg, Cu and Zn
309 levels than those captured in November 2008, whereas the opposite was observed for Pb levels
310 (all $F_{1,56}$≥5.51, all p≤0.022). However, none of these interannual differences biased the pattern
311 presented above. Grebes recaptured in November 2008 had higher Hg, Pb and Zn levels than
312 when they were initially captured in October 2008, whereas the opposite was observed for Cu (all
313 $F_{1,34}$≥7.37, all p≤0.010; **Fig. 3**). Age had no effects on metal levels.

314 Element concentrations in *A. parthenogenetica* from the Odiel Marshes are shown in **Table**
315 **2**.

316

317 *3.2. Association of health-indicative responses with metal accumulation in grebes*

318 Lutein-like carotenoids and *trans*-zeaxanthin were affected by metal exposure (**Table 3**).
319 Lutein-like carotenoids showed a negative relationship with Hg in August, and positive
320 relationships between October and December. Depending on the moult status and time of the
321 moulting period, *trans*-zeaxanthin showed a positive relationship with As levels (**Table 3**). On
322 contrary, eye redness, which was higher in adults than in juveniles, showed a negative relationship
323 with As accumulation both in adults and juveniles throughout the moulting period (**Table 3**). SMI

was positively related to As levels in moulted female grebes during November and December (**Table 3**).

4. Discussion

European populations of black-necked grebes congregate every year for moulting at the Odiel Marshes where they feed (primary) on the brine shrimp *Artemia parthenogenetica*. Brine shrimps are an important source of carotenoids for birds, thus influencing pigmentation, but our results show that they also accumulate high levels of metals. The way blood levels vary by month suggests that the relatively high blood levels of As, Hg and Zn (in comparison to toxicity thresholds) in grebes during their stay at the Odiel Marshes may be due to a food chain transfer, mainly from *A. parthenogenetica*. Our results also indicate that temporal variations due to changing physiological (e.g., moulting process) and environmental factors (e.g., diet) may complicate the interpretation of the health risks associated with this metal exposure scenario.

4.1. Temporal changes in metal exposure: The role of Artemia *and moulting*

Dietary exposure to metals is an important pathway for birds (Berglund, 2018), and *A. parthenogenetica* is the most important food item for grebes during their stay (for moulting) at the Odiel Marshes (Fox et al., 2013). Overall, we found that exposure (as assessed through blood levels) of grebes to As, Hg, Pb and Zn followed a temporal pattern similar to that of brine shrimp abundance for this period of the annual cycle (Varo et al., 2011), i.e., decreasing between August-September and the beginning of October and then increasing through October until November. This suggests that metal exposure is largely attributable to food chain transfer through a dependency on brine shrimp availability as a food resource (Conover and Vest, 2009; Darnall and Miles, 2009). Concentrations of As, Pb, Cu and Zn in *A. parthenogenetica* from the Odiel saltpans

are about 12, 2, 6 and 3-fold higher, respectively, than mean levels reported as reference values for these metals in *Artemia* spp. from unpolluted aquatic ecosystems (see references in **Table 2**). Thus, the relatively high loads found in *Artemia* (which indicate that this macroinvertebrate takes up and assimilates metals from polluted waters and sediments; Rainbow, 2002; Stewart et al., 2008; Adams et al., 2015; Huang, 2016), and the progressive increase in the consumption rate of adult brine shrimps expected during the late summer and early autumn (aging brine shrimp population accumulates higher concentrations of metals; Naftz et al., 2008), support this interpretation. Furthermore, the very low Cd levels found in Artemia agree with the fact that this metal was undetected in most grebes, and when detected (only in six grebes), it was found at very low blood concentrations. The comparison between Pb, Hg and Zn levels observed in grebes recaptured in November 2008 respect to those detected at the initial capture in October 2008 also support the food chain transfer of metals pollution to grebes from Artemia.

Metals in feathers tend to reflect levels in blood during feather growth, when the feather is connected to blood vessels and metals are incorporated into the keratin structure (Sánchez-Virosta et al., 2015; Ackerman et al., 2016). Thus, moult status may reflect the transfer of toxic metals into growing feathers during active moulting, which may (passively or actively) act as a metal detoxification pathway (Dauwe et al., 2003; Martínez et al., 2012; Finger et al., 2016; Hartman et al., 2017). Fluctuations in circulating levels of essential elements such as Cu and Zn would also be expected to respond to physiological processes such as breeding or moult, or to dietary changes (Stewart et al., 1999; Berglund et al., 2018). Here, we initially observed that temporal variation in As levels was influenced by the moult status, but this effect was not robust, probably because of the lack of a representative number of birds for certain subgroups. On the other hand, both the depletion of Zn levels in October and the increase in Cu in December (in blood pellets) might be linked to metal-dependent metabolic processes associated with the moulting process (e.g., keratinization during active moulting, thyroid regulation and feather

374 pigmentation), in which Zn and Cu have key roles (Honda et al., 1986; Stewart et al., 1999; Holman
375 et al., 2015; Borghesi et al., 2016). However, our results also failed to detect clear links
376 (interactions) between moult status and temporal changes in blood for these elements.

377 The comparison of samples from November 2008 and 2009 suggests that there may be
378 interannual differences in exposure of grebes to metals. Although this did not bias our analysis
379 regarding temporal/monthly variations, it may have ecotoxicological relevance to explain
380 variations in pollutants levels in the environment or fluctuations in the availability of sources of
381 exposure (Baos et al., 2006; Padula et al., 2010; Johns, 2012). Nieto et al. (2013) demonstrated
382 that the pollution load transported by the Tinto and Odiel rivers into the coastal salt marshes is
383 subject to strong interannual variation dependent on flood events associated with intense rainfall.
384 This would in turn influence interannual differences in bioaccumulation patterns of metals by the
385 brine shrimp and thus levels of exposure for consumers (Adams et al., 2015). On the other hand,
386 Varo et al. (2011) observed that brine shrimps were more abundant in 2009 in comparison with
387 2008, which might explain the lower levels of exposure to metals in grebes in 2008 vs. 2009, due
388 to changes in foraging intake rates between years. In this regard, it is also worth noting that data
389 for August (in the present study) were only obtained from grebes captured in 2009. For As, Hg and
390 Zn, August values were higher than in October 2008, which may be a consequence of these
391 interannual differences.

392

393 *4.2. Interpreting levels of exposure to metals in grebes*

394 Non-essential elements (As, Hg, Pb, and Cd here) have no known biological function, and
395 become toxic when they exceed certain limits. These metals are endocrine disruptors and can
396 affect a multitude of physiological systems, including the reproductive and immune systems. As
397 such, exposure can have important implications for wildlife population dynamics and fitness,
398 reproductive success, resistance to diseases and survival (Iavicoli et al., 2009). Therefore,

monitoring exposure of wildlife to these toxic metals and assessing their effects is important. However, inter- and intra-species related differences in metal absorption, accumulation, metabolism, excretion and resistance to toxic effects, together with ecophysiological population adaptations, often make it difficult to establish critical thresholds (Sánchez-Virosta et al., 2015). Although we did not measure metal levels in whole blood, simple estimations still allow us to compare our results (expressed in µg/g d.w. of blood pellet) with published data, i.e., when we assume that most of the metal burden is in the cell fraction, and consider average dry matter in our pellet samples (36.06%), average haematocrit values for grebes (≈50%; Kloskowski et al., 2017), and an approximate density of 1.1 g/ml for blood pellet (Ortiz-Santaliestra et al., 2015). Thus, we used conversion factors of 0,198 and 0,180 to convert our data to µg/ml (and then to ng/ml by multiplying by 1000) and µg/g w.w. in whole blood, respectively.

Inorganic As (arsenite and arsenate) is found at high concentrations in the Odiel Marshes (Sánchez-Rodas et al., 2005; Sarmiento et al., 2009), which potentially poses serious risks for this ecosystem, its wildlife and human health. A whole blood As level of 20 ng/ml has been suggested as a reference baseline value for birds inhabiting unpolluted environments (Benito et al., 1999); while Franson et al. (2000) suggested that blood As <0.10 µg/g (wet weight; w.w.) was related to unpolluted systems when studying common eiders (*Somateria mollissima*); and Grand et al. (2002) measured mean blood As of 0.16 µg/g w.w. in common and spectacled eiders (*S. fischeri*), suggesting that such levels were not likely to cause harm. Here, mean As in blood pellets in the moulting grebe population (mean±SE: 0.756±0.035 µg/g d.w.) would be equivalent to ≈150 ng/ml or ≈0.14 µg/g w.w. in whole blood. As such, levels suggest relatively elevated exposure to environmental As pollution, which is particularly apparent at the end of the moulting period (i.e., in December, when mean As was 1.162 µg/g d.w., equivalent to ≈230 ng/ml or ≈0.21 µg/g w.w. in whole blood). Arsenic is not a well-documented element when it comes to birds, thus it is

423 necessary a comprehensive overview of As exposure levels at which bird populations may start
424 suffering negative health consequences (Sánchez-Virosta et al., 2015).

425 Ackerman et al. (2016) showed that the lowest documented toxic effects associated with Hg
426 exposure in birds occurred at blood Hg levels of 0.2 µg/g w.w. Here, mean Hg levels in blood pellet
427 (mean±SE: 0.565±0.084 µg/g d.w.) would be equivalent to ≈112 ng/ml or ≈0.10 µg/g w.w. in whole
428 blood, i.e., below this baseline value. Specifically, 87% of birds would have Hg exposure below this
429 reference threshold, whereas 12% would have blood levels corresponding to a low risk of adverse
430 health effects (0.2-1.0 µg/g w.w.). Furthermore, three animals (outliers) had extremely elevated
431 Hg exposure (23.71, 28.38 and 85.76 µg/g d.w. in blood pellet, equivalent to ≈4.27, 5.11 and 15.44
432 µg/g w. w. in whole blood, respectively). This would represent a high risk of toxic consequences
433 (≥3.0 µg/g w.w. in whole blood; Ackerman et al., 2016; Hartman et al., 2017). Taking into account
434 that most of the Hg in avian blood is normally in the more toxic methylmercury form (Alvárez et
435 al., 2013; Rimmer et al., 2005), these three animals would also be above the threshold associated
436 with effects on reproduction for small-medium size birds (2.1 µg/g w.w. in whole blood; Fuschman
437 et al., 2017). The fact that these three individuals showed anomaly high levels of Hg could
438 contradict the hypothesis of the brine shrimp, which constitutes 60 to 90% of food items for the
439 grebe population form the Odiel marshes (Varo et al., 2011), as the main source of accumulation
440 of this elements in birds. However, Varo et al. (2011) also found, after examining gizzard contents
441 of six dead grebes from this population, other invertebrate groups capable of accumulating large
442 amounts of Hg like Hydrobia (Gastropoda) (Cardoso et al., 2013) and Corixidae (Hemiptera) (Hall et
443 al., 1998). Interestingly, the three grebes with outlying Hg concentrations had moulted recently,
444 thus they might have spread their feeding ranges to areas neighbouring salt ponds, to which
445 *Artemia* is restricted, and accessed other food items or zones with punctual accumulation of high
446 Hg inputs from the nearby industrial effluents. In fact, previous studies have described the spatial
447 variations of heavy metal concentrations in sediments of the Odiel river catchment area, including

448 the presence of points with remarkable levels of Pb and Hg (Santos Bermejo et al., 2003). Finally, it
449 must be considered that Hg is a persistent, cumulative metal that may have a long body retention
450 time (Gupta et al., 2018), and that part of these migrant birds might come from biological Hg
451 hotspots in European breeding areas (Lavoie et al., 2015).

452 Our results contrast with two previous studies that considered Hg exposure in little egret
453 (*Egretta garzetta*) nestlings and greater flamingo (*Phoenicopterus roseus*) fledglings in the Odiel
454 Marshes through the analysis of feathers (Rubio et al., 2016*;* Borghesi et al, 2011). Both
455 considered the Odiel Marshes to have some of the lowest Hg levels found among several
456 Mediterranean sites. Surprisingly, Borghesi et al. (2011) further suggested that Hg in the Odiel
457 Marshes might be considered at a "baseline" level for western Mediterranean greater flamingo
458 populations. Nevertheless, our results (for the 12% of grebes with Hg levels corresponding to a low
459 risk of adverse health effects and for those that had extremely elevated Hg exposure) would agree
460 with those found in three grebe species inhabiting freshwater environments subject to different
461 degrees of Hg contamination in western North America (Ackerman et al., 2016); wherein mean±SE
462 equivalent total mercury (THg) in whole blood ranged from 0.48±0.13 to 4.75±1.89 µg/g w.w. They
463 would also be comparable to those found by Álvarez et al. (2013) in several bird species from the
464 Doñana National Park (Southwest Spain) after a toxic mine spill accident (wherein mean total Hg in
465 whole blood ranged from 4.67 to 567 ng/ml in 11 species).

466 Whole blood Pb levels below 200 ng/ml are considered background, between 200 and 500
467 ng/ml subclinical exposure, and > 500 ng/ml clinical poisoning in waterbirds (Franson and Pain,
468 2011). However, Martinez-Haro et al. (2011) noted that a specific subclinical health effect
469 biomarker linked to Pb exposure (the enzyme δ-aminolevulinic acid dehydratase, ALAD), is
470 inhibited at blood Pb levels as low as 60 ng/ml in mallards (*Anas platyrhynchos*). This suggests that
471 the no-effect physiological level may well be lower than the background value frequently assumed
472 for birds. Grebes from the Odiel Marshes had Pb levels in blood pellets that would be well below

both possible reference value (mean±SE: 0.025±0.006 µg/g d.w., which would be equivalent to ≈4.95 ng/ml in whole blood). One grebe, considered an outlier, did display a particularly high Pb level (1.094 µg/g d.w.; ≈217 ng/ml in whole blood), but this could be explained by the ingestion of Pb shot or sinker, which would cause this level of subclinical exposure. With regard to Cd, blood levels ≤50 ng/ml are considered normal for wild birds, whereas birds with blood concentrations ≥260 ng/ml should be considered at risk of suffering toxic effects (Wayland and Scheuhammer, 2011). Here, only six grebes showed detectable blood Cd levels (mean±SE: 5.60±1.5 ng/g d.w.; equivalent to ≈1.11 ng/ml in whole blood), which would be, in all cases, within the normal reference threshold.

Although metals such as Cu and Zn are key to normal biological functions, when they are outside required ranges, deficiency or toxicity can lead to adverse health consequences. Tissue concentrations of both these essential elements are under homeostatic control, and normal physiological levels can range widely within and between species depending on several intrinsic factors associated with particular metabolic requirements (Goyer and Clarkson, 2001). Overall, whole blood Cu and Zn levels ranging from 80 to 500 ng/ml and from 1300 to 3400 ng/ml, respectively, may be considered within the reference threshold for birds (see reference values for blood of birds in Table 1 of Wyss et al., 2014, expressed in µg/dl). Mean Cu and Zn levels in blood pellets here in moulting grebes (mean±SE: 1.885±0.085 and 17.97±0.10 µg/g d.w., respectively; equivalent to ≈373 and 3558 ng/ml in whole blood, respectively) would thus be within the normal range for Cu. But, for Zn, 72% of birds would have blood levels higher than the upper limit of this normal range, which may be indicative of high environmental exposure. Our values for both metals are in line with those found by Benito et al. (1999) in several waterbird species from areas around the Doñana National Park affected by a toxic mine spill (wherein mean Cu and Zn in whole blood ranged from 133 to 586 and from 900 to 5900 ng/ml, respectively, in 11 species).

498 *4.3. Health biomarkers: Effects of metal exposure*

499 Carotenoids play key physiological functions as part of the antioxidant system in
500 vertebrates, acting as immune system enhancers and influencing social and sexual signalling in
501 birds through the production of yellow, orange and red traits (e.g., eye redness), which can
502 advertise the quality and condition of their bearers (Lifshitz and St Clair, 2016). They are a limited
503 resource that has to be acquired through the diet, and expression is sensitive to the cascade of
504 physiological effects produced by stressful events, such as exposure to metal pollution (Vallverdú-
505 Coll et al., 2016; García-Heras et al., 2017). For grebes at the Odiel Marshes, a key potential source
506 of carotenoids during the moulting period is likely *Artemia*, since it is widely recognized for its
507 carotenoid contribution in aquatic food webs (De Carvalho and Caramujo, 2017). Our results
508 showed a changing relationship between lutein-like carotenoids and Hg exposure and between
509 *trans*-zeaxanthin and As exposure, the latter potentially being linked to moult status, which were
510 accompanied by a negative relationship between eye redness and As accumulation throughout the
511 moulting period. The positive relationships between plasma carotenoids and metal exposure
512 support the idea of *Artemia* being the main source of both carotenoids and metals. Overall, our
513 findings likely reflect temporal differences in the availability and use of carotenoid-rich food items
514 (Eeva et al., 2012) and a possible allocation trade-off between using available carotenoids for
515 maintaining cellular redox balance (potentially disturbed by metal exposure) and for carotenoid-
516 based signalling (Giraudeau et al., 2015; Lifshitz and St Clair, 2016; Sumasgutner et al., 2018).
517 Ortiz-Santaliestra et al. (2015) observed that circulating levels of carotenoids were negatively
518 related to As exposure in nestlings of Bonelli's eagle (*Aquila fasciata*), which may occur when
519 organisms need to utilise carotenoids in redox processes to balance against increased oxidative
520 stress caused by pollutants. According to this, the carotenoids obtained by grebes from *Artemia*
521 should have been used, in part, to counteract the reactive oxygen substances generated from the
522 metabolism of the co-occuring metals, obtained also from *Artemia*, or to ameliorate the effect of

metals on other functions. This allocation of carotenoids might contribute to explain a potential altered coloration. White and Cristol (2014) found that belted kingfishers (*Megaceryle alcyon*) breeding on a Hg-contaminated river exhibited altered plumage coloration, suggesting that this might have consequences for behavioural signalling and fitness. On contrary, carotenoid levels and other biochemical parameters were not affected in great tit nestlings (*Parus major*) experimentally exposed to 0.2 or 1 µg/g-day of sodium arsenite, or in individuals from a metal-polluted area in comparison to controls (Sánchez-Virosta et al., 2018).

Body condition represents energy resources in the body and correlates with fitness through effects on reproduction and survival, and therefore is a meaningful morphometric biomarker of overall health in wildlife that can be affected by exposure to environmental pollution (Takekawa et al., 2002; Wayland et al. 2002). Our results showed that, in moulted female grebes, mass gain was positively related to an increased exposure to As during November and December, likely due to higher consumption rates of brine shrimps during these months (Sánchez et al., 2006; Varo et al., 2011; Fox et al., 2013). In other work, Conover and Vest (2009) did not detect an effect of relatively high blood Hg (mean±SE: 7.85±2.08 µg/g d.w.; equivalent to ≈3.57 µg/g d.w. in blood pellet) on body mass, concluding that even high levels of environmental pollution are unlikely to prevent grebes from increasing or maintaining mass in a normal way. In contrast, Ackerman et al. (2012) estimated a potential decrease in body mass of 5-7% over a range of blood Hg concentrations in Californian clapper rails (*Rallus longirostris*) that would be comparable with the range of exposure found in some grebes here (mean, range: 0.56, 0.15-1.43 µg/g w.w.; equivalent to ≈3.11, 0.83-10.06 µg/g d.w. in blood pellet).

5. Conclusions

Results here showed that black-necked grebes moulting at the Odiel Marshes had relatively high blood levels of As and Zn; while 12% of the monitored individuals had also elevated blood levels of Hg, in all cases in comparison to toxicity thresholds. These findings are not surprising given that they temporarily inhabit/moult in a highly polluted aquatic ecosystem, and suggest a risk of negative health consequences during this period. The temporal changes observed in blood pellets (as a marker of exposure/uptake) suggested that variable metal exposure may be mostly attributable to food chain transfer through the most important dietary component for grebes during their stay at the Odiel Marshes, *A. parthenogenetica*. Variations in plasma carotenoids, eye redness, and body condition may have been influenced by metals exposure. Monitoring metal levels in moult migrant grebes is useful in understanding possible health risks, and blood metal concentrations are reflective of local contamination and food chain transfer. However, many other factors, including food resource availability, moult status and timing, gender-related physiological differences, interannual variations in environmental contamination and seasonal adjustments in the biomarkers selected here should be taken into account to avoid misinterpretations. Especially, when studying birds during critical events within in their annual life cycle, such as moulting, breeding and egg laying.

Acknowledgements

We thank the many volunteers that participated in the capture of the grebes, which was organized by Equipo de Seguimiento de Procesos Naturales and led by Luis García (Estación Biológica de Doñana – CSIC) and SEO/BirdLife. The help of Manuel Vázquez was essential in taking blood samples. Dr George Britton kindly provided material for carotenoid analyses. We also appreciate the assistance of Pablo R. Camarero with metal analysis. Enrique Martínez, Director of Paraje Natural Marismas del Odiel (Consejería de Medio Ambiente, Junta de Andalucía), authorised and facilitated the field work. J. Rodríguez-Estival was supported by a Research and

572 Innovation Strategy for Smart Specialisation (RIS3) postdoctoral contract (2017/11747) from the
573 Junta de Comunidades de Castilla-La Mancha. The study was financially supported by Consejería
574 de Innovación, Ciencia y Empresa (Junta de Andalucía, Project P07-CVI-02700).

575

576 **Conflict of interest**

577 Authors have no competing interests to declare.

578

579 **References**

580

581 Ackerman, J. T., Eagles-Smith, C. A., Herzog, M. P., Hartman, C. A., Peterson, S. H., Evers, D. C.,
582 Jackson, A. K., Elliott, J. E., Vander Pol, S.S., Bryan, C.E. 2016. Avian mercury exposure and
583 toxicological risk across western North America: A synthesis. Sci. Total Environ. 568, 749-769.
584 Ackerman, J. T., Overton, C. T., Casazza, M. L., Takekawa, J. Y., Eagles-Smith, C. A., Keister, R. A.,
585 Herzog, M. P. 2012. Does mercury contamination reduce body condition of endangered
586 California clapper rails? Environ. Pollut. 162, 439-448.
587 Adams, W. J., DeForest, D. K., Tear, L. M., Payne, K., Brix, K. V. 2015. Long-term monitoring of
588 arsenic, copper, selenium, and other elements in Great Salt Lake (Utah, USA) surface water,
589 brine shrimp, and brine flies. Environ. Monit. Assess. 187:118, 13 p.
590 Aloui, N., Amorri, M., Azaza, M., Chouba, L. 2012. Study of trace metals (Hg, Cd, Pb, Cu, and Zn) in
591 cysts and biomass of *Artemia salina* (Linnaeus, 1758) (Branchiopoda, Anostraca) from the salt
592 work of Sfax (Tunisia). Crustaceana 85, 1-10.
593 Alvárez, C. R., Moreno, M. J., Alonso, L. L., Gómara, B., Bernardo, F. J. G., Martín-Doimeadios, R. C.
594 R., González, M. J. 2013. Mercury, methylmercury, and selenium in blood of bird species from
595 Doñana National Park (Southwestern Spain) after a mining accident. Environ. Sci. Pollut. Res.
596 20, 5361-5372.

597 Amat, J. A., Varo, N., Sánchez, M. I., Green, A. J., Ramo, C. 2014. Female-biased sex ratio in
598 moulting black-necked grebes *Podiceps nigricollis* in Southern Spain. Ardea 102, 207-212.
599 Baos, R., Jovani, R., Forero, M. G., Tella, J. L., Gómez, G., Jiménez, B., González, M. J., Hiraldo, F.
600 2006. Relationships between T-cell-mediated immune response and Pb, Zn, Cu, Cd, and as
601 concentrations in blood of nestling white storks (*Ciconia ciconia*) and black kites (*Milvus*
602 *migrans*) from Doñana (southwestern Spain) after the Aznalcóllar toxic spill. Environ. Toxicol.
603 Chem. 25, 1153-1159.
604 Barta, Z., McNamara, J. M., Houston, A. I., Weber, T. P, Hedenström, A., Feró, O. 2008. Optimal
605 moult strategies in migratory birds. Philos. T. R. Soc. B. 363, 211-229.
606 Battin, J. 2004. When good animals love bad habitats: Ecological traps and the conservation of
607 animal populations. Conserv. Biol. 18, 1482-1491.
608 Beltrán, R., de la Rosa, J. D., Santos, J. C., Beltrán, M., Gómez-Ariza, J. L. 2010. Heavy metal
609 mobility assessment in sediments from the Odiel River (Iberian Pyritic Belt) using sequential
610 extraction. Environ. Earth Sci. 61, 1493-1503.
611 Benito, V., Devesa, V., Muñoz, O., Suñer, M. A., Montoro, R., Baos, R., Hiraldo, F., Ferrer, M.,
612 Fernández, M., González, M. J. 1999. Trace elements in blood collected from birds feeding in
613 the area around Doñana National Park affected by the toxic spill from the Aznalcóllar mine. Sci.
614 Total Environ. 242, 309-323.
615 Berglund, Å. M. M. 2018. Evaluating blood and excrement as bioindicators for metal accumulation
616 in birds. Environ. Pollut. 233, 1198-1206.
617 Blanco, G., Hornero-Mendez, D., Lambertucci, S.A., Bautista, L. M., Wiemeyer, G., Sanchez-Zapata,
618 J. A., Garrido-Fernandez, J., Hiraldo, F., Donázar, J. A. 2013. Need and seek for dietary
619 micronutrients: endogenous regulation, external signalling and food sources of carotenoids in
620 New World vultures. PLoS ONE, 8: e65562.

621 Borghesi, F., Andreotti, A., Baccetti, N., Bianchi, N., Birke, M., Migani, F., Dinelli, E. 2011. Flamingo
622 feathers to monitor metal contamination of coastal wetlands: methods and initial results
623 concerning the presence of mercury at six Mediterranean sites. Chem. Ecol. 27, 137-151.
624 Borghesi, F., Migani, F., Andreotti, A., Baccetti, N., Bianchi, N., Birke, M., Dinelli, E. 2016. Metals
625 and trace elements in feathers: A geochemical approach to avoid misinterpretation of analytical
626 responses. Sci. Total Environ. 544, 476-494.
627 Britton, G. 1991. Carotenoids. In: Dey, P. M., Harborne, J. B. (Eds). Methods in Plant Biochemistry.
628 Academic Press, London. Pp. 473-518.
629 Britton, G. 1995. UV/visible spectroscopy. In: Britton, G., Liaaen-Jensen, S., Pfander, H. (Eds.).
630 Carotenoids. Volume 1B: Spectroscopy. Birkhäuser Verlag, Basel (Switzerland). Pp. 13-62.
631 Britton, G. Liaanen-Jensen, S., Pfander, H. 2004. Carotenoid Handbook. Birkhäuser Verlag, Basel
632 (Switzerland).
633 Butler, M. W., McGraw, K. J. 2010. Relationships between dietary carotenoids, body tissue
634 carotenoids, parasite burden, and health state in wild mallard (*Anas platyrhynchos*) ducklings.
635 Arch. Biochem. Biophys. 504, 154-160.
636 Cardoso P. G., Sousa E., Matos P., Henriques B., Pereira E., Duarte A. C., Pardal M. A. 2013. Impact
637 of mercury contamination on the population dynamics of *Peringia ulvae* (Gastropoda):
638 Implications on metal transfer through the trophic web. Estuar. Coast. Shelf S., 129, 189-197.
639 Conover, M. R., Vest, J. L. 2009. Concentrations of selenium and mercury in eared grebes (*Podiceps*
640 *nigricollis*) from Utah's Great Salt Lake, USA. Environ. Toxicol. Chem. 28, 1319-1323.
641 Darnall, N. L., Miles, K. 2009. Dynamics of mercury in eared grebes on Great Salt Lake. Nat. Res.
642 Environ. Issues 15, Article 6.
643 Dauwe, T., Bervoets, L., Pinxten, R., Blust, R., Eens, M. 2003. Variation of heavy metals within and
644 among feathers of birds of prey: Effects of molt and external contamination. Environ. Pollut.
645 124, 429-436.

646 De Carvalho, C. C. C. R., Caramujo, M. J. 2017. Carotenoids in aquatic ecosystems and aquaculture:
647 A colorful business with implications for human health. Front. Mar. Sci. 4, 93.
648 Eeva, T., Rainio, M., Kanerva, M., Salminen, J. -P. 2012. Plasma carotenoid levels are not directly
649 related to heavy metal exposure or reproductive success in three insectivorous passerines.
650 Environ. Toxicol. Chem. 31, 1363-1369.
651 Eugster, C. H. 1995. Chemical derivatization: Microscale tests for the presence of common
652 functional groups in carotenoids. In: Britton, G., Liaaen-Jensen, S., Pfander, H. (Eds.).
653 Carotenoids. Volume 1A: Isolation and analysis. Birkhäuser Verlag, Basel (Switzerland). Pp. 71-
654 80.
655 Finger, A., Lavers, J. L., Orbell, J. D., Dann, P., Nugegoda, D., Scarpaci, C. 2016. Seasonal variation
656 and annual trends of metals and metalloids in the blood of the Little Penguin (*Eudyptula minor*).
657 Mar. Pollut. Bull. 110, 261-273.
658 Fox, A. D., Kahlert, J. 2005. Changes in body mass and organ size during wing moult in non-
659 breeding Greylag Geese *Anser anser*. J. Avian Biol. 36, 538-548.
660 Fox, A. D., Ramo, C., Varo, N., Sánchez, M. I., Amat, J. A., Green, A. J. 2013. Late-moulting Black-
661 necked Grebes *Podiceps nigricollis* show greater body mass in the face of failing food supply.
662 Ibis 155, 814-822.
663 Franson, J. C., Hollmen, T., Poppenga, R. H., Hario, M., Kilpi, M., Smith, M. R. 2000. Selected trace
664 elements and organochlorines: Some findings in blood and eggs of nesting common eiders
665 (*Somateria mollissima*) from Finland. Environ Toxicol Chem 19, 1340-1347.
666 Franson, J. C., Pain, D. J. 2011. Lead in birds. In: Beyer, W.N., Meador, J.P. (Eds.), Environmental
667 Contaminants in Biota: Interpreting Tissue Concentrations, 2nd ed. CRC Press, Boca Raton,
668 Florida, USA. Pp. 563-594.

669 Funes, V., Alhama, J., Navas, J. I., López-Barea, J., Peinado, J. 2006. Ecotoxicological effects of
670 metal pollution in two mollusc species from the Spanish South Atlantic littoral. Environ. Pollut.
671 139, 214-223.
672 Fuchsman, P. C. Brown, L. E., Henning, M. H., Bock, M. J., Magar, V. S. 2017. Toxicity reference
673 values for methylmercury effects on avian reproduction: Critical review and analysis. Environ.
674 Toxicol. Chem. 36, 294-319.
675 García-Heras, M. S., Arroyo, B., Simmons, R. E., Camarero, P. R., Mateo, R., García, J. T., &
676 Mougeot, F. 2017. Pollutants and diet influence carotenoid levels and integument coloration in
677 nestlings of an endangered raptor. Sci. Total Environ. 603, 299-307.
678 Gehrold, A., Köhler, P. 2013. Wing-moulting waterbirds maintain body condition under good
679 environmental conditions: A case study of Gadwalls (*Anas strepera*). J. Ornithol. 154, 783-793.
680 Giraudeau, M., Mateos-Gonzalez, F., Cotín, J., Pagani-Nuñez, E., Torné-Noguera, A., Senar, J.C.
681 2015. Metal exposure influences the melanin and carotenoid-based colorations in great tits. Sci.
682 Total Environ. 532, 512-516.
683 Gómez, J., Liñán-Cembrano, G. 2017. SpotEgg: an image-processing tool for automatised analysis
684 of colouration and spottiness. J. Avian Biol. 48, 502-512.
685 Goyer, R. A., Clarkson, T. 2001. Toxic effects of metals. In: Klaassen, C. D. (Ed.). Casarett and Doull's
686 Toxicology the Basic Science of Poisons, 6th Ed. McGraw-Hill, Kansas. Pp. 811-867.
687 Grand, J. B., Franson, J. C., Flint, P. L., Petersen, M. R. 2002. Concentrations of trace elements in
688 eggs and blood of spectacled and common eiders on the Yukon-Kuskokwim Delta, Alaska.
689 Environ Toxicol Chem 21, 1673-1678.
690 Gupta, R. C., Milatovic, D., Lall, R., Srivastava, A. 2018. Chapter 31 – Mercury. In: Gupta, R. C. (Ed.).
691 Veterinary Toxicology: Basic and clinical principles, Third Edition. Elsevier & AP Academic Press.
692 Pp. 455-462.

693 Hall B. D., Rosenberg D. M., Wiens A. P. 1998. Methyl mercury in aquatic insects from an
694 experimental reservoir. Can. J. Fish. Aquat. Sci., 55, 2036-2047.
695 Hartman, A. A., Ackerman, J. T., Herzog, M. P., Eagles-Smith, C. A. 2017. Season, molt, and body
696 size influence mercury concentrations in grebes. Environ. Pollut. 229, 29-39.
697 Holman, K. L., Schummer, M. L., Petrie, S. A., Chen. Y. -W., Belzile, N. 2015. Inorganic
698 contaminants, nutrient reserves and molt intensity in autumn migrant red-necked grebes
699 (*Podiceps grisegena*). Arch. Environ. Con. Tox. 69, 399-410.
700 Honda, K., Min, B. L., Tatsukawa, R. 1986. Distribution of heavy metals and their age-related
701 changes in the Eastern Great White Egret, *Egretta alba modesta*, in Korea. Arch. Environ. Con.
702 Tox. 15, 185-197.
703 Huang, J. -H. 2016. Arsenic trophodynamics along the food chains/webs of different ecosystems: a
704 review. Chem. Ecol. 32, 803-828.
705 Iavicoli, I., Fontana, L., Bergamaschi, A. 2009. The effects of metals as endocrine disruptors. J.
706 Toxicol. Env. Heal B 12, 206-223.
707 Johns, C. 2012. Trends of total cadmium, copper, and zinc in the zebra mussel (*Dreissena*
708 *polymorpha*) along the upper reach of the St. Lawrence River: 1994-2005. Environ. Monit.
709 Assess. 184, 5371-5385.
710 Kingsford, R. T., Basset, A., Jackson, L. 2016. Wetlands: conservation's poor cousins. Aquat.
711 Conserv. 26, 892-916.
712 Kloskowski, J., Kaczanowska, E., Krogulec, J., Grela, P. 2017. Hematological indicators of habitat
713 quality: Erythrocyte parameters reflect greater parental effort of Red-necked Grebes under
714 ecological trap conditions. Condor 119, 239-250.
715 Lavoie, R. A., Kyser, T. K., Friesen, V. L., Campbell, L. M. 2015. Tracking overwintering areas of fish-
716 eating birds to identify mercury exposure. Environ. Sci. Technol. 49, 863-872.

717 Leonova, G. A., Bobrov, V. A., Bogush, A. A., Bychinskii, V. A., Anoshin, G. N. 2007. Geochemical
718 characteristics of the modern state of salt lakes in Altai Krai. Geochem. Int. 45, 1025-1039.
719 Lifshitz, N., St Clair, C. C. 2016. Coloured ornamental traits could be effective and non-invasive
720 indicators of pollution exposure for wildlife. Conserv. Physiol. 4,cow028.
721 Martínez, A., Crespo, D., Fernández, J. Á., Aboal, J. R., Carballeira, A. 2012. Selection of flight
722 feathers from *Buteo buteo* and *Accipiter gentilis* for use in biomonitoring heavy metal
723 contamination. Sci. Total Environ. 425, 254-261.
724 Martinez-Haro, M., Green, A. J., Mateo, R. 2011. Effects of lead exposure on oxidative stress
725 biomarkers and plasma biochemistry in waterbirds in the field. Environ. Res. 111, 530-538.
726 Mínguez-Mosquera, M. I., Hornero-Méndez, D. 1993. Separation and quantification of the
727 carotenoid pigments in red peppers (*Capsicum annuum* L.), paprika, and oleoresin by reversed-
728 phase HPLC. J. Agric. Food Chem. 41, 1616-1620.
729 Naftz, D., Angeroth, C., Kenney, T., Waddell, B., Darnall, N., Silva, S., Perschon, C., Whitehead, J.
730 2008. Anthropogenic influences on the input and biogeochemical cycling of nutrients and
731 mercury in Great Salt Lake, Utah, USA. Appl. Geochem. 23, 1731-1744.
732 Nieto, J. M., Sarmiento, A. M., Canovas, C. R., Olias, M., Ayora, C. 2013. Acid mine drainage in the
733 Iberian Pyrite Belt: 1. Hydrochemical characteristics and pollutant load of the Tinto and Odiel
734 rivers. Environ. Sci. Pollut. R. 20, 7509-7519.
735 Olney, C. E., Schauer, P. S., McLean, S., Lu, Y., Simpson, K. L. 1980. International study of *Artemia*
736 VIII. Comparison of the chlorinated hydrocarbons and heavy metals in five different strains of
737 newly hatched *Artemia* and a laboratory-reared marine fish. U.S. Environmental Protection
738 Agency, Washington, D.C., EPA/600/D-82/219.
739 Ortiz-Santaliestra, M. E., Resano-Mayor, J., Hernández-Matías, A., Rodríguez-Estival, J., Camarero,
740 P. R., Moleón, M., Real, J., Mateo, R. 2015. Pollutant accumulation patterns in nestlings of an
741 avian top predator: Biochemical and metabolic effects. Sci. Total Environ. 538, 692-702.

742 Padula, V., Burger, J., Newman, S. H., Elbin, S., Jeitner, C. 2010. Metals in feathers of black-
743 crowned night-heron (Nycticorax nycticorax) chicks from the New York Harbor Estuary. Arch.
744 Environ. Con. Tox. 59, 157-165.
745 Peig. J., Green, A. J. 2009. New perspectives for estimating body condition from mass/length data:
746 the scaled mass index as an alternative method. Oikos 118, 1883-1891.
747 Pérez-López, R., Nieto, J. M., López-Cascajosa, M. J., Díaz-Blanco, J., Sarmiento, A. M., Oliveira, V.,
748 Sánchez-Rodas, D. 2011. Evaluation of heavy metals and arsenic speciation discharged by the
749 industrial activity on the Tinto-Odiel estuary, SW Spain. Mar. Pollut. Bull. 62, 405-411.
750 Petrucci, F., Caimi, S., Mura, G., Caroli, S. 1995. *Artemia* as a bioindicator of environmental
751 contamination by trace elements. Microchem. J. 51, 181-186.
752 Portugal, S. J., Green, J. A., Butler, P. J. 2007. Annual changes in body mass and resting metabolism
753 in captive Barnacle Geese (*Branta leucopsis*): the importance of wing moult. J. Exp. Biol. 210,
754 1391-1397.
755 Preacher, K. J., MacCallum, R. C., 2002. Exploratory factor analysis in behavior genetics research:
756 factor recovery with small sample sizes. Behav. Genet. 32, 153-161.
757 Rainbow, P. S. 2002. Trace metal concentrations in aquatic invertebrates: Why and so what?
758 Environ. Pollut. 120, 497-507.
759 Rimmer, C. C., Mcfarland, K. P., Evers, D. C., Miller, E. K., Aubry, Y., Busby, D., Taylor, R. J. 2005.
760 Mercury concentrations in Bicknell's thrush and other insectivorous passerines in Montane
761 forests of northeastern North America. Ecotoxicology 14, 223-240.
762 Rodríguez-Estival, J., Martinez-Haro, M., Monsalve-González, L., Mateo, R. 2011. Interactions
763 between endogenous and dietary antioxidants against Pb-induced oxidative stress in wild
764 ungulates from a Pb polluted mining area. Sci. Total Environ. 409, 2725-2733.

765 Rubio, I., Martinez-Madrid, M., Méndez-Fernández, L., Galarza, A., Rodriguez, P. 2016. Heavy
766 metal concentration in feathers of Little Egret (*Egretta garzetta*) nestlings in three coastal
767 breeding colonies in Spain. Ecotoxicology 25, 30-40.
768 Sáez-Gómez, P., Camacho, C., Álvarez, A., Varo, N., Ramo, C., Green, A. J. 2017. Sexual dimorphism
769 and sex determination in Black-necked Grebes *Podiceps nigricollis*: on the need to account for
770 geographical variation in morphology and sex ratio. Bird Study 64, 502-512.
771 Sánchez, M. I., Green, A. J., Castellanos, E. M. 2006. Temporal and spatial variation of an aquatic
772 invertebrate community subjected to avian predation at the Odiel salt pans (SW Spain). Arch.
773 Hydrobiol. 166, 199-223.
774 Sánchez-Rodas, D., Gomez-Ariza, Giraldez, J. L., I., Velasco, A., Morales, E. 2005. Arsenic speciation
775 in river and estuarine Waters from southwest Spain. Sci. Total Environ. 345, 207-217.
776 Sánchez-Virosta, P., Espín, S., García-Fernández, A. J., Eeva, T. 2015. A review on exposure and
777 effects of arsenic in passerine birds. Sci. Total Environ. 512-513, 506-525.
778 Sánchez-Virosta, P., Espín, S., Ruiz, S., Salminen, J.-P., García-Fernández, A. J., Eeva, T. 2018.
779 Experimental manipulation of dietary arsenic levels in great tit nestlings: Accumulation pattern
780 and effects on growth, survival and plasma biochemistry. Sci. Total Environ. 233, 764-773.
781 Santos Bermejo, J. C., Beltran, R., Gomez Ariza, J. L. 2003. Spatial variations of heavy metals
782 contamination in sediments from Odiel river (Southwest Spain). Environ. Int., 29, 69-77.
783 Sarmiento, A. M., Nieto, J. M., Casiot, C., Elbaz-Poulichet, F., Egal, M. 2009. Inorganic arsenic
784 speciation at river basin scales: the Tinto and Odiel Rivers in the Iberian Pyrite Belt, SW Spain
785 Environ. Pollut. 157, 1202-1209.
786 Schiedt, K., Liaaen-Jensen, S. 1995. Isolation and analysis. In: Britton, G., Liaaen-Jensen, S.,
787 Pfander, H. (Eds.). Carotenoids. Vol. 1A: Isolation and analysis. Birkhäuser Verlag, Basel
788 (Switzerland). Pp. 81-108.

789 Shlosberg, A., Rumbeiha, W. K., Lublin, A., Kannan, K. 2011. A database of avian spot examinations
790 for exposure of wild birds to environmental toxicants: the DABSE biomonitoring project. J.
791 Environ. Monit. 13, 1547-1558.
792 Stankovic, S., Kalaba, P., Stankovic, A. R. 2014. Biota as toxic metal indicators. Environ. Chem. Lett.
793 12, 63-84.
794 Storer, R. W., Jehl, J.R. 1985. Molt patterns and molt migration in the Black-necked Grebe
795 Podiceps nigricollis. Ornis Scand. 16, 253-260.
796 Stewart, F. M., Phillips, R. A., Bartle, J. A., Craig, J., Shooter, D. 1999. Influence of phylogeny, diet,
797 moult schedule and sex on heavy metal concentrations in New Zealand Procellariiformes. Mar.
798 Ecol. Prog. Ser. 178, 295-305.
799 Stewart, A. R., Saiki, M. K., Kuwabara, J. S., Alpers, C. N., Marvin-DiPasquale, M., Krabbenhoft, D. P.
800 2008. Influence of plankton mercury dynamics and trophic pathways on mercury
801 concentrations of top predator fish of a mining-impacted reservoir. Can. J. Fish Aquat. Sci. 65,
802 2351-2366.
803 Sumasgutner, P., Adrion, M., Gamauf, A. 2018. Carotenoid coloration and health status of urban
804 Eurasian kestrels (*Falco tinnunculus*). PloS One 13, e0191956.
805 Takekawa, J. Y., Wainwright-De La Cruz, S. E., Hothem, R. L., Yee, J. 2002. Relating body condition
806 to inorganic contaminant concentrations of diving ducks wintering in coastal California. Arch.
807 Environ. Con. Tox. 42, 60-70.
808 Usero, J., Izquierdo, C., Morillo, J., Gracia, I. 2004. Heavy metals in fish (*Solea vulgaris*, *Anguilla*
809 *anguilla* and *Liza aurata*) from salt marshes on the southern Atlantic coast of Spain. Environ.
810 Int. 29, 949-956.
811 Vallés, S. M., Cambrollé, J., Castillo, J. M., Curado, G., Mancilla-Leytón, J. M. Figueroa-Clemente, E.
812 2017. Handling high soil trace elements pollution: Case study of the Odiel and Tinto Rivers
813 Estuary and the accompanying salt marshes (Southwest Iberian Peninsula). In: Finkl, C. W.,

814 Makowski, C. (Eds.). Coastal Wetlands: Alteration and remediation. Volume 21 – Coastal
815 Research Library. Springer International Publishing AG. Cham, Switzerland. Pp. 215-241.
816 Vallverdú-Coll, N., Mougeot, F., Ortiz-Santaliestra, M. E., Rodríguez-Estival, J. López-Antia, A.,
817 Mateo, R. 2016. Lead exposure reduces carotenoid-based coloration and constitutive immunity
818 in wild mallards. Environ. Toxicol. Chem. 35, 1516-1525.
819 Varo, N., Green, A. J., Sánchez, M. I., Ramo, C., Gómez, J., Amat, J. A. 2011. Behavioural and
820 population responses to changing availability of *Artemia* prey by moulting black-necked grebes,
821 *Podiceps nigricollis*. Hydrobiologia 664, 163-171.
822 Vittinghoff, E., Shiboski, S. C., Glidden, D. V., McCulloch, C. E. 2005. Regression methods in
823 biostatistics: Linear, logistic, survival and repeated measures models. In: Dietz, K., Gail, M.,
824 Krickeberg, K., Samet, J., Tsiatis, A. (Series Editors). Statistics for Biology and Health Series.
825 Springer, USA. Pp. 340.
826 White, A. E., Cristol, D. A. 2014. Plumage coloration in belted kingfishers (*Megaceryle alcyon*) at a
827 mercury-contaminated river. Waterbirds 37, 144-152.
828 Wayland, M., Gilchrist, H. G., Marchant, T., Keating, J., Smits, J.E. 2002. Immune function, stress
829 response, and body condition in Arctic-breeding common eiders in relation to cadmium,
830 mercury, and selenium concentrations. Environ. Res. 90, 47-60.
831 Wayland, M., Scheuhammer, A. M. 2011. Cadmium in birds. In: Beyer, W.N., Meador, J.P. (Eds.),
832 Environmental Contaminants in Biota: Interpreting Tissue Concentrations, 2nd ed. CRC Press,
833 Boca Raton, Florida, USA. Pp. 645-666.
834 Wyss, F., Wolf, P., Wenker, C., Hoby, S., Schumacher, V., Béchet, A., Robert, N., Liesegang, A. 2014.
835 Comparison of plasma vitamin A and E, copper and zinc levels in free-ranging and captive
836 greater flamingos (*Phoenicopterus roseus*) and their relation to pododermatitis. J. Anim.
837 Physiol. An. N. 98, 1102-1109.

838 Zhang, W. W., Ma, J. Z. 2011. Waterbirds as bioindicators of wetland heavy metal pollution.
839 Procedia Environ. Sci. 10 (PART C), 2769-2774.

840

841 **Figure captions**

842

843 **Fig. 1.** Temporal variation in blood pellet As level (n values; mean±SE) in black-necked grebes
844 (*Podiceps nicricollis*) from the Odiel Marshes during the moulting period, as a function of moult
845 status. Letters indicate significant differences ($p \leq 0.05$) between months for each category of moult
846 status. Smallest subgroups of individuals (1 unmoulted and 2 moulted birds in August, 2
847 unmoulted and 1 moulting birds in November, and 1 unmoulted bird in December) were excluded
848 from the statistical analysis in order to avoid misinterpretation in the effect of moult status on As
849 blood levels.

850

851 **Fig. 2.** Temporal variation in blood pellet Zn level (n values; mean±SE) in black-necked grebes
852 (*Podiceps nicricollis*) from the Odiel Marshes during the moulting period, as a function of gender.
853 Letters indicate significant differences ($p \leq 0.05$) between months for each gender, asterisks (*)
854 indicate significant differences between males and females within the same month, and numbers
855 indicate the specific n values.

856

857 **Fig. 3**. Blood As, Hg, Pb, Cu and Zn levels (mean±SE) in grebes captured in October 2008 (initial
858 capture) and recaptured in November 2008 (n=18). Superscript letters indicate significant
859 differences ($p \leq 0.05$) between months for each metal.

Highlights

- Moult migrant grebe was used as sentinel of metal pollution in the Odiel Marshes.
- Grebes had high blood levels of As, Hg and Zn in comparison to toxicity thresholds.
- Metals in *Artemia* and temporal changes in exposure suggest a food chain transfer.
- Plasma carotenoids, eye redness and body condition were influenced by metal exposure.

Tables

Table 1. Concentrations (geometric mean, GM) of metals in blood pellets (n=176), expressed in µg/g d.w., of black-necked grebes (*Podiceps nigricollis*) from the Odiel Marshes during the months comprising the moulting period. Levels of Cd were above the analytical LOD in only six grebes, thus this metal was not included in the table.*

		Moulting period							
Metals		N	**August**	N	**October**	N	**November**	N	**December**
As	GM (95%CI)	29	0.619 (0.519-0.738) [ab]	63	0.522 (0.464-0.587) [a]	58	0.719 (0.665-0.778) [b]	30	1.162 (1.033-1.307) [c]
	Range		0.174-1.954		0.154-1.448		0.307-1.310		0.691-2.293
Hg	GM (95%CI)	29	0.719 (0.557-0.928) [b]	62	0.383 (0.291-0.504) [a]	56	0.719 (0.615-0.841) [b]	30	0.440 (0.377-0.515) [a]
	Range		0.027-1.916		0.027-28,50 [+]		0.106-85.63 [+]		0.162-0.923
Pb	GM (95%CI)	29	<LOD [a]	62	0.024 (0.021-0.028) [b]	58	0.033 (0.028-0.038) [c]	30	0.024 (0.020-0.029) [b]
	Range		-		<LOD-1.094 [+]		<LOD-0.115		<LOD-0.077
Cu	GM (95%CI)	29	1.649 (1.229-2.212) [a]	63	1.859 (1.621-2.132) [a]	58	1.522 (1.301-1.780) [a]	30	2.509 (2.320-2.714) [b]
	Range		0.733-8.935		0.733-4.221		0.733-4.015		1.477-3.669
Zn	GM (95%CI)	29	19.11 (18.74-19.48) [c]	63	16.95 (16.62-17.28) [a]	58	18.17 (17.82-18.53) [b]	30	17.64 (17.30-17.99) [b]
	Range		16.77-20.29		14.44-19.89		14.88-20.49		16.12-19.30

* Superscript letters indicate significant differences (Tukey tests: $p \leq 0.05$) between months for each element.

<LOD: below the analytical limit of detection.

[+] Maximum values in the ranges include outliers, but they were excluded from the calculation of the GM (hence N value decrease).

Table 2. Concentrations of metals in *Artemia parthenogenetica* (μg/g, dry weight) collected from 4 different points in a channel at the Odiel saltpans and comparison with reference values obtained from other studies. For metals analyzed in the grebes' blood, those showing high concentrations in *Artemia* with respect to the reference values are highlighted.

	Present study		Reference values *		
Metal	**Mean±SE**	**Range**	**Mean±SE**	**Range**	**References**
As	43.14 ± 4.28	33.13 - 50.37	3.56 ± 0.79	2.5- 5.1	[2]; [3]
Hg	*Not analyzed*	-	0.13 ± 0.07	0.07 - 0.21	[2]; [3]; [4]
Pb	5.89 ± 1.04	3.67 - 7.74	3.74 ± 0.91	0.25 - 6.20	[1]; [2]; [3]; [4]
Cd	0.11 ± 0.02	0.08 - 0.14	0.17 ± 0.04	0.10 - 0.37	[1]; [2]; [3]; [4]
Cu	48.70 ± 6.72	33.84 - 60.22	8.26 ± 1.11	4.9 - 12.9	[1]; [2]; [3]; [4]
Zn	250.9 ± 16.9	206.3 - 278.8	85.4 ± 12.25	42.90 - 144.0	[1]; [2]; [3]; [4]
Co	1.88 ± 0.28	1.27 - 2.72	2.60 ± 0.30	2.30 - 2.90	[3]
Cr	3.14 ± 0.51	2.05 - 4.11	12.55 ± 0.07	12.50 - 12.60	[3]
Mn	58.25 ± 9.12	39.44 - 75.37	-	-	-
Mo	1.63 ± 1.06	0.40 - 4.79	0.55 ± 0.25	0.30 - 0.80	[3]
Ni	2.44 ± 0.35	1.65 - 3.07	14.85 ± 1.95	12.90 - 16.80	[3]
Se	1.40 ± 0.10	1.26 - 1.68	1.7	-	[2]

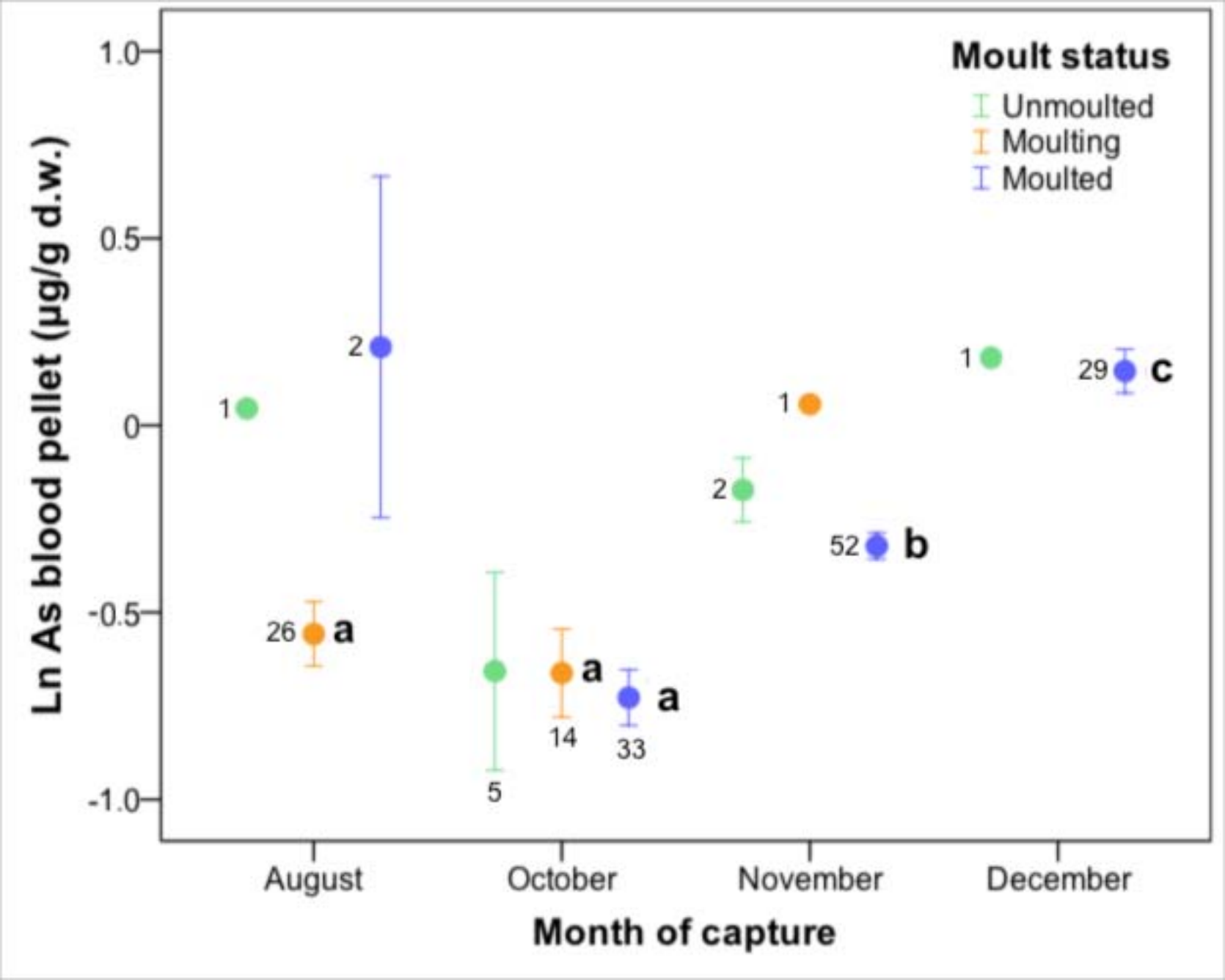
* Concentrations of elements in *Artemia* spp. (mean±SE) recorded at different localities (considered as unpolluted aquatic ecosystems: lakes, saltworks and coastal lagoons) around the world from strains commercialized and used in aquaculture for fish farming, therefore assumed as reference values: [1] Olney et al., 1980; [2] Petrucci et al., 1995; [3] Leonova et al., 2007; [4] Aloui et al., 2012.

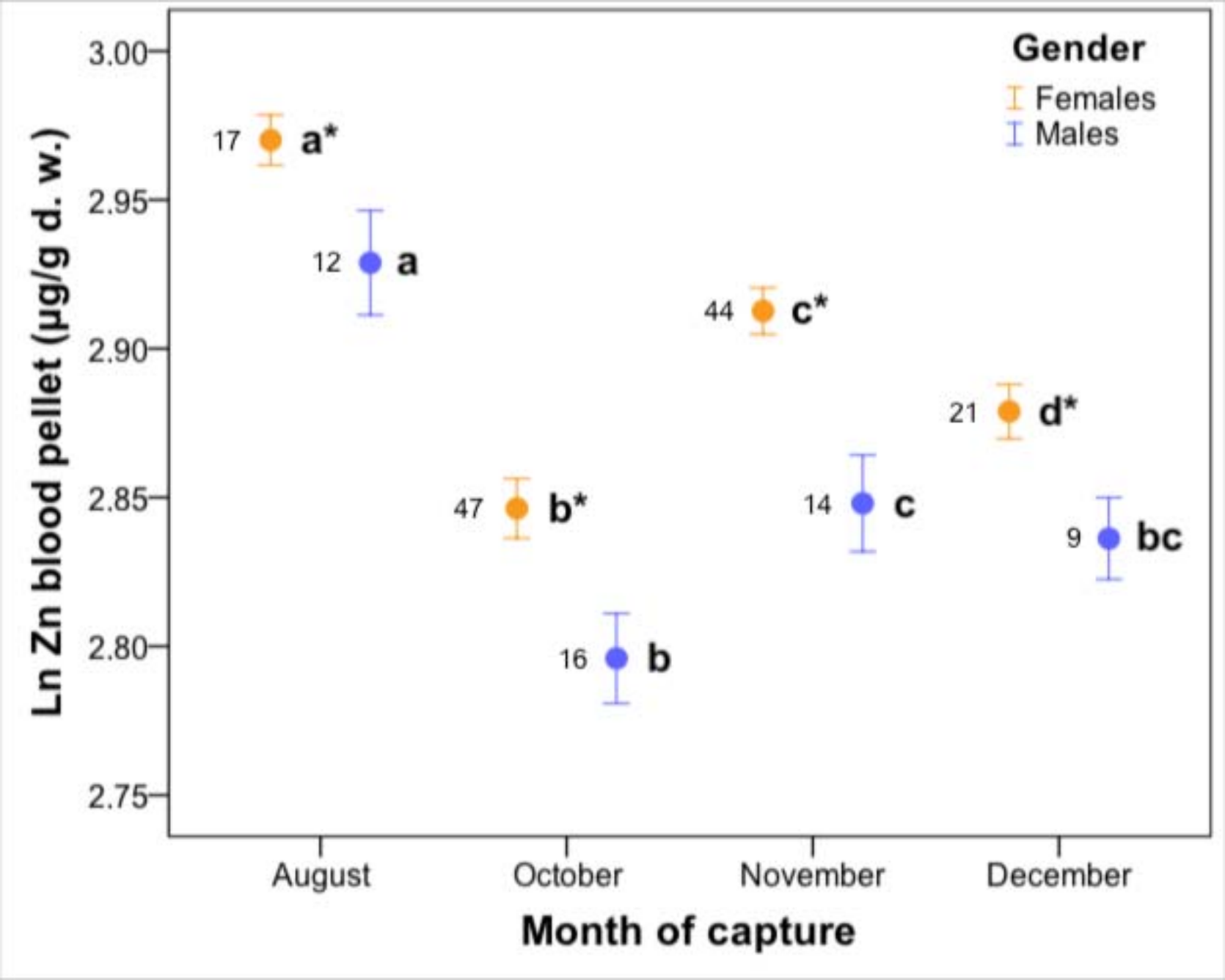
Table 3. Best-fit models explaining the effect of metal accumulation on the studied biomarkers of effect in black-necked grebes (*Podiceps nigricollis*) from the Odiel Marshes during the moulting period. *

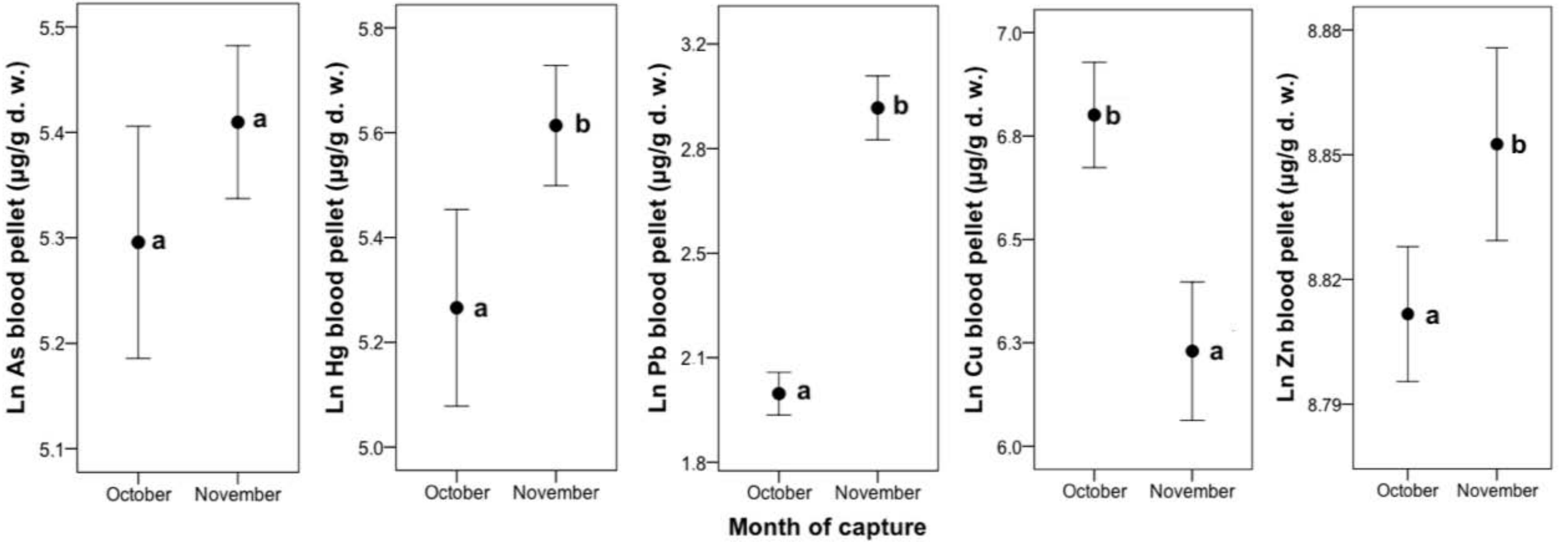
Dependent variable	Terms in the model	Effects of metals	*F* statistic	d. f.	*p*-values	*Adjusted-R^2*
Lutein-like carotenoids	Month of capture	(Aug) = - 0.329 × **Hg (Log)** + 0.538	13.77	3, 118	0.010	0.58
(Log)	**Hg (Log)**	(Oct) = 0.040 × **Hg (Log)** - 0.024	2.80	1, 118	0.069	
	Month of capture × **Hg (Log)**	(Nov) = 0.543 × **Hg (Log)** + 1.302	2.74	3, 118	0.047	
		(Dic) = 1.023 × **Hg (Log)** - 1.403				
***trans*-Zeaxanthin**	Month of capture	(Aug, Moulting) =	0.76	3, 108	0.519	0.43
(Log)	Moult status	0.357 × **As (Log)** + 1.425	0.87	2, 108	0.421	
	As (Log)	(Oct, Unmoulted) =	1.44	1, 108	0.233	
	Month of capture × Moult status	0.583 × **As (Log)** + 1.941	1.83	2, 108	0.166	
	Month of capture × **As (Log)**	(Nov, Moulted) =	3.42	3, 108	0.020	
	Moult status × **As (Log)**	0.161 × **As (Log)** + 1.965	2.59	2, 108	0.080	
	Month of capture × Moult status × **As (Log)**	(Dec, Moulted) =	5.41	2, 108	0.006	
		0.650 × **As (Log)** + 1.407				
Eye redness	Month of capture	(Oct, Juveniles) =	10.46	3, 133	<0.001	0.51
(Log)	Age	- 0.016 × **As (Log)** - 0.251	89.26	1, 133	<0.001	
	As (Log)	(Aug, Adults) =	5.10	1, 133	0.026	
		- 0.016 × **As (Log)** - 0.162				
		(Oct, Adults) =				
		- 0.016 × **As (Log)** - 0.164				
		(Nov, Adults) =				

		- 0.016 × **As (Log)** - 0.125 (Dec, Adults) = - 0.016 × **As (Log)** - 0.123				
Scaled Mass Index (Log)	Month of capture	(Nov, Moulted females) = 18.55 × **As (Log)** + 416.6 (Dec, Moulted females) = 18.55 × **As (Log)** + 397.5	9.23	3, 149	<0.001	0.29
	Moult status		0.07	2, 149	0.936	
	Gender		46.39	1, 149	<0.001	
	As (Log)		18.09	1, 149	<0.001	
	Month of capture × Moult status		4.50	5, 149	0.001	
	Moult status × **As (Log)**		4.69	2, 149	0.011	

* Metals having an effect on each biomarker have been marked in bold. The column showing the effects of metals includes the model equations for each particular case, where the positive or negative direction of the effects of metal accumulation is marked. Only significant effects have been included in this column.


Moult status
Unmoulted
Moulting
Moulted
Ln As blood pellet (µg/g d.w.)
1.0
0.5
0
-0.5
-1.0
1
2
26
a
5
14
a
33
a
2
1
52
b
1
29
c
August
October
November
December
Month of capture


Gender
Females
Males
3.00
2.95
2.90
2.85
2.80
2.75
Ln Zn blood pellet (µg/g d. w.)
17 a*
12 a
47 b*
16 b
44 c*
14 c
21 d*
9 bc
August
October
November
December
Month of capture


Ln As blood pellet (µg/g d. w.)
5.5
5.4
5.3
5.2
5.1
a
a
October
November
Ln Hg blood pellet (µg/g d. w.)
5.8
5.6
5.4
5.2
5.0
a
b
October
November
Ln Pb blood pellet (µg/g d. w.)
3.2
2.8
2.5
2.1
1.8
a
b
October
November
Ln Cu blood pellet (µg/g d. w.)
7.0
6.8
6.5
6.3
6.0
b
a
October
November
Ln Zn blood pellet (µg/g d. w.)
8.88
8.85
8.82
8.79
a
b
October
November
Month of capture

The Odiel Marshes (SW Spain)

Mining area
(Iberian Pyrite Belt)

Metal pollution
(As, Hg, Pb, Cd, Cu, Zn)

Artemia parthenogenetica

Blood metals
Plasma carotenoids
Eye redness
Body condition

Black-necked grebes
(Podiceps nigricollis)