Phthalate diversity in eggs and associations with oxidative stress in the European herring gull (*Larus argentatus*)

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Running header: Phthalates in gull eggs

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Abstract

Phthalates are plastic-derived contaminants that are ubiquitous in natural environments and function as pro-oxidants. The extent to which phthalates bioaccumulate in wild animals and associations with oxidative stress are poorly understood. Here, we describe relationships between maternally-derived phthalates, lipid peroxidation (malondialdehyde, MDA) and the dietary antioxidant α-tocopherol in eggs of European herring gulls (*Larus argentatus*) in Cornwall, UK. Up to six phthalate parent compounds and four phthalate metabolites were detected. Egg concentrations of MDA were positively associated with dicyclohexyl phthalate (DCHP) and negatively associated with α-tocopherol, suggesting that DCHP is associated with oxidative stress in gulls. The consequences of phthalate exposure *in ovo* for offspring development warrants study.

Plastic pollution is ubiquitous in the marine environment. Indeed, an estimated 4.8-12.7 million tonnes of plastic debris enter the ocean each year (Jambeck *et al.*, 2015). Mechanical effects of plastic debris on animals, including entanglement and ingestion, have been well-documented (reviewed by Laist, 1987; Senko *et al*., 2020). Increasingly, however, it is becoming clear that plastic debris can also present less obvious, non-mechanical threats. Chemicals bound or adsorbed to plastic can leach into the surrounding environment, or enter tissues if plastic is ingested, with sublethal physiological effects. One such group of plastics additives are phthalates, organic chemicals that function as plasticisers and are present in a multitude of products (Schettler, 2006). Phthalates are not chemically bound to the polymer matrix, and can therefore readily leach out into the surrounding environment during manufacturing, use, or disposal (Hermabessiere *et al.*, 2017). As a result, phthalates have been found in a wide range of environmental matrices (Net *et al.*, 2015). This is of particular concern because phthalates are lipophilic, and therefore have potential to bioaccumulate (Mathieu-Denoncourt *et al.*, 2016).

Impacts of phthalate exposure on development and reproduction have been documented in a range of taxa in the laboratory (e.g. Aly *et al.*, 2016; Wu *et al.*, 2019; Xu and Gye, 2018). One important pathway of disruption involves phthalates acting as pro-oxidants, causing increased formation of reactive oxygen species (ROS) during oxidative phosphorylation (Tetz et al., 2013). ROS are highly reactive, though antioxidant defences normally prevent them from causing excessive damage to important biomolecules. However, an imbalance between ROS and antioxidants in favour of the former leads to a state of oxidative stress, resulting in damage to DNA, lipids and proteins (Sies, 1993). While biological effects of phthalates have been extensively studied in the laboratory, much less is known about their impacts on wildlife, and data for vertebrates are especially scarce (Oehlmann *et al*. 2009). To date, most studies of phthalates in wildlife have focussed on documenting their presence in tissues (e.g. Baini *et al.*, 2017; Liu *et al.*, 2019; Savoca *et al.*, 2018). However, few studies have explored their physiological effects (e.g. Martins *et al*., 2016).

Pollutants are commonly deposited into the eggs of oviparous species as an unavoidable consequence of the transfer of lipids and proteins (Pajurek *et al.*, 2019; Verreault *et al.*, 2006). Indeed, phthalates have been reported in eggs of free-living reptiles (Liu *et al.*, 2019) and seabirds (Huber *et al.*, 2015). However, we are not aware of any previous studies that have examined associations between phthalates, antioxidants and oxidative damage in eggs. Such data would be informative for two reasons. Firstly, the eggs of oviparous species are a semi-closed system where egg contents are derived almost entirely from the mother. As such the composition of eggs provides a window to maternal physiological state during breeding (Surai *et al.*, 2016). Secondly, since the egg contains all of the nutrients required for embryo development in a self-contained package, egg composition can be used as a means of examining transfer of nutrients and other constituents (e.g. pollutants) from mother to offspring. Such data would be an important first step to understanding whether phthalate exposure can transmit across generations (i.e. an intergenerational effect).

In this study, concentrations of phthalates were measured in eggs of European herring gulls (*Larus argentatus*). We also examined associations between phthalates, and maternally-derived concentrations of the dietary antioxidant α-tocopherol (vitamin E – the primary antioxidant in egg yolk) and malondialdehyde (MDA - a marker of lipid peroxidation). Herring gulls frequently encounter and ingest plastics (e.g. Caldwell *et al.*, 2020). They occupy a high trophic level and have a relatively long lifespan, making them susceptible to bioaccumulation and biomagnification of contaminants (Burger and Gochfeld, 2004). We predicted that (i) phthalates would be present in eggs. In addition, if phthalates are associated with oxidative stress *in vivo*, we predicted that (ii) yolk concentrations of MDA would be positively related to yolk concentrations of individual phthalates, and negatively related to yolk concentrations of α-tocopherol (i.e. reflecting levels of oxidative stress in the mother).

In May 2019, 13 eggs were collected from 7 nests at 3 semi-urban sites in Cornwall, UK (Table 1). Eggs were frozen at -80°C until biochemical analysis. For phthalate analysis, eggs

were removed from storage and while still frozen the yolks were separated from albumen and shell using a stainless steel knife in a Pyrex dish. Yolks were cut into small pieces and then placed in 50 ml glass centrifuge tubes. Each yolk sample was analysed in triplicate. To provide an internal standard, to each ~5g yolk aliquot, 50 µl of deuterated phthalate parent mix and 50 µl of carbon-labelled phthalate metabolite mix were added using a Drummond micro dispenser with a glass tip. The phthalate parent mix contained 100 ppb of D_4-dimethyl phthalate (DMP), D_4-diethyl phthalate (DEP), D_4-di-*n*-butyl phthalate (DnBP) and D_4-bis(2-ethylhexyl) phthalate (DEHP) in isooctane. The phthalate metabolite mix contained 100 ppb of $^{13}C_4$-monomethyl phthalate (MMP), $^{13}C_4$-monoethyl phthalate (MEP), $^{13}C_4$-monocyclohexyl phthalate (MCHP), $^{13}C_4$-mono-*n*-butyl phthalate (MnBP), $^{13}C_4$-monobenzyl phthalate (MBzP), $^{13}C_4$-mono (2-ethylhexyl)phthalate (MEHP), $^{13}C_4$-mono (2-ethyl-5-hydroxyhexyl)phthalate (MEHHP), $^{13}C_4$-mono (2-ethyl-5-oxyhexyl)phthalate (MEOHP), $^{13}C_4$-mono(2-ethyl-5-carboxypentyl) phthalate (MECPP), $^{13}C_4$-mono-*n*-octyl phthalate (MnOP), $^{13}C_4$-mono(3-carboxypropyl) phthalate (MCPP) and $^{13}C_4$-mono-iso-nonyl phthalate (MiNP) in methanol. Acetonitrile (10 ml) was added to each sample using a glass pipette, then briefly vortexed to form an emulsion. A QuEChERs extraction kit (Agilent part number: 5982-5650) containing 4 g of $MgSO_4$, 1 g NaCl, 1 g Na Citrate, and 0.5 g disodium citrate sesquihydrate was added to each sample. Samples were then immediately vortexed for 30 sec, then shaken vigorously for 5 min, before being centrifuged at 1000rpm at 4°C for 10 min, and finally placed in a -20°C freezer for at least 4 hours. An aliquot of the supernatant (1 ml) was transferred to a 2 ml autosampler vial and dried under a stream of nitrogen of high purity, before being reconstituted with 1 ml acetonitrile.

Table 1

Colony locations, and numbers of nests and eggs of European herring gulls sampled in Cornwall, UK.

Colony (UK grid reference)	Nest	N eggs
SW7933	1	2
SW7933	2	1
SW7932	3	1
SW8261	4	2
SW8261	5	2
SW8261	6	3
SW8261	7	2

An aliquot (800 µl) of each sample was transferred to a 15 ml glass centrifuge tube, to which an Agilent d-SPE clean-up kit was added (Agilent Part number 5982-5122CH) containing 50 mg of PSA, 50 mg of C18EC, and 150 mg of $MgSO_4$. Samples were vortexed for 30 sec and then shaken vigorously for 5 min, before being centrifuged at 4000 rpm at 4°C for 10 min. An aliquot (200 µl) of the supernatant was then transferred to an autosampler vial, evaporated under a gentle stream of high-purity nitrogen and reconstituted in 100 µl of isooctane containing 40 ppb $^{13}C_{12}$-PCB141 as an instrument standard for monitoring the instrument performance and calculating the recoveries of the spiked internal standards. For phthalate parent compounds, samples were analysed using a Q Exactive™ GC Orbitrap™ (Thermo Fisher Scientific Inc., Mass., USA) in electron impact ionisation mode, with the resolution set to 60,000 and the scan range set from 50 to 750 m z^{-1}. Injection of samples occurred in splitless mode, with the injection port at 250°C. The transfer line and source were maintained at 280°C. A TraceGOLD™ TG-5SilMS column (30 m x 0.25 mm x 0.25 µm, Thermo Scientific™) was used, with helium as the carrier gas at a flow rate of 1 ml min^{-1}. For each target analyte and internal standard, 2 fragments were monitored: one for quantification and one for confirmation. For quantification of phthalate metabolites, a 1ml solution of acetonitrile containing 0.1% formic acid was added to a 3ml captiva non-drip liquid column

(Agilent part number A5300635). The remaining 200 µl of reconstituted sample was added, and the solution was then filtered through a 13 mm PTFE 0.2 µm syringe filter (Agilent part number 5190-5265) into a glass screw-capped vial before being centrifuged for 10 min at 3283 x g and 4°C. This was then evaporated under a stream of high-purity nitrogen until near dryness. The sample was reconstituted using 100 µl of 20% MeOH in water, and transferred to an autosampler vial. Samples were analysed using a UHPLC system (Shimadzu, Nexera 2 UHPLC system, Kyoto, Japan) coupled with a tandem mass spectrometer equipped with an IonDrive source (SCIEX QTRAP® 6500+, Ontario, Canada). A Kinetex® F5 column (2.6 µm) was used. Oven temperature was kept constant at 45°C. Two mobile phases were used, consisting of 1% MeOH and 0.1% acetic acid in MilliQ water (solvent A) and 95% MeOH and 0.1% acetic acid in MilliQ water (solvent B). The binary eluting gradient was 10% solvent B from 0 to 1 min, increasing to 75% solvent B from 1 to 11.5 min and to 98% until 14 min. This was then decreased to 10% solvent B until 14.1 min and held for 1.5 min. The mass spectrometer was operated with scheduled multiple reaction monitoring (sMRM). The source temperature was 430°C and the ionspray voltage was set to -4500. Two ion transitions were monitored for each target analyte and internal standard.

Initial target compounds included 8 phthalate parent compounds and 13 phthalate metabolites (Table S1). Strict quality assurance/control (QA/QC) procedures were conducted including background level examination, and analytical precision and accuracy assessment. 6 parent phthalate compounds and 5 phthalate metabolites were considered to have passed the QA/QC criteria. Details of QA/QC including blank concentration, method detection limit (MDL), and analytical accuracy and precision are provided in the SI.

Quantification of MDA was performed by HPLC, as previously described with some modifications (Plummer *et al.*, 2013). An aliquot (40-50 mg) of yolk was placed in phosphate-buffered saline solution at 5% w v^{-1}, then homogenised for 30 sec with a T18 Basic Ultra-Turrax® homogeniser (IKA® England LTD). An aliquot of the homogenate (200 µl) was transferred to a reaction tube. Samples were vortexed for 5 sec, then placed on a hot plate

at 37°C standing on a shaker set at 100 rpm for 20 min. Following this, the samples were cooled on ice for 5 min. After cooling, a 20 µl aliquot was transferred to a reaction tube pre-loaded with 20 µl of butylated hydroxytoluene (BHT). To this were added 40 µl of thiobarbituric acid (TBA) and 160 µl of phosphoric acid. These reaction tubes were vortexed for 5 seconds, and then placed in a dry heat bath at 100°C for 1 hour, before being cooled on ice. After cooling, 100 µl of butanol was added and samples were vortexed for 20 sec, then centrifuged at 4°C at 12,000 x g for 3 min. An aliquot (60 µl) of the upper phase, which contained MDA-TBA adducts, was drawn off ready for HPLC, which was carried out as described previously (Plummer *et al.*, 2013).

For α-tocopherol analysis, 40-50 mg of yolk was vortexed in 0.7 ml of 5% NaCl solution for 5 sec. This was then homogenised with 1 ml EtOH for 20 sec before the addition of 1.5 ml hexane, followed by further homogenisation for 10 sec. Samples were then centrifuged for 4 min at 8,000 x g. The hexane phase containing the antioxidants was then drawn off and reserved, while a further 1.5 ml of hexane was added to the remaining precipitate for a second extraction. The two hexane extracts were combined, and a 500 µl aliquot was dried under a stream of high purity nitrogen before being re-dissolved in 150 µl DCM and 150 µl MeOH ready for HPLC, which was carried out as described previously (Plummer *et al.*, 2013).

Statistical analyses were performed using R version 4.0.1 (R Core Team, 2020). As metabolites are unlikely to be independent of parent compounds (Hu *et al.*, 2016), inferential statistical tests were performed using parent compounds only. To maximise statistical power, only phthalate parent compounds with sufficient data points (n >10) were included to test for their associations with MDA and α-tocopherol. The compounds modelled were dicyclohexyl phthalate (DCHP), benzyl butyl phthalate (BBzP), and diisobutyl phthalate (DiBP). Relationships between phthalates, MDA and α-tocopherol were assessed using linear mixed-effects models in the R package ‘lme4’, where MDA was the response variable, and phthalate and α-Tocopherol concentrations (and their interaction) were included as

predictors. Nest of origin was included as a random factor to control for non-independence of samples from the same nests. Models were developed by backwards elimination of non-significant ($p < 0.05$) terms, starting with the interaction.

Phthalate parent compounds and phthalate metabolites were present at concentrations greater than the MDL in all 13 eggs. All phthalate parent compounds were found in at least 1 egg at concentrations greater than the MDL (Figure 1). The mean (± SE) concentrations of phthalate metabolites were 12.89 ± 1.17 ng g^{-1} for MnBP (found in 12 eggs), 0.32 ± 0.06 ng g^{-1} for MECPP (found in all 13 eggs), and 31.10 ± 3.07 ng g^{-1} for Monoisobutyl phthalate (MiBP, found in all 13 eggs). MnOP was only present in 1 egg at a concentration of 0.20 ng g^{-1}. MBzP and MEOHP were either not present in eggs, or only present at concentrations lower than the MDL. The metabolite MEHP was only present at concentrations lower than the MDL.

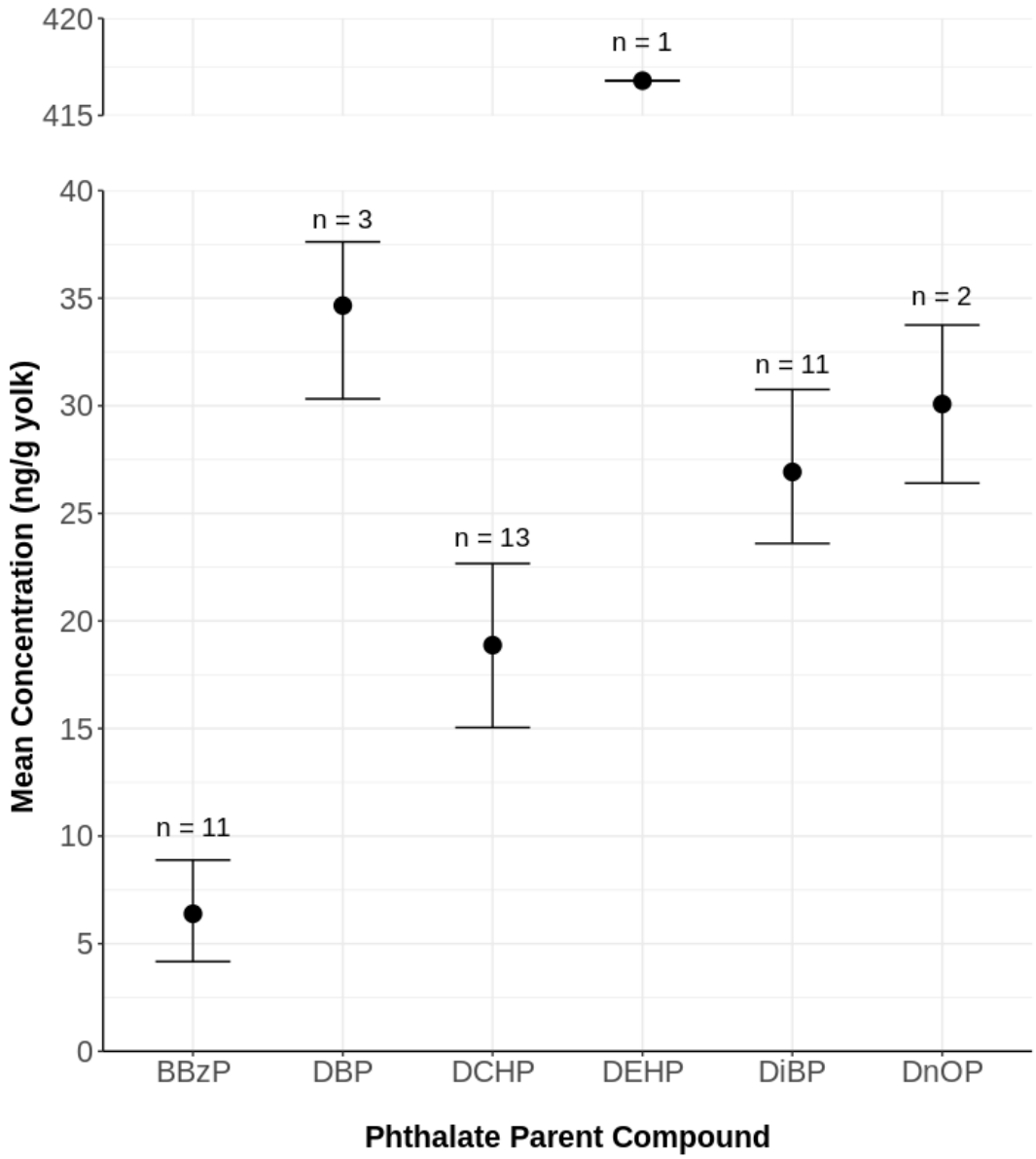

Fig. 1. Concentrations of phthalate parent compound in the yolks of eggs collected from three sites in Cornwall, UK. Points represent the mean concentration for each phthalate, while error bars denote the range of values detected across eggs. Sample sizes represent the number of eggs where the measured concentration was greater than the MDL.

Yolk concentrations of MDA were not significantly predicted by BBzP or α-tocopherol (BBzP: $\chi^2_1 = 0.19$, $p = 0.66$; α-tocopherol: $\chi^2_1 = 2.74$, $p = 0.10$; BBzP x α-tocopherol interaction: $\chi^2_1 = 0.00$, $p = 0.97$). Similarly, there was no significant relationship between yolk

levels of MDA and DiBP or α-tocopherol (DiBP: $\chi^2_1 = 1.26$, $p = 0.26$; α-tocopherol: $\chi^2_1 = 0.02$, $p = 0.88$; DiBP x α-tocopherol interaction: $\chi^2_1 = 0.97$, $p = 0.33$). However, yolk MDA was positively associated with DCHP ($\chi^2_1 = 7.94$, $p = 0.01$; Figure 2a) and negatively associated with α-tocopherol (α-tocopherol: $\chi^2_1 = 5.64$, $p = 0.02$, Figure 2b; DCHP x α-tocopherol interaction: $\chi^2_1 = 0.0047$, $p = 0.95$).

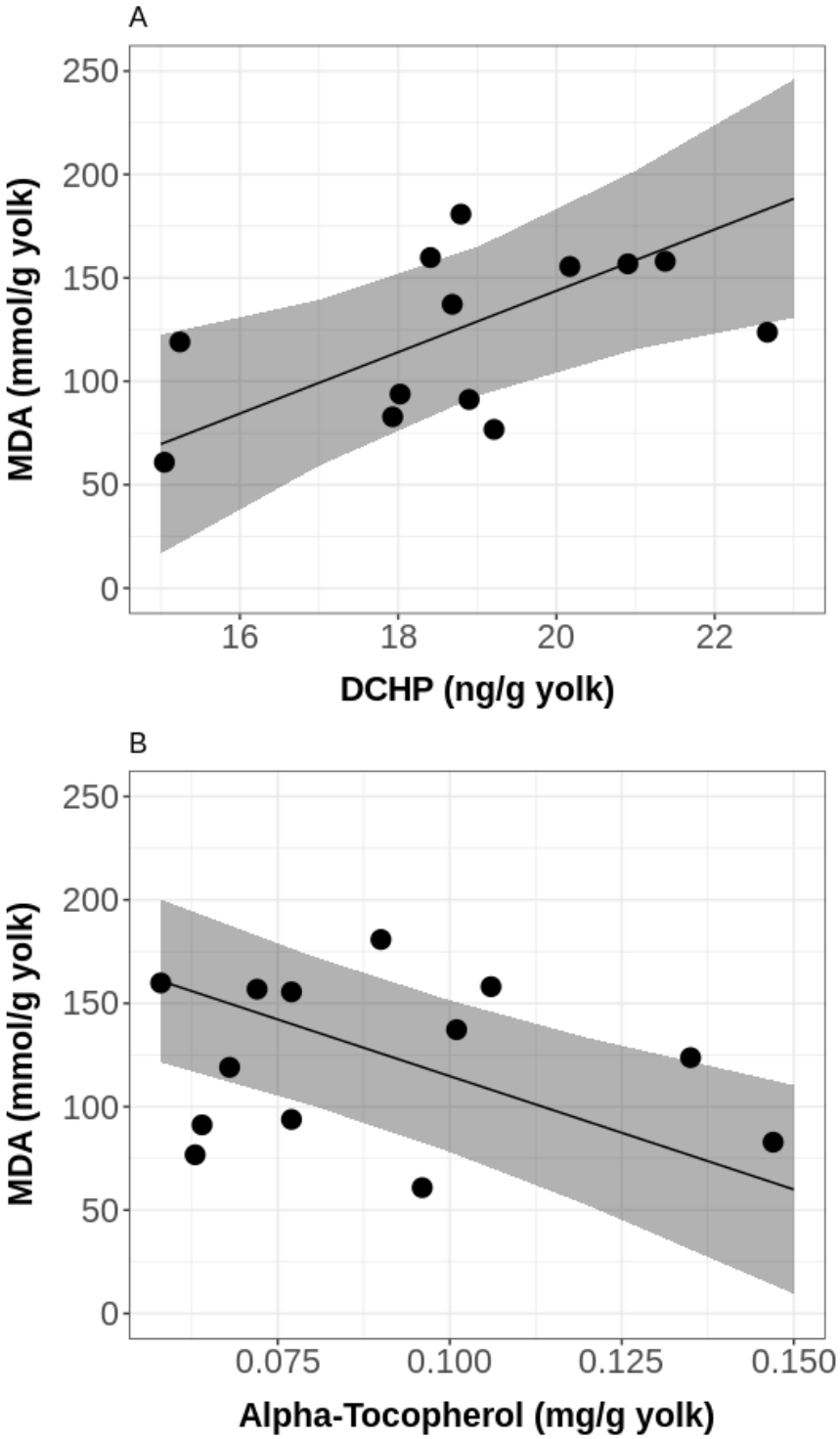

Fig. 2. Relationships between yolk concentrations of malondialdehyde (MDA) and: A) DCHP; and B) α-tocopherol, based on linear mixed-effects models. Data points represent individual herring gull eggs (n = 13) collected in Cornwall, UK. Shaded areas represent the 95% confidence interval of the model estimates.

Overall, a combination of up to 6 phthalate parent compounds (BBzP, DnBP, DCHP, DEHP, DiBP, DnOP) and up to 4 phthalate metabolites (MnBP, MECPP, MiBP, MnOP) were present in herring gull eggs. MnBP was present in 12 eggs, but its parent compound DnBP was present in only 3 eggs. Similarly, DiBP was detected in 3 eggs while its metabolite MiBP was found in all eggs. This could at least in part be due to the relatively high MDLs of these parent compounds compared to their metabolites, but may also indicate that phthalate parent compounds were metabolised in female gulls *in vivo*. Biotransformation of phthalates has been reported in mammals (Albro and Lavenhar, 1989) and fish (Barron *et al.*, 1995). Biotransformation time differs between compounds, but available data indicate a relatively fast biotransformation of DnBP (Seckin *et al.*, 2009). Alternatively, phthalate metabolites could have been sequestered by gulls in their diet and transferred directly to their eggs.

Phthalates have been detected in several prey species of the herring gull, including fishes, crabs, and shrimps (Munshi *et al.*, 2013). Lipophilic molecules such as phthalates may be readily co-acquired with lipids in the diet, and thus transferred through the food chain. In addition, gulls may directly acquire phthalates by ingesting plastics. Like other seabirds, gulls are known to ingest microplastic particles and even relatively large plastic items such as fragments of discarded fishing net (Lindborg *et al.*, 2012). Interestingly, a previous study of phthalates in herring gull eggs collected at two remote colonies in Norway found DEHP in all 18 eggs sampled (Huber *et al*. 2015), whereas we detected DEHP in only 1 of 13 eggs. It is clear that phthalate ingestion and deposition in gull eggs can vary over macro- and even microgeographic scales, likely due to differences in local phthalate exposure and/or foraging preferences.

The extent to which phthalates bioaccumulate in birds, or biomagnify across food chains, is poorly understood (Net *et al.*, 2015). Bioaccumulation and biomagnification of chemically similar compounds such as polychlorinated biphenyls (PCBs) have been linked to deleterious health effects in many marine top predators (e.g. Desforges *et al.*, 2016; Jepson *et al.*, 2016). Since herring gulls occupy a relatively high trophic position, they may be at

greater risk of phthalate bioaccumulation than species at lower trophic levels. Several factors must be quantified in order to estimate bioaccumulation of phthalates, including retention times of plastics and species' ability to excrete these compounds. Similarly, how concentrations of phthalates deposited into egg yolk compare with concentrations of the same compounds in mothers awaits study. Nevertheless, as for nutrients and some other xenobiotics (e.g. Blount *et al.*, 2002; Li *et al.*, 2019; Zheng *et al.*, 2018), it seems unlikely that mothers are able to adjust levels of phthalates deposited into eggs independently of levels in their own tissues and in circulation. Indeed, gulls extensively catabolise stored lipids and proteins in order to produce a clutch of eggs (Blount *et al*. 2002). As such, the phenotype of newly laid eggs and the concentrations of pollutants in them reflect maternal physiological state.

There was a significant positive association between yolk concentrations of MDA and DCHP. DCHP is considered a substance of very high concern by the EU due to its endocrine-disrupting properties and reproductive toxicity (Bieńkowska, 2018). Exposure to DCHP *in utero* caused downstream genotoxic effects and apoptosis in testicular cells during puberty in rats (Ahbab *et al.*, 2014), and DCHP was associated with oxidative stress in goldfish (*Carassius auratus*) (Qu, 2015). It is therefore possible that DCHP exposure *in ovo* may have deleterious effects on health in gulls.

Oxidative damage to lipids may be especially likely to occur during egg production, when metabolic demands are at their highest (e.g. Vézina and Williams, 2002). Therefore, gulls exposed to DCHP may experience even greater oxidative stress than would normally be associated with reproduction. This could result in reduced levels of α-tocopherol and increased levels of MDA in maternal circulation and deposited into egg yolk. Malondialdehyde (MDA) is highly reactive, and its deposition in egg yolk could trigger oxidative stress during embryogenesis. A combination of poor antioxidant protection and high exposure to maternally-derived MDA could be especially damaging for the development and survival of offspring, since avian hatchlings are inherently susceptible to oxidative stress

upon exposure to atmospheric concentrations of oxygen at hatching (Surai *et al.*, 2016). The presence of phthalates in egg yolk – perhaps especially DCHP - may present an additional risk of invoking oxidative stress in offspring.

Aside from DCHP, we found no other significant relationships between yolk levels of phthalates and MDA. However, other studies have found effects of such phthalates on tissue levels of oxidative stress at concentrations comparable to those observed in egg yolk in this study (e.g. Franken *et al.*, 2017; Van'T Erve *et al.*, 2019; Xu and Gye, 2018). The consequences of phthalate exposure for the health and fecundity of breeding gulls, and intergenerational impacts on hatchlings, warrant further study.

CRediT Authorship Contribution Statement

Simon Allen: Data curation, Formal analysis, Writing – original draft.

Francesca Ellis: Data curation, Formal analysis, Writing – original draft.

Christopher Mitchell: Methodology, Investigation, Data curation, Validation, Writing – original draft.

Xianyu Wang: Conceptualisation, Funding acquisition, Methodology, Supervision, Investigation, Data curation, Validation, Writing – original draft.

Neeltje Boogert: Conceptualization, Funding acquisition, Investigation, Writing – review and editing.

Chun-Yin Lin: Investigation, Writing – review and editing.

Joseph Clokey: Investigation, Writing – review and editing.

Kevin Thomas: Conceptualisation, Funding acquisition, Methodology, Resources, Writing – review and editing, Project administration.

Jonathan Blount: Conceptualisation, Funding acquisition, Methodology, Resources, Supervision, Investigation, Writing – original draft, Project administration.

Declaration of Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

Ahbab, M.A., Ündeğer, Ü., Barlas, N., Başaran, N., 2014. In utero exposure to dicyclohexyl and di-n-hexyl phthalate possess genotoxic effects on testicular cells of male rats after birth in the comet and TUNEL assays. Hum. Exp. Toxicol. 33, 230–239. https://doi.org/10.1177/0960327113494903

Albro, P.W., Lavenhar, S.R., 1989. Metabolism of Di(2=ethylhexyl)phthalate. Drug Metab. Rev. 21, 13–34. https://doi.org/10.3109/03602538909029953

Aly, H.A., Hassan, M.H., El-Beshbishy, H.A., Alahdal, A.M., Osman, A.-M.M., 2016. Dibutyl phthalate induces oxidative stress and impairs spermatogenesis in adult rats. Toxicol. Ind. Health 32, 1467–1477. https://doi.org/10.1177/0748233714566877

Baini, M., Martellini, T., Cincinelli, A., Campani, T., Minutoli, R., Panti, C., Finoia, M.G., Fossi, M.C., 2017. First detection of seven phthalate esters (PAEs) as plastic tracers in superficial neustonic/planktonic samples and cetacean blubber. Anal. Methods 9, 1512–1520. https://doi.org/10.1039/c6ay02674e

Barron, M.G., Albro, P.W., Hayton, W.L., 1995. Biotransformation of di (2-ethylhexyl) phthalate by rainbow trout. Environ. Toxicol. Chem. 14, 873–876. https://doi.org/10.1002/etc.5620140519

Bieńkowska, E., 2018. COMMISSION IMPLEMENTING DECISION (EU) 2018/636 of 17 April 2018 on the identification of dicyclohexyl phthalate (DCHP) as a substance of very high concern according to Article 57(c) and (f) of Regulation (EC) No 1907/2006 of the European Parliament and of, Official Journal of the European Union.

Blount, J.D., Surai, P.F., Nager, R.G., Houston, D.C., Møller, A.P., Trewby, M.L., Kennedy, M.W., 2002. Carotenoids and egg quality in the lesser black-backed gull *Larus fuscus*: A supplemental feeding study of maternal effects. Proc. R. Soc. B Biol. Sci. 269, 29–36. https://doi.org/10.1098/rspb.2001.1840

Burger, J., Gochfeld, M., 2004. Marine birds as sentinels of environmental pollution. Ecohealth 1, 263–274. https://doi.org/10.1007/s10393-004-0096-4

Caldwell, A., Seavey, J., Craig, E., 2020. Foraging strategy impacts plastic ingestion risk in seabirds. Limnol. Oceanogr. Lett. 5, 163–168. https://doi.org/10.1002/lol2.10126

Desforges, J.P.W., Sonne, C., Levin, M., Siebert, U., De Guise, S., Dietz, R., 2016. Immunotoxic effects of environmental pollutants in marine mammals. Environ. Int. https://doi.org/10.1016/j.envint.2015.10.007

Franken, C., Lambrechts, N., Govarts, E., Koppen, G., Den Hond, E., Ooms, D., Voorspoels, S., Bruckers, L., Loots, I., Nelen, V., Sioen, I., Nawrot, T.S., Baeyens, W., Van Larebeke, N.,

Schoeters, G., 2017. Phthalate-induced oxidative stress and association with asthma-related airway inflammation in adolescents. Int. J. Hyg. Environ. Health 220, 468–477. https://doi.org/10.1016/j.ijheh.2017.01.006

Hermabessiere, L., Dehaut, A., Paul-Pont, I., Lacroix, C., Jezequel, R., Soudant, P., Duflos, G., 2017. Occurrence and effects of plastic additives on marine environments and organisms: A review. Chemosphere 182, 781–793. https://doi.org/10.1016/j.chemosphere.2017.05.096

Hu, X., Gu, Y., Huang, W., Yin, D., 2016. Phthalate monoesters as markers of phthalate contamination in wild marine organisms. Environ. Pollut. 218, 410–418. https://doi.org/10.1016/j.envpol.2016.07.020

Huber, S., Warner, N.A., Nygård, T., Remberger, M., Harju, M., Uggerud, H.T., Kaj, L., Hanssen, L., 2015. A broad cocktail of environmental pollutants found in eggs of three seabird species from remote colonies in Norway. Environ. Toxicol. Chem. 34, 1296–1308. https://doi.org/10.1002/etc.2956

Jambeck, J.R., Geyer, R., Wilcox, C., Siegler, T.R., Perryman, M., Andrady, A., Narayan, R., Law, K.L., 2015. Plastic waste inputs from land into the ocean. Science 347, 768–771. https://doi.org/10.1126/science.1260352

Jepson, P.D., Deaville, R., Barber, J.L., Aguilar, À., Borrell, A., Murphy, S., Barry, J., Brownlow, A., Barnett, J., Berrow, S., Cunningham, A.A., Davison, N.J., Ten Doeschate, M., Esteban, R., Ferreira, M., Foote, A.D., Genov, T., Giménez, J., Loveridge, J., Llavona, Á., Martin, V., Maxwell, D.L., Papachlimitzou, A., Penrose, R., Perkins, M.W., Smith, B., De Stephanis, R., Tregenza, N., Verborgh, P., Fernandez, A., Law, R.J., 2016. PCB pollution continues to impact populations of orcas and other dolphins in European waters. Sci. Rep. 6, 1–17. https://doi.org/10.1038/srep18573

Laist, D.W., 1987. Overview of the biological effects of lost and discarded plastic debris in the marine environment. Mar. Pollut. Bull. 18, 319–326. https://doi.org/10.1016/S0025-326X(87)80019-X

Li, F., Yang, Y., Yang, X., Shan, M., Gao, X., Zhang, Y., Hu, J., Shan, A., 2019. Dietary intake of broiler breeder hens during the laying period affects amino acid and fatty acid profiles in eggs. Rev. Bras. Zootec. 48. https://doi.org/10.1590/RBZ4820180292

Lindborg, V.A., Ledbetter, J.F., Walat, J.M., Moffett, C., 2012. Plastic consumption and diet of Glaucous-winged Gulls (*Larus glaucescens*). Mar. Pollut. Bull. 64, 2351–2356. https://doi.org/10.1016/j.marpolbul.2012.08.020

Liu, Y.E., Tang, B., Liu, Y., Luo, X.J., Mai, B.X., Covaci, A., Poma, G., 2019. Occurrence, biomagnification and maternal transfer of legacy and emerging organophosphorus flame retardants and plasticizers in water snake from an e-waste site. Environ. Int. 133, 105240. https://doi.org/10.1016/j.envint.2019.105240

Martins, K., Hagedorn, B., Ali, S., Kennish, J., Applegate, B., Leu, M., Epp, L., Pallister, C., Zwollo, P., 2016. Tissue phthalate levels correlate with changes in immune gene expression in a population of juvenile wild salmon. Arch. Environ. Contam. Toxicol. 71, 35–47. https://doi.org/10.1007/s00244-016-0283-7

Mathieu-Denoncourt, J., Wallace, S.J., de Solla, S.R., Langlois, V.S., 2016. Influence of lipophilicity on the toxicity of bisphenol A and phthalates to aquatic organisms. Bull. Environ. Contam. Toxicol. 97, 4–10. https://doi.org/10.1007/s00128-016-1812-9

Munshi, A.B., Karim, N., Shaukat, S., Hashmi, D., Boardman, G.D., Flick, G.J., 2013. Toxicity of phthalate esters in fish and shellfish from Virginia beach using Matrix Solid Phase Dispersion (MSPD) and GC-MS. J. Chem. Soc. Pakistan 35, 1463–1471.

Net, S., Sempéré, R., Delmont, A., Paluselli, A., Ouddane, B., 2015. Occurrence, fate, behavior and ecotoxicological state of phthalates in different environmental matrices. Environ. Sci. Technol. 49, 4019–4035. https://doi.org/10.1021/es505233b

Oehlmann, J., Schulte-Oehlmann, U., Kloas, W., Jagnytsch, O., Lutz, I., Kusk, K.O., Wollenberger, L., Santos, E.M., Paull, G.C., VanLook, K.J.W., Tyler, C.R., 2009. A critical analysis of the biological impacts of plasticizers on wildlife. Philos. Trans. R. Soc. B Biol. Sci. https://doi.org/10.1098/rstb.2008.0242

Pajurek, M., Pietron, W., Maszewski, S., Mikolajczyk, S., Piskorska-Pliszczynska, J., 2019. Poultry eggs as a source of PCDD/Fs, PCBs, PBDEs and PBDD/Fs. Chemosphere 223, 651–658. https://doi.org/10.1016/j.chemosphere.2019.02.023

Plummer, K.E., Bearhop, S., Leech, D.I., Chamberlain, D.E., Blount, J.D., 2013. Fat provisioning in winter impairs egg production during the following spring: a landscape-scale study of blue tits. J. Anim. Ecol. 82, 673–682. https://doi.org/10.1111/1365-2656.12025@10.1111/(ISSN)1365-2656.NUTRITIONALECOLOGY

Qu, R., Feng, M., Sun, P., Wang, Z., 2015. A comparative study on antioxidant status combined with integrated biomarker response in *Carassius auratus* fish exposed to nine phthalates. Environ. Toxicol. 30, 1125–1134. https://doi.org/10.1002/tox.21985

R Core Team, 2020. R: A language and environment for statistical computing.

Savoca, D., Arculeo, M., Barreca, S., Buscemi, S., Caracappa, S., Gentile, A., Persichetti, M.F., Pace, A., 2018. Chasing phthalates in tissues of marine turtles from the Mediterranean sea. Mar. Pollut. Bull. 127, 165–169. https://doi.org/10.1016/j.marpolbul.2017.11.069

Schettler, T., 2006. Human exposure to phthalates via consumer products. Int. J. Androl. 29, 134–139. https://doi.org/10.1111/j.1365-2605.2005.00567.x

Seckin, E., Fromme, H., Völkel, W., 2009. Determination of total and free mono-n-butyl phthalate in human urine samples after medication of a di-n-butyl phthalate containing capsule. Toxicol. Lett. 188, 33–37. https://doi.org/10.1016/j.toxlet.2009.03.002

Senko, J., Nelms, S., Reavis, J., Witherington, B., Godley, B., Wallace, B., 2020. Understanding individual and population-level effects of plastic pollution on marine megafauna. Endanger. Species Res. 43, 234–252. https://doi.org/10.3354/esr01064

Sies, H., 1993. Strategies of antioxidant defense. Eur. J. Biochem. 215, 213–219. https://doi.org/10.1111/j.1432-1033.1993.tb18025.x

Surai, P.F., Fisinin, V.I., Karadas, F., 2016. Antioxidant systems in chick embryo development. Part 1. Vitamin E, carotenoids and selenium. Anim. Nutr. 2, 1–11. https://doi.org/10.1016/j.aninu.2016.01.001

Tetz, L.M., Cheng, A.A., Korte, C.S., Giese, R.W., Wang, P., Harris, C., Meeker, J.D., Loch-Caruso, R., 2013. Mono-2-ethylhexyl phthalate induces oxidative stress responses in human placental cells in vitro. Toxicol. Appl. Pharmacol. 268, 47–54. https://doi.org/10.1016/j.taap.2013.01.020

Van'T Erve, T.J., Rosen, E.M., Barrett, E.S., Nguyen, R.H.N., Sathyanarayana, S., Milne, G.L., Calafat, A.M., Swan, S.H., Ferguson, K.K., 2019. Phthalates and phthalate alternatives have diverse associations with oxidative stress and inflammation in pregnant women. Environ. Sci. Technol. 53, 3258–3267. https://doi.org/10.1021/acs.est.8b05729

Verreault, J., Villa, R.A., Gabrielsen, G.W., Skaare, J.U., Letcher, R.J., 2006. Maternal transfer of organohalogen contaminants and metabolites to eggs of Arctic-breeding glaucous gulls. Environ. Pollut. 144, 1053–1060. https://doi.org/10.1016/j.envpol.2005.10.055

Vézina, F., Williams, T.D., 2002. Metabolic costs of egg production in the European starling (Sturnus vulgaris). Physiol. Biochem. Zool. 75, 377–385. https://doi.org/10.1086/343137

Wu, M., Xu, L., Teng, C., Xiao, X., Hu, W., Chen, J., Tu, W., 2019. Involvement of oxidative stress in di-2-ethylhexyl phthalate (DEHP)-induced apoptosis of mouse NE-4C neural stem cells. Neurotoxicology 70, 41–47. https://doi.org/10.1016/j.neuro.2018.10.013

Xu, Y., Gye, M.C., 2018. Developmental toxicity of dibutyl phthalate and citrate ester plasticizers in *Xenopus laevis* embryos. Chemosphere 204, 523–534. https://doi.org/10.1016/j.chemosphere.2018.04.077

Zheng, S., Wang, P., Sun, H., Matsiko, J., Hao, Y., Meng, D., Li, Y., Zhang, G., Zhang, Q., Jiang, G., 2018. Tissue distribution and maternal transfer of persistent organic pollutants in Kentish Plovers (*Charadrius alexandrines*) from Cangzhou Wetland, Bohai Bay, China. Sci. Total Environ. 612, 1105–1113. https://doi.org/10.1016/j.scitotenv.2017.08.323

Supporting Information

Quality assurance and quality control (QA/QC).

Blank samples and method detection limits (MDLs). Demineralised (MilliQ) water (5 ml) was employed as blank samples (n = 5 for phthalates and phthalate metabolites respectively) to determine the background level during sample preparation. A method detection limit (MDL) was calculated for each phthalate parent and metabolite compound, defined as the mean value in the blank sample +3 x standard deviations. When a compound was not detected in a blank sample, a value of half the instrument detection limits (IDLs) was used instead. Data for blank samples and MDLs are outlined in Table S1. Values below the MDL were removed prior to statistical analyses.

MDLs for phthalate parent compounds ranged from 2.0 – 290 ng g^{-1} and for phthalate metabolites this value ranged from 0.0094 – 100 ng g^{-1}. It should be noted that DEHP concentration in blank samples was relatively high (160 ng g^{-1}) and less consistent between these blank samples compared to other compounds, resulting in a relatively high standard deviation (43 ng g^{-1}) and thus a high MDL (290 ng g^{-1}).

Analytical precision and accuracy. Native-fortified samples were prepared by spiking known amounts of targeted compounds (phthalate parent compounds and phthalate metabolites) into pre-homogenised chicken eggs, with a resultant concentration of 9.7 ng g^{-1} for each target compound. Four of these fortified samples, together with two aliquots of the same sample prior to native fortification, were analysed together with the real samples to assess the analytical accuracy. The analytical precision was

also assessed using these samples, expressed as relative standard deviation (RSD) for the measured concentration from each native-fortified sample.

Three phthalate parent compounds (DiBP, DCHP, DnOP) and four phthalate metabolites (MEOHP, MiNP, MCHP, MnOP) passed the QA/QC criteria (i.e. accuracy between 0.80 and 1.2 and precision/variation <25%) (Table S1).

Three other parent compounds (BBzP, DnBP, DEHP) and one other metabolite (MnBP) were also included in the data analysis and discussion. We are aware that QA/QC results (Table S1) for these four compounds were outside of the range of typical criteria as stated above. However, the accuracy assessment for BBzP, DnBP and MnBP was off only by 25-50% from the upper boundary. We therefore considered that the benefit of including data for these compounds outweighed the risk associated with uncertainties of the data. Duplicates or triplicates for each individual sample were analysed and final results were shown as the mean of these replicates. For the high analytical variability for BBzP and DnBP, we hypothesise that the above approach may reduce the analytical uncertainties. The QA/QC assessment cannot be conducted for DEHP due to the relatively high level in the blank samples/background. However, the only sample we reported for DEHP had a concentration above the MDL, which was calculated based on the concentrations (and variations) in the blank samples. We therefore considered this data point acceptable. Overall, we suggest that caution should be applied when comparing our results to those of other studies.

Table S1. List of target compounds and QA/QC data

Compound	Abbreviation	Blank concentration (SD) (ng g^{-1})	MDL (ng g^{-1})	Accuracy	Precision	QA/QC passed?
Di-methyl phthalate	DMP	0.88 (0.38)	2.0	NC	NC	N
Di-ethyl phthalate	DEP	8.2 (1.0)	11	0.29	24%	N
Di-*n*-butyl phthalate	DnBP	19 (2)	25	1.5	62%	N
Di-iso-butyl phthalate	DiBP	17 (2)	23	1.1	21%	**Y**
Butylbenzyl phthalate	BBzP	2.2 (0.3)	3.3	1.5	83%	N
Di(2-ethylhexyl) phthalate	DEHP	160 (43)	290	NC	NC	N
Di-cyclohexyl phthalate	DCHP	ND	3.7	1.2	18%	**Y**
Di-*n*-octyl phthalate	DnOP	ND	3.7	1.1	6.0%	**Y**
Monoethyl phthalate	MEP	36 (20)	95	7.7	95%	N
Mono-*n*-butyl phthalate	MnBP	6.0 (0.4)	7.4	1.8	20%	N
Mono(3-carboxypropyl) phthalate	MCPP	0.050 (0.003)	0.060	0.12	98%	N
Mono(2-ethylhexyl) phthalate	MEHP	40 (16)	88	1.3	35%	N
Mono(2-ethyl-5-carboxypentyl) phthalate	MECPP	0.021 (0.005)	0.036	0.17	89%	N
Mono(2-ethyl-5-hydroxyhexyl) phthalate	MEHHP	0.11 (0.02)	0.18	0.76	38%	N
Mono(2-ethyl-5-oxohexyl) phthalate	MEOHP	0.090 (0.011)	0.12	0.93	23%	Y
Monobenzyl phthalate	MBzP	0.15 (0.02)	0.22	0.84	32%	N
Mono-iso-butyl phthalate	MiBP	12 (1)	14	2.8	29%	N
Mono-iso-nonyl phthalate	MiNP	0.0019*	0.0019	0.98	14%	Y
Monocyclohexyl phthalate	MCHP	0.0028*	0.0028	0.87	21%	Y
Mono-*n*-octyl phthalate	MnOP	0.0022*	0.0022	0.83	21%	Y
Mono-iso-decyl phthalate	MiDP	0.31 (0.17)	0.82	0.61	32%	N

ND: not detected; NC: not calculated due to poor quality of the data; * SD not calculable

Table S2. List of phthalate parent compounds and associated metabolites.

Parent Compound	Associated Metabolites
DMP*	MMP
DEP*	MEP*
DnBP*†	MnBP*†
DCPP	MCPP*
DEHP*	MEHP*
	MECPP*
	MEHHP*
	MEOHP*†
BBzP*†	MBzP*
DiBP*†	MiBP*
DiNP	MiNP*†
DCHP*†	MCHP*†
DnOP*†	MnOP*†
DiDP	MiDP*

*** Target compound, † Included in manuscript**