Master's thesis

NTNU
Norwegian University of Science and Technology
Faculty of Natural Sciences
Department of Biology

Wasique Zawad Prian

Analysis of Plastic Associated Chemicals in Cinereous Vultures (*Aegypius monachus*).

Master's thesis in Environmental Toxicology and Chemistry
Supervisor: Veerle Jaspers
Co-supervisor: Laura Monclús Anglada and Junjie Zhang
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Wasique Zawad Prian

Abstract

Plastic is an invention of science and technology that is closely associated with our modern lives. No matter which aspect we refer to, plastic has a significant influence making things easier, faster and cheaper. This has led to an immense amount of production of plastics. The production industries are continuously trying to advance their production techniques, increasing the amount while decreasing the cost. The management of plastic waster however has not increased compared to production and this leads to tons of plastic waste being stranded in the environment and it is increasing every year with increasing production. These plastic products in many cases contain various additives to increase its value as a product. These additives sometimes end up causing severe toxic effects to organisms after being exposed. Oxidative stress, endocrine disruption are some of the damage caused by these types of additives. These additives are known as plastic associated chemicals. Phthalates and bisphenols are two commonly used such chemicals and these leach into the environment becoming available to the wildlife as well as humans. These compounds also contaminate the organisms after direct ingestion of plastics and many cases have been reported of such behavior. Thus these plasticizers contaminate the food web and make their ways to the top of the food chain. There is still a lack of study regarding the contamination and damage to various organisms caused by these plasticizers. This includes birds which are generally used as a biomonitoring agent because of their high trophic positions and sensitivity to the environmental change.

This study is aimed at to investigate the presence of phthalate metabolites and bisphenols in cinereous vulture nestlings in Spain exposed to landfills. As the plastic contamination of these individuals were previously investigated, the aim of this study mainly focused on the contamination of the plasticizers and their correlation with each other and biological factors. The three biological factors investigated include, weight of the individuals, development days and days of incubations. Among the phthalate metabolites studied, four of them were observed to have concentrations above the LOQ (limit of quantification) levels. These four metabolites are mono methyl phthalate (mMP), mono ethyl phthalate (mEP), mono iso butyl phthalate (mIBP), and mono butyl phthalate (mBP) having mean values of 14.79 ± 4.65, 611.69 ± 191.73, 34.8 ± 16.78, and 37.27 ± 14.87 ng g^{-1} respectively. In case of bisphenols, bisphenol A (BPA), bisphenol S (BPS), and bisphenol Z (BPZ) were found to have the highest detection frequency (DF). While calculating correlation between the biological factors and chemicals, no significant correlations were observed. The chemicals themselves did not show any correlations between them as the Spearman's coefficient value ranged from -0.0242 to 0.2396. Overall, the presence of phthalate metabolites and bisphenols were observed but due to lack of studies conducted upon vultures or raptors as a whole, it became quite difficult to compare the levels of these two plastic associated chemicals and draw a definite conclusion of the state of contamination and potential risks of exposures in the vultures.

Sammendrag

Plast er en oppfinnelse av vitenskap og teknologi som er nært knyttet til våre moderne liv. Uansett hvilket aspekt vi referer til, har plast en betydelig innflytelse som gjør ting enklere, raskere og billigere. Dette har ført til en enorm mengde produksjon av plast. Produksjonsindustrien prøver kontinuerlig å fremme produksjonsteknikkene sine, øker mengden samtidig som kostnadene reduseres. Håndteringen av plastavfall har imidlertid ikke økt sammenlignet med produksjon, og dette fører til at tonnevis med plastavfall strander i miljøet, og det øker hvert år med økende produksjon. Disse plastproduktene inneholder i mange tilfeller ulike tilsetningsstoffer for å øke verdien som produkt. Disse tilsetningsstoffene ender noen ganger opp med å forårsake alvorlige toksiske effekter på organismer etter å ha blitt eksponert. Oksidativt stress, hormonforstyrrelser er noen av skadene forårsaket av denne typen tilsetningsstoffer. Disse tilsetningsstoffene er kjent som plasttilknyttede kjemikalier. Ftalater og bisfenoler er to ofte brukte slike kjemikalier, og disse lekker ut i miljøet og blir tilgjengelig for både dyrelivet og mennesker. Disse forbindelsene forurenser også organismene etter direkte inntak av plast, og mange tilfeller er rapportert om slik oppførsel. Dermed forurenser disse myknere næringsnettet og kommer seg til toppen av næringskjeden. Det er fortsatt mangel på studier angående forurensning og skade på ulike organismer forårsaket av disse myknere. Dette inkluderer fugler som vanligvis brukes som et bioovervåkingsmiddel på grunn av deres høye trofiske posisjoner og følsomhet for miljøendringene.

Denne studien er rettet mot å undersøke tilstedeværelsen av ftalatmetabolitter og bisfenoler i gribbeunger i Spania utsatt for deponier. Siden plastisk forurensning av disse individene tidligere ble undersøkt, fokuserte målet med denne studien hovedsakelig på forurensning av myknere og deres korrelasjon med hverandre og biologiske faktorer. De tre biologiske faktorene som er undersøkt inkluderer individenes vekt, utviklingsdager og inkubasjonsdager. Blant de studerte ftalatmetabolittene ble fire av dem observert å ha konsentrasjoner over LOQ-nivåene (grense for kvantifisering). Disse fire metabolittene er monometylftalat (mMP), monoetylftalat (mEP), monoisobutylftalat (mIBP) og monobutylftalat (mBP) med gjennomsnittsverdier på 14,79 ± 4,65, 611,69 ± 191,73, 34,78 og 34,78 ± 37,27 ± 14,87 ng g-1 henholdsvis. Når det gjelder bisfenoler, ble bisfenol A (BPA), bisfenol S (BPS) og bisfenol Z (BPZ) funnet å ha den høyeste deteksjonsfrekvensen (DF). Ved beregning av korrelasjon mellom de biologiske faktorene og kjemikaliene ble det ikke observert noen signifikante korrelasjoner. Kjemikaliene i seg selv viste ingen korrelasjoner mellom dem da Spearmans koeffisientverdi varierte fra -0,0242 til 0,2396. Totalt sett ble tilstedeværelsen av ftalatmetabolitter og bisfenoler observert, men på grunn av mangel på studier utført på gribber eller rovfugler som helhet, ble det ganske vanskelig å sammenligne nivåene av disse to plasttilknyttede kjemikaliene og trekke en sikker konklusjon om forurensningstilstanden og potensiell risiko for eksponering i gribbene.

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List of abbreviations

mMP……Mono methyl phthalate

mEP……Mono ethyl phthalate

mIBP……..Mono iso butyl phthalate

mBP……Mono butyl phthalate

mPeP……Mono-n-pentyl phthalate

mIPeP……Mono iso pentyl phthalate

mHxP……Mono-n-hexyl phthalate

mCHP……..Mono cyclo hexyl phthalate

mHpP……Mono-n-heptyl phthalate

mBzP……..Mono benzyl phthalate

mOP……..Mono-n-octyl phthalate

mEHP……Mono (2-ethyl-1-hexyl) phthalate

mEOHP……Mono (2-ethyl-5-oxy hexyl) phthalate

mEHHP……Mono (2-ethyl-5-hydroxy hexyl) phthalate

mDP……..Mono-n-decyl phthalate

mNP……Mono-n-nonyl phthalate

DEHP……Di (-ethyl hexyl) phthalate

mCPP……Mono (3-carboxy propyl) phthalate

BPA……..Bisphenol A

BPS……..Bisphenol S

BPZ……..Bisphenol Z

BPB……..Bisphenol B

BPAF……Bisphenol AF

BPAP……..Bisphenol AP

BPP……Bisphenol P

BPM……Bisphenol M

DF……Detection frequency

HPA-axis……Hypothalamic-pituitary-adrenal-axis

IS……Internal standard

LC-MS/MS……Liquid chromatographic mass spectrometry

UPLC……Ultra performance liquid chromatography

LOQ……Limit of quantification

NTNU……Norwegian University of Science and Technology

RB……Reagent Blank

SD……Standard deviation

SP……Spiked sample

TA……Target analyte

PCA……Principle component analysis

Introduction

Modern science and technology have provided us with inventions which have made our lives smoother and easier. Plastic is such an invention with various positive aspects for example, non-reactivity, lightweight, easier and cheaper production, heat and electricity resistance, and so on. These have made plastics a crucial ingredient in our daily lives such as water bottles to more sophisticated uses such as medicinal or research purposes. These necessities and availabilities have made plastics one of if not the biggest struggles against sustainability. Its non-reactivity, for example, has made it so difficult to dispose of resulting in a massive 8.3 billion tons of production reported by Harald Franzen (2017). The report also mentions that among those 8.3 billion tons, 6.3 billion tons were waste products and approximately 79 percent of it reached landfills and the environment and only 9 percent was recycled. These plastics come with different additives for better flexibility, utilization, and apperance. But along with plastics, these additives sometimes result in various toxic effects on humans and wildlife (Verma et al., 2016). This study will focus on two such additives, phthalates and bisphenols, and their metabolites.

1.1. Phthalates

Phthalates are esters of benzene-1.2- dicarboxylic acid, also known as phthalic acid, and in their structure, contain two functional ester groups associated with the benzene ring (figure 1). With the increasing length of the carbon chain, the solubility of phthalate in water decreases (Przybylińska & Wyszkowski, 2016). Some of their characteristics include having high boiling temperatures, solubility in organic solvents, and oily texture.

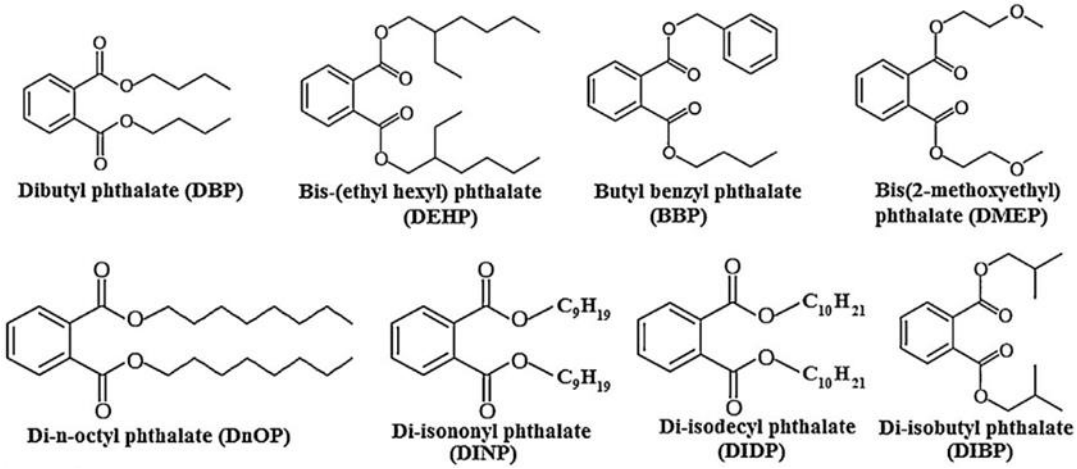


Figure 1: Different metabolites of phthalates (Natsheh et al., 2015).

In general, phthalates are classified into two categories: low molecular-weight phthalates like di-n-butyl phthalate, and high molecular weight phthalate, such as diisononyl phthalate (Ventrice et al., 2013).

The first commercial utilization of phthalate started in 1931 after their introduction in 1921. Their uses have been increasing ever since then due to their ability to make polyvinyl chloride (PVC) products more flexible and durable (Peijnenburg et al., 2008). Phthalates are also used in the manufacture of cosmetics, printing ink, paper, and packaging industries, and Benjamin et al. (2017) estimated the global value of phthalates to reach approximately 10 billion USD by the year 2020.

1.1.1. **Exposure:** Due to their weak bonding to the substrates, phthalates contaminate the environment through leaching, migration, and oxidation during the use and storage of products. These leachates from daily use contaminate the water and reach wastewater treatment plants and have been reported to remain even after the treatment process (Clara et al., 2010). Przybylińska & Wyszkowski (2016) reported soil to be contaminated by phthalates through the application of organic fertilizers, oil leakage from farm machineries, and wet deposition from atmospheric air. Additionally, phthalates reach crop plants (Zorníková et al., 2011) from the soil through nutrients by roots and contaminate the food web.

1.1.2. **Effects:** Phthalates have been reported to impair reproductive organs in animals. Lyche (2009) reported that phthalates decrease the production of estradiol and Ventrice et al. (2013) reported lower testicular weight, reduced sperm production, and atrophy of seminiferous tubules in male rodents. Scientists have reported damage to Leydig and Sertoli cells of Male Sprague-Dawley rats by DEHP (di (2-ethylhexyl) phthalate) and DBP (dibutyl phthalate) (Sun et al., 2018; Wang et al., 2017). Such damages to Leydig and Sertoli cells lead to decreased weight, spermatogenesis impairment, and external genital malformations in humans (Schiffer et al., 2014). Studies done using frog (*Xenopus*) embryos have shown developmental alterations caused by dibutyl phthalate (DBP) (Lee et al., 2005). Whereas, in fish phthalates have been reported to cause both estrogenic and non-estrogenic symptoms (Jobling et al., 1995; Kim et al., 2002). Additionally, their similarity in chemical structure to steroid hormones cause interference and lead to imbalance and impairing effects (Asai et al., 2000). Phthalates have also been reported by Dvořáková et al. (2018) to have anti-androgenic and anti-estrogenic effects (DEHP), meaning they can bind to nuclear receptors and block the effects of estrogens and androgens.

1.2. Bisphenols

Bisphenols are related to diphenylmethane and contain two hydroxyphenyl functional groups linked by a methylene bridge (figure 2). Among the variations of bisphenols, bisphenol A was the most popular representative (Fiege et al., 2000). BPA (bisphenol A) is a colorless, phenolic, synthetic organic compound that is soluble in most common organic solvents but has very poor solubility in water (Fiege et al., 2000; Ohore & Zhang 2019;

Shareef et al., 2006). It dissolves in a broad range of organic solvents including toluene, ethanol, and ethyl acetate (Haynes et al., 2017).

1.2.1. **Exposure:** The sources of bisphenol contamination in the environment are through wastewater treatment plants, landfills, domestic waste, and natural breakdown processes during chemical manufacturing, transporting and processing and during post-consumer release (Oehlmann et al., 2009 and Kang et al., 2007). Bisphenols are also found in marine and surface water due to them being moderately soluble in water. The concentrations are higher in marine systems as it works as a sink of plastic waste (Crain et al., 2007). As mentioned earlier BPA is the most used bisphenols among others but due to its toxic effects on wildlife (Zoeller et al., 2012, Oehlmann et al., 2009), its use is restricted in the recent years in Europe (EFSA, 2023) and being replaced by other metabolites of bisphenols, for example BPS and BPF. But these compounds have similar chemical structure and a potency to cause similar toxic effects on organisms (Thoene et al., 2020; Ullah et al., 2019).

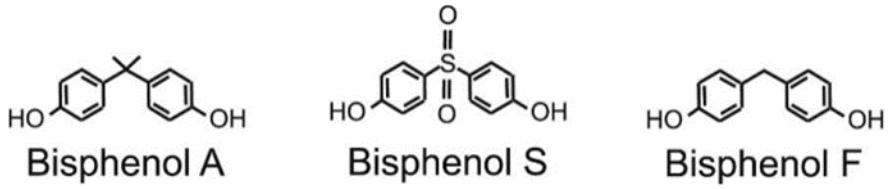


Figure 2: Chemical structures of Bisphenol A, S and F (Eladak et al., 2015)

1.2.2. **Effects:** Bisphenols cause endocrine disruption by acting as both agonist and antagonist of receptors. BPA especially has been reported to block the thyroid-induced metamorphosis of Xenopus at larval stages (Iwamuro et al., 2006) as it has a similar structure to thyroid hormones. Goto et al. (2006) conducted an experiment involving *Rana rugose* (Japanese wrinkled frog) tadpoles where the tails of the species had apoptotic features by triiodothyronine with a simultaneous presence of BPA. BPA has also been reported to suppress the release of TSH (Thyroid stimulating hormones) and prolactin from the pituitary gland. But this antagonism is more effective in suppressing the TR (Thyroid receptor)-mediated transcription, rather than on the receptor level in mammals (Mathieu-Denoncourt et al., 2014).

BPA was reported to act as estrogen agonist in fish (Gibert et al., 2011) and frogs (Suzuki et al., 2004) by binding to the ER (estrogen receptors). Ekman et al. (2012) showed androgen antagonistic effects of BPA on hepatic metabolome in female fathead minnows. BPA has also been reported to be responsible for activating PPARγ induced lipid accumulation in zebrafish (*Danio rerio*) (Riu et al., 2014), and increased superoxide activity leading to oxidative stress in sterlet (*Acipenser ruthenus*) (Hulak et al., 2013). Bisphenol S (BPS) have been reported to cause deteriorating effects on rates of egg hatching in

zebrafish (*Danio rerio*) as well as cause developmental toxicity (Kyunghee et al., 2013) whereas, Bisphenol F (BPF) has been reported to cause oxidative stress and protein imbalance in Sprague-dawley rats (*Rattus norvegicus*) by Ullah et al. (2019). These BPs are not as extensively studied as BPA but they in some cases show similar detrimental effects on wildlife.

1.3. Birds

1.3.1. **Birds as bioindicating organisms:** "Bioindicators are species used to appraise the health conditions of the environment or ecosystem and they are usually capable of determining the environmental integrity using their functions and populations" (Egwumah et al., 2017). They also help to understand complex relationships various compounds have among themselves and how they might affect the wildlife not only as a singular entity but as the whole community and ecosystem. Additionally, birds are good sentinel species because they are observable, sensitive to contaminants, and spread across different trophic positions (Ferreira, 2011). Thus birds are considered to be good bioindicator as they represent their concerning habitat and the interactions between metabolic pathways and the compound is relatively easy to understand. Also, from the methodological point of view, collecting bird samples (for example, preen oil, feathers) are non-destructive.

1.3.2. **Plastic ingestion by birds:** Our uses in daily life and lack of a proper management system, plastic waste has reached a massive value of 400 million tons annually (UNEP, 2022), and 75-199 million tons are found in the ocean. Figure 3 shows how plastics flow through various environmental elements and ends up in food web. Through breakdown, these plastics reach macro and micro sizes and are wrongfully taken up by marine preying animals like fish, shellfish, and so on (Waring et al., 2018).

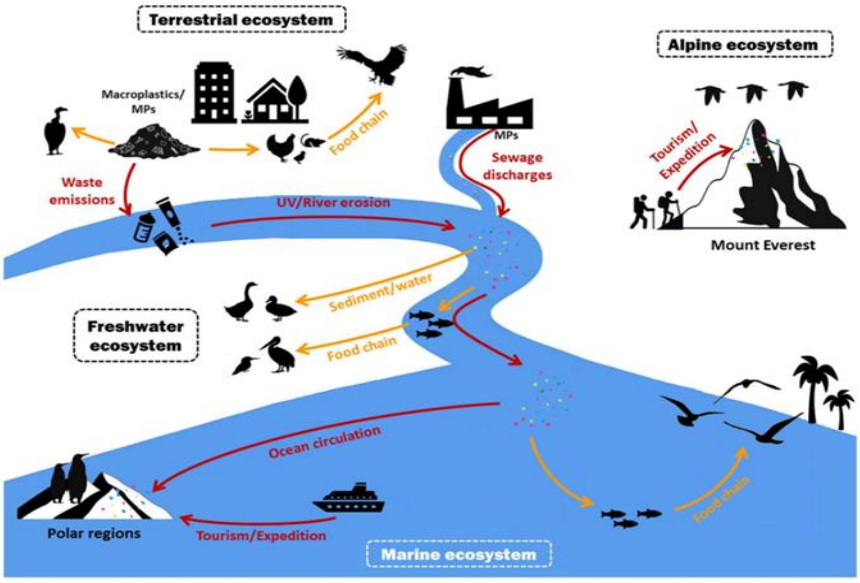


Figure 3: The cycling process of macro and microplastics in different ecosystems (red arrow) and potential uptake ways by birds from different ecological groups (orange arrow) (Wang et al., 2021).

Marine birds, through these animals get caught up in the plastic pollution. The terrestrial birds are also not secured from the pollution. Houston et al. (2007) reported ingestion of plastics by California Condor (*Gymongyps californianus)*, and was linked to be one of the major causes of death in nestlings (Rideout et al., 2012). Another study showed that microplastics were significantly more abundant in the digestive tract tissues of Red Shrouded Hawk (*Buteo haliaetus*), which lives on small mammals, snakes and amphibians, when compared to fish feeding Osprey (*Pandion haliaetus*) (Carlin et al., 2020).

1.3.3. **Effects of phthalates and bisphenols on birds:** Birds are susceptible to phthalates and bisphenols by both direct ingestion of plastics and indirect exposure through the food chain. Species that birds usually feed on, for instance, crabs, oysters, and shrimps have been detected to be contaminated by phthalates (Munshi et al., 2013). Phthalates being a lipophilic compound, helps it to circulate through the food chain (Allen et al., 2021). Additionally, raptors are highly vulnerable to phthalates due to their bioaccumulation and biomagnification properties and their positions in the food web. Huber et al. (2015) even reported the transmission of phthalates to eggs indicating its generational impact. Its metabolites are capable of causing oxidative stress to birds through the production of reactive oxygen species (ROS). Allen et al. (2021) reported a positive correlation between egg DCHP (dicyclohexyl phthalate) levels and yolk MDA (malondialdehyde) levels, linking to maternal oxidative stress in European herring gulls (*Larus argentatus*). Phthalates have also been reported to cause neurotoxicity by altering brain NXR (nuclear xenobiotic receptor) leading to damaging the CYP (cytochrome p450) enzyme systems in quails (Du et al., 2017).

Bisphenols have been reported to cause the feminization of male birds (Berg et al., 2001) due to their estrogen-like behavior. On a deeper analysis, bisphenols have been found to cause more damage on the histological level than on gross morphological level (Jessl et al., 2018). Mentor et al. (2020) concluded that bisphenol metabolites, BPAF, and BPF induce similar effects on the chicken embryo as other estrogenic compounds like DES (diethylstilbestrol), EE_2 (ethinylestradiol), 17β-estradiol and genistein.

1.3.4. **Vulture as bioindicating organism for plastics:** Vultures are birds of prey feeding on carcasses and their 'clean up' duties have earned the nickname of 'nature's garbage collector' and thus signify their role in the ecosystem (Duke et al., 2021). These birds of prey usually show a wide range foraging behaviour and along with their position in food web and extended abundance (Ogada et al., 2011), hold significant potential as a terrestrial biomonitoring agent. Previously vulture was used as for investigating bisphenols and benzophenones and their relationship with stress hormone corticosterone (CORT) (Nilsen, 2021)

1.4. Aims and objectives:

This study aims to quantify the concentrations of phthalate metabolites in chicks of Cinereous vulture (*Aegypius monachus)* exposed to plastics in their environment. To study this, blood samples were collected from the chicks and measured for phthalate metabolite concentrations. This study is a follow-up of a previous study that quantified the plastic exposure in the vulture nestlings as well as analyzed blood samples for bisphenols (Nilsen, 2022). I will combine these previous data with my own results to get an overall picture of the exposure to vultures to plastics associated chemicals.

The main objectives of this study are:

1. Determine concentrations of different phthalate metabolites in blood from cinereous vulture (*Aegypius monachus*) chicks
2. Compare concentrations of phthalate metabolites with previous data on bisphenols in the vulture

The main hypothesis of this study is that there will be a positive correlation between the concentrations of phthalate metabolites and the biological factors.

2. Methods and materials

2.1. The study species and sampling area

The cinereous vulture (*Aegypius monachus*) belongs to the family of Accipitridae (del Hoyo, 1994) and spread across Eurasia (Fergusson-Lees and Christie, 2001). Its population is distributed among five countries of Europe; namely, Spain, Portugal, France, Greece, and Ukraine, and is estimated to be around 2,536 – 2,838 breeding pairs (Andevski et al., 2017). These large birds of prey mainly feed on carcasses of for example rabbits, livestock and so on (Donázar, 1993). The population used for this study is located in Sierra Guadarrama, Madrid, Spain. These birds are exposed to plastic materials through landfill wastes around their habitat. Some adult birds that have been tracked are often observed in such landfill sites and may have been exposed while eating the organic matter (OM) from the plastic bags or other trash adhering to OM. The food is then brought back to their nests and ingested by the nestlings. The cinereous vultures have one chick per nest (breeding season: from May to September).

Figure 4: Cinereous vulture chick on forest ground with banding on foot. Picture taken by Javier de da Puente 2020.

2.2. Collection of samples: In this project, 91 nests of cinereous vultures in Sierra Guadarrama (Madrid, Spain) were analyzed. All samples were collected within the framework of monitoring program of a breeding colony of the Cinereous vultures. When the chicks were between 30 – 80 days, they were picked from the nest for sampling, and banding. Incubation days (days from egg laying to hatchling) and days of development (days from hatching to fledgling) together with

weight (g) at sampling were collected. Blood samples (n= 46) were also collected from chicks of the same population in Spain during the summer of 2020. Biological data (weight at sampling, days of incubation and developing days) were also recorded. After collection the samples were shipped to Norway for analyzing at NTNU. The same samples were previously utilized for master's thesis project at NTNU (Nilsen, 2022).

2.3. Extraction procedure for phthalates metabolites

Whole blood taken from the chicks of cinereous vulture (n= 46) in each nest was used in this experiment. As mentioned before, the samples collected from the chicks were previously used for master's thesis project, resulting in a fewer number of samples being available for current study than originally collected. Extraction protocols of phthalate metabolites in blood was carried out according to the procedures described by Gonzalez-Rubio et al. 2020 and afterwards analyzed by mass spectrometry at the Chemistry Department of NTNU.

The extraction procedure was conducted over three days. On day 1, blood samples were taken out of the freezer and thawed on ice until liquid. The samples were then vortexed for homogenizing. After thawing, approximately 0.1 g (≈100 µL) of samples were taken out in a well-marked 15 mL polypropylene (pp) tube. 1 mL of 1.0 M ammonium acetate aqueous buffer and 10 µL of 500 µg/L internal standards were added to each sample. The mix consisted of isotope of mono ethyl phthalate (mEP d4), mono butyl phthalate (mBP d4), and mono nonyl phthalate (mNP d4). The samples were then put in an ultrasonic bath for 45 minutes. After ultra-sonication another 1 mL of 1 M ammonium acetate (aqueous buffer) containing 44 units of *β-glucoronidase* (prepared by spiking 100 µL of *β-glucoronidase* into 100 mL of 1.0 M ammonium acetate solution was added. Then the samples were left for incubation (Innova 44/44R) overnight at 37°C at 220 rpm.

On the second day, after approximately 20 hours of incubation, liquid-liquid extraction was performed by the addition of 4 mL of ethyl acetate to the incubated samples and put in ultrasonic bath for 60 minutes after having vortexed the mixture. These were then centrifuged (Eppendorf centrifuge 5810) at 3500 rpm for 5 minutes. The supernatant were then extracted into a new 15 mL tube and the extraction process was repeated 3 times which resulted in a total volume of 11-12 mL of supernatant. All the 3 batches of supernatant were pooled together and 2 mL of milli-Q-water was added. The samples were then centrifuged at 3500 rpm for 10 minutes and transferred at well-marked PP tubes and stored at -20°C.

When ready for evaporation, samples were taken out of the -20°C freezer and left in room temperature for about 30 minutes for thawing. A nitrogen (N_2(g)) evaporator was used with cleaned needles, and a 37°C on the heat block (needles were previously cleaned using acetone, methanol and treated with high temperature of 200°C for two hours). Tubes were placed under each needle, and a gentle stream of nitrogen (5-10 psi) was switched on. Samples were carefully checked individually until only 50 µL of liquid was left in the PP tube. Samples were then removed and 450 µL methanol was added to each tube. This was followed by vortexing, and 0.5 mL of the

sample was added into a marked brown 2 mL glass vial. All samples were stored in -20°C before mass spectrometry reading.

For quality assurance and control, procedural blanks which followed the whole procedure were analyzed to control for background and preparation contamination. Three spiked samples (SP), two matrix match (MM), two blanks made using blood from 4-5 random blood samples, and one reagent blank (RB). The same protocol as with other samples was followed, but target analytes (TA) and IS were added according to Table-1. The TA´s included standards of Mono carboxy propyl phthalate, mono methyl phthalate, mono ethyl phthalate, mono isobutyl phthalate, mono butyl phthalate, mono-n-pentyl phthalate, mono isopentyl phthalate, mono cyclohexyl phthalate, mono-n-hexyl phthalate, mono benzyl phthalate, mono-n-heptyl phthalate, mono-n-octyl phthalate, mono-2-ethyl-1-hexyl phthalate, mono-2-ethyl-5-oxy hexyl phthalate, mono nonyl phthalate, mono-2-ethyl-5-hydroxyhexyl phthalate, mono-n-decyl phthalate, and mono-3-carboxy propyl phthalate, purchased from Sigma-Aldrich (St.Louis MO, USA).

Table 1: Making of quality controls with target analytes (TA) and internal standards (IS). Reagent blank (RB) without any sample, blanks (B) without TA, and spiked samples (SP) with IS and TA added before evaporation. Matrix match (MM) with TA and IS after evaporation.

	Sample	**TA (addition pre-extraction)**	**IS (addition pre-extraction)**	**TA (addition post-extraction)**	**IS (addition post-extraction)**
Reagent blank	No		X		
Blank 1	Yes		X		
Blank 2	Yes		X		
Spike 1	Yes	X	X		
Spike 2	Yes	X	X		
Spike 3	Yes	X	X		
Matrix matched 1	Yes			X	X
Matrix matched 2	Yes			X	X

2.4. Liquid Chromatographic Mass spectrometry (LC-MS/MS)

Equipment and training were provided by Junjie Zhang at the Department of Chemistry, at NTNU. To carry out the liquid chromatographic mass spectrometry (LC-MS/MS) and Acquity (Ultra Performance Liquid Chromatography) UPLC I-Class system (Waters, Milford, U.S.), together with a triple quadruple mass analyzer (QqQ; Xevo TQ-S) and a ZSpray ESI ion Source (Waters, milford, U.S.) was used. The column temperature was set to 30°C and the column used in the LC was a Kinetex C18 (50 x 2.1 mm, 1.3 µm) connected to a Phenomenex C18 guard column (2.0 x 2.1 mm). An injection volume of 4 µL and a mobile phase containing of solvents acetic acid (A) and

methanol (B) were used for the LC, while nitrogen (gas N_2) was used as the gas in the collision cell.

2.5. Data analysis

The LC-MS/MS data obtained using the MassLynx and TargetLynx software packages (version 4.1, Waters Corporation, Milford MA, USA). Data was processed in a spreadsheet (Excel, 2016).

A calibration curve containing 12 points was made with concentrations ranging from 0.01 to 50 ng mL^{-1} (0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1, 2, 5, 10, 20, 50 ng mL^{-1}). To quantify the target analytes the internal standard method with relevant matrix-matched calibration standards was used (Asimakopoulos et al., 2016).

The determination of LOQ was based on the equations described by Shrivastava and Gupta (2011) from the standard curve. The equation is given below:

LOQ = (10 X standard deviation)/slope

Both the slope and standard deviation is derived from the standard sample calculation.

2.6. Analyzing Procedure for Bisphenols

The data for bisphenols were collected from a previous study conducted by Sarah Nilsen, in 2022. The protocol was followed according to the description in the paper by Gonzalez-Rubio et al. (2020).

2.7. Statistical analysis

The statistical analysis of the data sampled was performed using Microsoft Excel. Mean estimates, standard deviations (SD), median and range for each parameter measured was calculated. A Shapiro-Wilk test was completed prior to analyses to check for normality ($p<0.05$). Only the phthalate metabolites and BPs with a DF above 60% were used in the statistical analysis. A Spearman's correlation test along with T test was also provided for all variables mMP, mEP, mBP, mIBP, BPS, BPA, BPZ and sum of BPs (∑BPs), developmental days, and incubation days, weight to evaluate correlation of data. A PCA was conducted using python module (version 3.9 13150.1013) to test the correlation among the phthalate metabolites and BPs.

3. Results

In this study, blood samples were collected from vulture chicks at Sierra Guadarrama Madrid, Spain. The blood samples were analyzed (total 42 samples) for 18 phthalate and 8 bisphenol metabolites.

3.1. Phthalate metabolite concentrations in blood samples:

With the exception of mono cyclo-hexyl phthalate, all other metabolites were quantified in blood samples of vulture chicks. Among them, twelve of the metabolites had concentrations below LOQ and two of the metabolites had only one sample above the LOQ level (see table 2). Among the eighteen phthalate metabolites analyzed, only four of them had more than one concentrations above the LOQ value.

Mono butyl phthalate (mBP), and its isomer mono iso butyl phthalate (mIBP) had above 60% detection frequency (97.56 and 90.24% respectively). The average sum concentrations of these four metabolites is presented in figure 1 (also in appendix). The average highest concentrations were observed in case of mEP (mono ethyl phthalate) but had a lower detection frequency than the mBP and mIBP. Among the metabolites, mono butyl phthalates had the highest detection frequency and the highest concentration was detected for mono ethyl phthalate but was only detected in four of the samples.

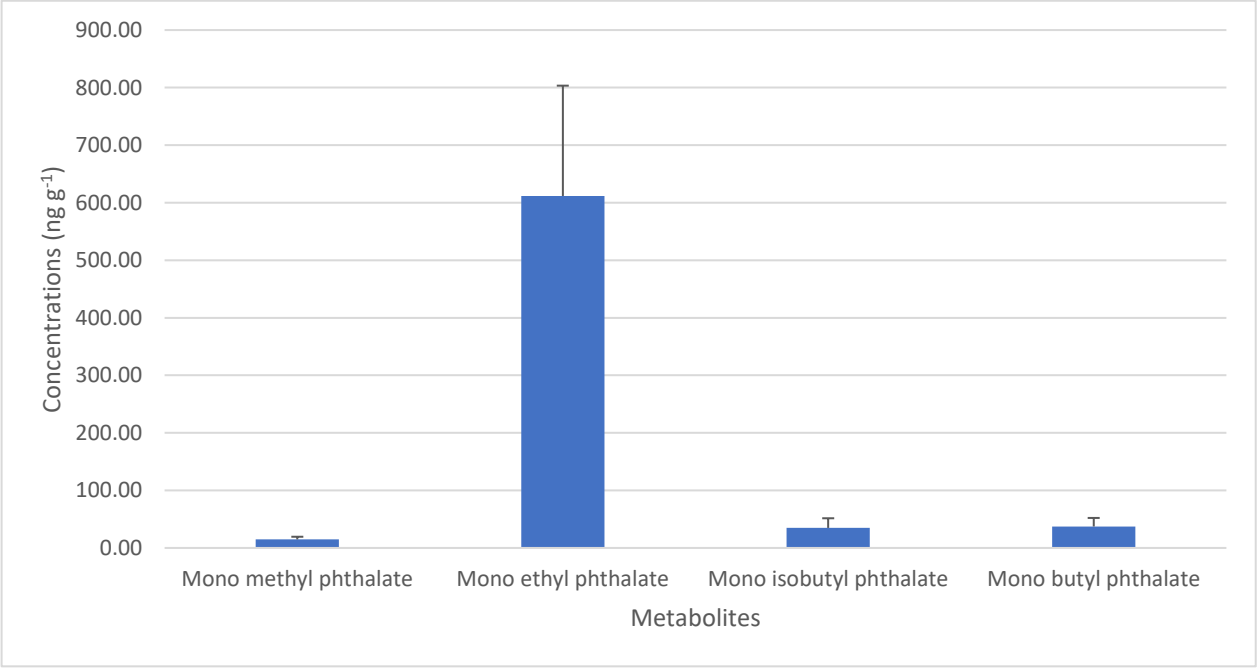


Figure 5: Mean concentrations of the phthalate metabolites detected above LOQ levels in more than one sample (total blood samples 41). The error bars illustrate the standard deviation.

Figure 5 shows the mean concentrations of mono methyl, mono ethyl, mono iso butyl and mono butyl phthalates above the LOQ levels. Most of the high density phthalates were detected at a concentrations lower than the LOQ levels. Figure 6 represents the concentrations found in individual samples without regarding the LOQ concentration.

Table 2: Overview of phthalate metabolites analyzed in blood samples of vulture chicks, including the common name of the metabolites, the limit of quantification (LOQ), detection frequency (DF, %), number (n) of individuals with concentrations detected above the LOQ, the mean ng g^{-1}, standard deviation (SD, ng g^{-1}), median (ng g^{-1}), range (min and max ng g^{-1}) detected for each metabolite.

Common name	LOQ	DF	n	Mean	Median	Standard deviation	Min	Max
Mono methyl phthalate	1.91	9.76	4	14.79	14.9	4.65	8.84	20.53
Mono ethyl phthalate	3.37	9.76	4	611.69	638.235	191.73	344.4	825.88
Mono iso butyl phthalate	10.24	90.24	37	34.8	32.24	16.78	14.21	74.49
Mono butyl phthalate	12.6	97.56	40	37.27	36.1	14.87	17.06	72.43
Mono-n-pentyl phthalate	43.09	-	-	-	-	-	-	-
Mono iso pentyl phthalate	27.16	-	-	-	-	-	-	-
Mono-n-hexyl phthalate	35.52	2.44	1	66.2	66.2	-	66.2	66.2
Mono cyclo hexyl phthalate	28.7	-	-	-	-	-	-	-
Mono benzyl phthalate	28.44	-	-	-	-	-	-	-
Mono-n-heptyl phthalate	30.01	-	-	-	-	-	-	-
Mono (2-ethyl-1-hexyl) phthalate	35.21	-	-	-	-	-	-	-
Mono-n-octyl phthalate	25.14	-	-	-	-	-	-	-
Mono (2-ethyl-5-oxyhexyl) phthalate	12.83	-	-	-	-	-	-	-
Mono nonyl phthalate	8.62	-	-	-	-	-	-	-
Mono (2-ethyl-5-hydroxy hexyl) phthalate	6.4	2.44	1	7.77	7.77	-	1.21	1.21
DEHP	119.29	-	-	-	-	-	-	-
Mono (3-carboxy propyl) phthalate	717.49	-	-	-	-	-	-	-

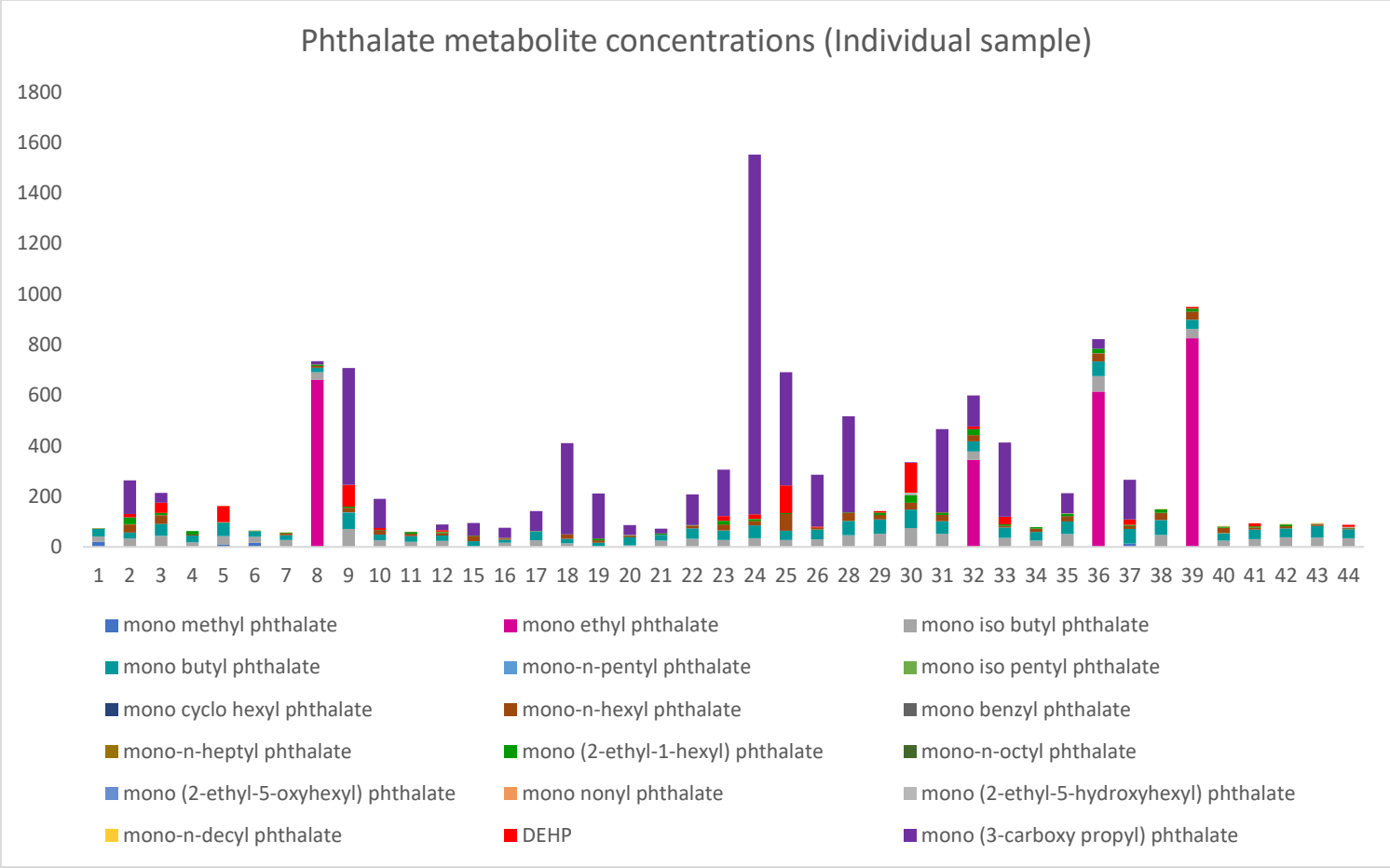


Figure 6: Phthalate metabolite concentrations found in individual samples (without taking into account the LOQ levels.

Most individual samples showed a lower concentrations of metabolites. Sample 8, 9, 24, 25, 32, 36, and 39 had higher levels of metabolites showing a high level of mono ethyl, mono (3-carboxy propyl), and DEHP concentrations.

3.2. Bisphenol concentrations in blood sample:

A table summarizing concentrations of all measures of BPs (bisphenols) can be found in the appendix.

The data of the BPs was collected from the thesis of Sarah Nilsen which was conducted in the year 2022. The thesis included benzophenones as well but only the data of BPs are used in this research. A summary of the calculated LOQ, along with other parameters for example, detection frequency, mean, median and so on is given in table 3. In the previous thesis, results from 46 individual samples were included, whereas in this one, 40 samples similar to both of the research for BPs are included.

Table 3: Overview of bisphenols analyzed in blood samples of vulture chicks, including the common name of the metabolites, the limit of quantification (LOQ), detection frequency (DF, %), number (n) of individuals with concentrations detected above the LOQ, the mean ng g^{-1}, standard deviation (SD, ng g^{-1}), median (ng g^{-1}), range (min and max ng g^{-1}) detected for each compound.

Common name	LOQ	DF	n	Mean	Median	Standard deviation	Min	Max
Bisphenol A	0.33	87.5	35	40.56	38.08	25.32	0.34	125.08
Bisphenol B	0.67	0	0	-	-	-	-	-
Bisphenol S	0.56	25	10	2.77	0.86	3.69	0.59	10.02
Bisphenol Z	3.40	10	4	5.10	5.10	2.68	3.57	9.11
Bisphenol AP	3.36	0	0	-	-	-	-	-
Bisphenol AF	0.91	0	0	-	-	-	-	-
Bisphenol M	0.92	0	0	-	-	-	-	-
Bisphenol P	0.87	0	0	-	-	-	-	-

Out of all the bisphenols (n=8), only three BPs (Bisphenol A or BPA, Bisphenol S or BPS, Bisphenol Z or BPZ) were quantified above the LOQ (table 2). Additionally, in this research only BPA was found to have a higher detection frequency than 60% unlike the previous one where it was the case for both BPA and BPS. The highest BPA concentrations was found to be 125.08 ng g^{-1}, with a mean value of 40.56 ± 25.32 ng g^{-1}.

Figure 7 shows mean concentrations of three bisphenols (BPA, BPS and BPZ) found to have individuals having a higher value than LOQ.

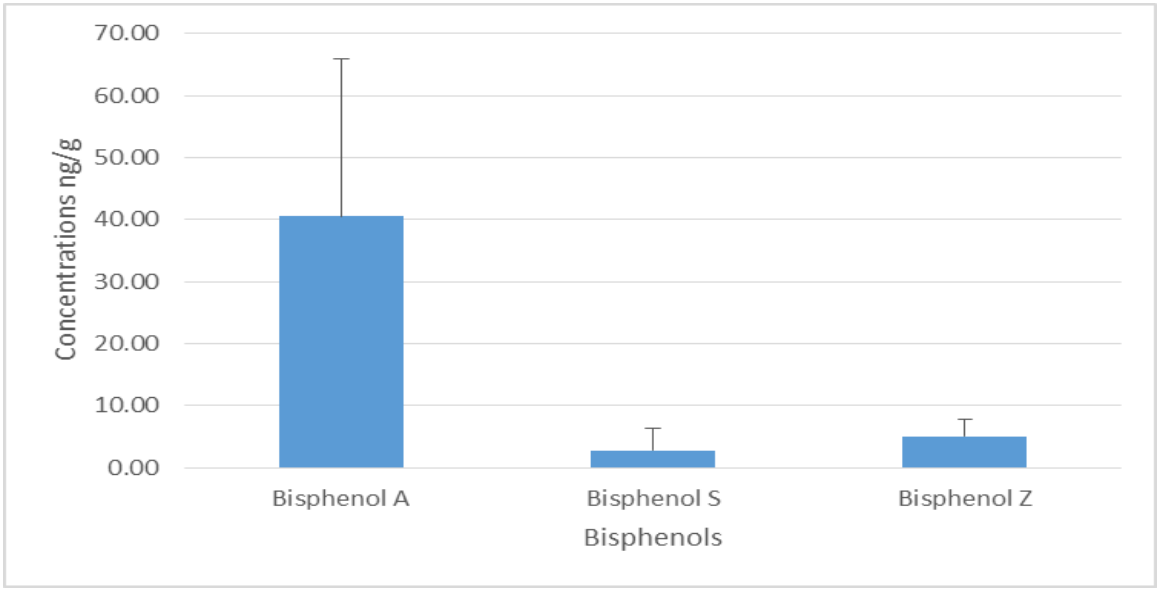


Figure 7: Mean concentrations of the bisphenols detected above LOQ levels in more than one sample (total blood samples 40). The error bars illustrate the standard deviation.

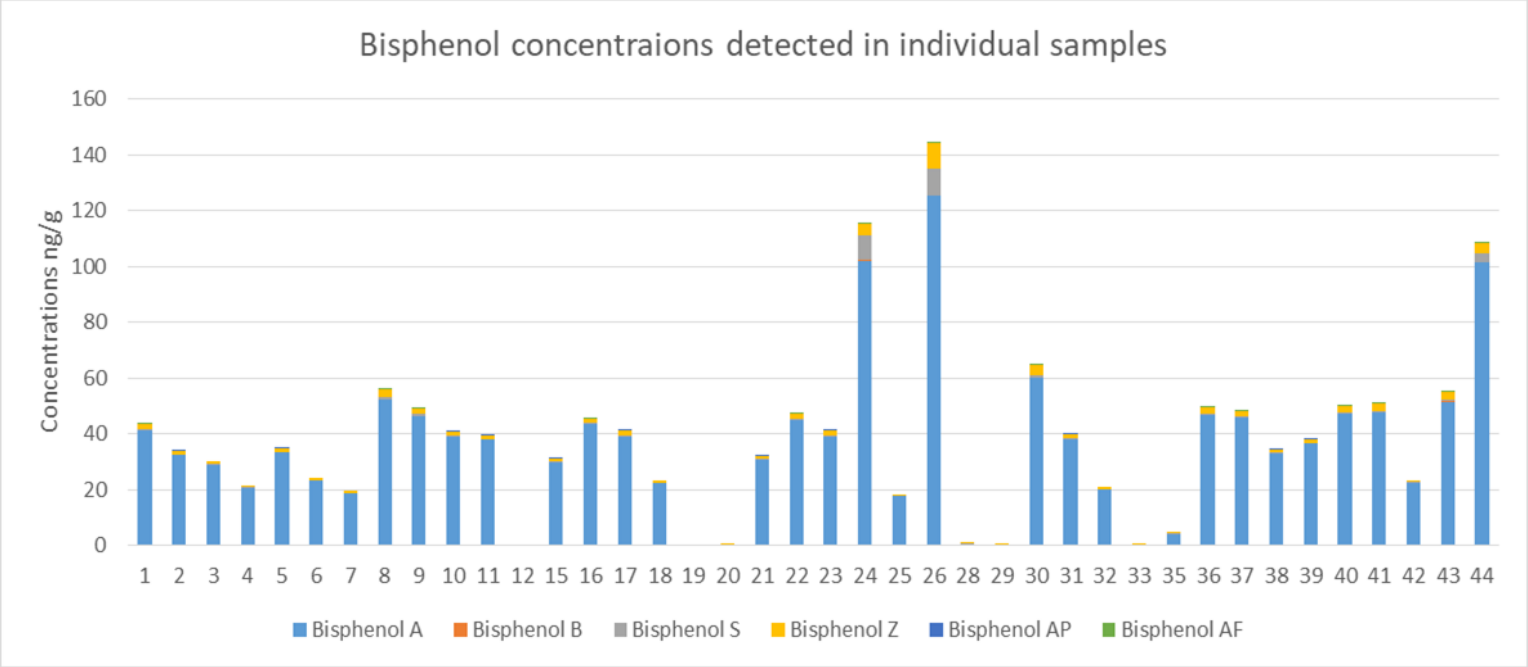


Figure 8: Bisphenol concentrations found in individual samples without regarding LOQ levels.

The total BP concentrations found in the sample is illustrated in figure 8. In this figure all concentrations and analogues wee included (without taking into account the LOQ). The maximum total concentrations for BPs was observed in sample 26 and the lowest was observed for sample 19 (only contains BPS). In appendix sections, the values of the concentrations are presented in table A5.

3.3 Correlation measured between bisphenols and phthalate metabolites and biological factors

Correlations study between biological factors and 4 phthalate metabolites was conducted through Spearman's coefficient analysis along with T-value and p-value. The result is represented in table 4. Table 5 illustrates correlation between phthalate metabolites and BPs.

Table 4: Spearman correlation coefficients between different metabolites and biological factors. All concentrations for individuals (n=41) are measured in ng g^{-1}

Compound name	Factor	Spearman's coefficient	T-Value	P-Value
Mono methyl phthalate	**Days of incubation**	0.15	0.95	0.35
	Development days	0.07	0.46	0.65
	Weight of birds (g)	-0.28	-1.68	0.10
Mono ethyl phthalate	**Days of incubation**	0.22	1.34	0.19
	Development days	-0.08	-0.49	0.63
	Weight of birds (g)	0.12	0.73	0.47
Mono butyl phthalate	**Days of incubation**	0.19	1.16	0.25
	Development days	0.06	0.36	0.72
	Weight of birds (g)	0.23	1.42	0.16
Mono iso butyl phthalate	**Days of incubation**	0.30	1.77	0.08
	Development days	0.10	0.59	0.56
	Weight of birds (g)	0.29	1.71	0.10

The highest coefficient value was observed between mIBP and days of incubation days whereas the lowest was observed between mBP and development days. The overall coefficient values is not high for a significant statistical relationship between the variables.

Table 5: Spearman's correlation coefficients between different variables (three bisphenols and four phthalate metabolites). All concentrations for individuals (n=40) are measured in ng g^{-1}

BPA + mMP	-0.0242	-0.1489	0.8824
BPA +mEP	0.1292	0.7897	0.4346
BPA + mIBP	0.1682	1.0223	0.3131
BPA + mBP	0.2392	1.4319	0.1604
BPS + mMP	-0.0242	-0.1489	0.8824
BPS + mEP	0.1292	0.7896	0.4346
BPS + mIBP	0.1691	1.0272	0.3108
BPS + mBP	0.2396	1.4339	0.1598
BPZ + mMP	-0.0242	-0.1489	0.8824
BPZ + mEP	0.1292	0.7897	0.4346
BPZ + mIBP	0.1682	1.0223	0.3131
BPZ + mBP	0.2392	1.4319	0.1604

The correlation coefficient is low for the compounds with respect to high p-values. The maximum coefficient was found between BPS and mBP (0.2396). These low values indicate that there is no visible correlation among the measured samples regarding the compounds.

4. Discussions

4.1 Phthalate metabolites in blood samples

There is only a few number of studies conducted on the contamination of phthalate metabolites on wildlife and even fewer studies on birds. Most studies are conducted on either humans or on other matrices like liver (Rian et al., 2020) or urine (Hart et al., 2018). Although a different matrix, the current study conducted on vulture blood shows a similar trend to previous studies conducted on wildlife. Rian et al. (2020) showed a detection rates of more than 85% for low molecular weight metabolites in harbor porpoise liver (*Phocoena phocoena)*. The difference lies in the detection frequency and concentrations of mEP which in case of harbor porpoises, was detected in all of the liver samples (n=100). The average concentrations for mBP and mIBP in vulture blood is also closely similar to that of porpoise liver matrix (41.1 & 37.2 ng g^{-1} in case of harbor porpoise and 34.8 and 37.27 ng g^{-1} in case of vulture chicks for mBP and mIBP respectively). The different matrices could be responsible for this varying detection of phthalate metabolites. Rian et al. (2020) conducted the study focusing on three different matrices (liver, blubber, and muscle of harbor porpoise) and number of metabolites detected in the other two was lower than the liver (10 for the liver and 9 and 6 for muscle and blubber respectively). Hart et al. (2018) reported a different trend of phthalate metabolite in dolphins. The author reported an absence of mBP and highest concentration in case of mEP. Such high concentration was also visible in the present study where the maximum concentration was observed to be 825.88 ng g^{-1}. Although in the study mentioned, the high concentrations were observed in urine showing evidence that mEP might be excreted rather than accumulate within body. This could also prove why many of the samples in the current study did not show any mEP concentrations. The concentrations of mBP and mIBP was also observed in European herring gull (*Larus argentatus*) eggs. The author also compared metabolites with their parent compound and reported some metabolites like mHP to be present in more samples (12 eggs) than its parent compound (3 eggs). The presence of metabolites in eggs also provides evidence that transmission of phthalate metabolites from parents to next generation is possible which can also be the case for the present study.

Detection of mEP, mBP and mIBP was also observed in human urinary samples (Yang et al., 2015; and Asimakopoulos et al., 2016). A different trend was mentioned by Hart et al. (2018) focusing on the concentrations of mEHP (mono (2-ethyl hexyl) phthalate) and mEP in bottlenose dolphins (*Tursiops truncates*). This similar trend of mEHP and mEP was mentioned by other studies as well (blubber of fin whale, *Balaenoptera physalus* by Fossi et al. (2014), blubber and skin of Risso's dolphin, *Grampus griseus* by Baini et al. (2017) and so on). Fredrikesen et al. (2010) reported

serum mBP, mEP and DEHP level to be lower than urinary level in study conducted on Danish men.

This study includes relationship between metabolites and the biological factors like weight of the birds, days of incubation and development days, but there is no significant relationship between the variables according to the Spearman's correlation coefficient and p-values (table 1). The same trend was also mentioned in the harbor porpoise study (Rian et al., 2020) where the only correlation was observed in case of phthalic acid (PA). Between the metabolites themselves, this study shows a significant relationship between mBP and mIBP with a Spearman's coefficient of 0.75 and p-value of 0.004. This proves a similar pattern of biotransformation of these two metabolites. This type of correlation was also mentioned in previous studies (Rian et al., 2020; Fourgous et al., 2016 and Rocha et al., 2017).

4.2 Bisphenols observed in blood samples

Studies conducted on contamination of bisphenols in blood samples of raptors are scarce. Most studies involve analyzing other matrices predominantly in liver, kidney for example Gonzalez-Rubio et al. (2020) studied liver, kidney, preen gland, brain, muscle and adipose tissues of three raptors, Oró-Nolla et al. (2021) studied liver samples from white –tailed eagles (*Haliaeetus albicilla*), Staniszweska et al. (2014) studied guano samples from European herring gulls (*Larus argentatus*) and great black-backed gull (*Larus marinus*), Castro et al. (2022) studied on brown mussles (*Perna perna*) and so on. There is also another study conducted on a similar conditions as the current one where blood samples were analyzed for bisphenol contamination (Elliott et al., 2019).

Among the eight bisphenol analyzed, only three of them were found to have concentrations higher than LOQ and BPA (bisphenol A) to have more than 60% of samples having higher concentrations than LOQ. The other two bisphenols are BPS (bisphenol S) and BPZ (bisphenol z). In previous studies on raptors, the trend for DF (detection frequency) is different than the current study. Gonzalez-Rubio reported BPM/BPP to have the highest DF than other BPs and BPF to have the second highest value, where in this case BPM and BPP was not detected (BPF was not among the analogues analyzed). However they reported BPA having the highest median concentrations. Oró-Nolla et al. (2021) on the other hand reported BPAF to be the most abundant in the livers of white-tailed eagle (32 samples were detected to have BPAF among 38), whereas in current study, BPAF was not detected. The author also reported BPA to have the highest mean concentrations among the detected BPs but also have the highest standard deviation. Only 8 out of 38 samples were found to have BPA and was ranging from 3.36 to 33.8 ng g^{-1} w. w. ; i.e. the concentration changed tenfold among only 8 individuals.

The similar trend of having high DF of BPA was reported by Castro et al. (2022), who reported a hundred percent DF for BPA in brown mussels. Additionally, the author reported BPS to have the

second highest DF (91% and n=138) among the BPs. The median concentration found in the current study also matches with the above mentioned study (38.08 ng g^{-1} and 32.1 ng g^{-1} respectively). High levels of BPA was also mentioned by Salgueiro-Gonzalez et al. (2016) in wild mussels (*Mytilus galloprovincialis*) from Spain.

The varying trend for concentrations for BP analogues might be attributed to location and species variety. From the year 20111 and onwards, application of BPA has been restricted in packaging and food contact materials (European-parliament, 2011) which may reflect the reduced detected BPA concentrations. Additionally, different species have different mechanisms of 'dealing with' foreign materials, either storing at a different site or excreting out of the body. Elliott et al. (2019) examined BPA levels on blood samples of bald eagles and found very low concentrations compared to the present study.

The scientists previously reported a correlation between total BP concentrations with increasing body mass, for instance, Gonzalez-Rubio et al. (2020) reported white-tailed eagle to have the highest order of total BPs compared to the other two raptors of the study (Eurasian sparrowhawk, *Accipiter nisus*; and long-eared owl, *Asio otus*). In the present study however, there was no correlation found between mass of the species with BP concentrations (table A6).

Also, in the study of Gonzalez-Rubio et al. (2020) highest detection frequency was found in preen glands when compared to other tissues. The comparison between different matrices is scarce as most of the studies include analysis of BPs in one matrix (predominantly in liver). The study also mentioned observing a pattern between detection frequency and median concentrations of BPs with trophic position as white-tailed eagle (apex predator) showed the higher median concentrations compared to Eurasian sparrowhawk and long eyed owl. This may have been due to having longer life-expectancy leading to higher bioaccumulation potential. However in the present study, the cinereous vulture species does have longer life expectancy, but the study is conducted on nestling so high BP (especially BPA) concentrations may be either caused by availability of BP induced plastics or its maternal transmission ability.

It is also important to note the rate of biotransformation of chemicals in blood as many of the compounds can have relatively short half-lives in blood providing evidence for a lower concentrations compared to other matrices (Gonzalez-Rubio et al., 2021). This might have attributed to varying levels of BPs in other studies and 'not detectable' levels of other BPs in this study.

4.3 Relationship between phthalate metabolites and bisphenols and biological factors

Phthalates and bisphenols are both plasticizers and a study based on both of their separate and combined effects on the organism is quite rare. In a general sense, concentrations of BPs and phthalates could correlate with each other and with increasing plastic content taken up by

organism. In an attempt to support this hypothesis, Spearman's correlation was conducted (table 3) between the three BP analogues and four phthalate metabolites in this study that were detected to have the largest DF. However, there was no visible correlation between them as the correlation coefficient had low values (ranging from -0.0242 to 0.2396) along with high p-values (ranging from 0.1598 to 0.8824). The maximum coefficient values was observed between BPs and mBP. In case of mono methyl phthalate, there were observed negative coefficient values. Additionally, the total concentrations of phthalate metabolites and BPs did not follow a pattern; i.e. the individual sample which showed higher concentration of phthalate metabolite did not show a high BP concentration. This could be because of different levels of plasticizer being added in different products and the vulture chicks being exposed to a variety of plastic products.

A study conducted on urine of pregnant woman in the year 2004-05 in Netherlands, showed high DF of BPS and mono butyl phthalate (Philips et al., 2018), although there were no correlation analysis available for the study. Another study conducted on human urine and hair samples showed high DF of BPA, and BPS associated with mMP, mEP, and mEHP (Fäys et al., 2021). Interestingly, the author tried to show correlation among the compounds with response to two different excretory matrices but there was no correlation. The lack of correlation may have been attributed by the differences in the chemical foundation of the compounds, different utilization of plasticizers, different rate of intake, their different pathways and excretion rate as well as internal mechanism with the matrix.

4.4 Limitations and future perspectives

The studies conducted on wildlife usually incorporate several limitations for example, sample size, choice of sampling matrix, statistical procedure, quality assurance and so on. The present study also possess some of these limitations. It would have been more fulfilling to compare and analyze different matrices theoretically. Additionally, there is a lack of study conducted on contaminants in blood sample which made it difficult to compare the levels of phthalate metabolites and BPs and come with a better conclusion. This requires more research on wildlife comprising different trophic levels on different matrices.

Additionally, research on the effects of such compounds in different organisms is also lacking. And it results in lack of knowledge on how these compounds may interact with the organism at environmental level. An example of such situation may be whether environmental levels of phthalate metabolite may cause detrimental effects on terrestrial birds considering its low stability, relationship between dietary behavior and contamination level, multigenerational effects of phthalate metabolites (if there is any), changes in behavioral pattern (if there is any), how they transmit from one trophic level to another and so on could help the industries to optimize the plastic production, and its management resulting a lesser contamination.

In practical sense, this broad study might be difficult to accomplish as it involves working with living organisms. Working with dead samples might have helped with that issue but it has its own problems, for example, cause of death, habitat, food source, and so on. While analyzing relationship between two different chemicals, some different procedure (for example, analyzing cause and effect relationship of such chemicals with other biomolecule) could be developed rather than comparing them directly. Choice of sampling matrix is also an important factor that needs to be considered. Different matrix depending on the species and compound may show different trends of contamination. Analyzing different matrices and formation of a model depicting how a compound may behave after ingestion till excretion, their site of accumulation and so on might be an interesting approach. This may help us to have a solid knowledge on the compound and its interaction with the organism. Also, studying the contaminants with respect to surrounding environment might be useful. For example in this study, what type of plastics were present surrounding the nests of vulture chicks could evaluate the lack of correlation between the phthalates and BPs. Moreover, considering the environmental condition may help us to find out how the organisms are exposed to the contaminants.

Additionally, effects of different metabolites should be studied as the novel compounds are replacing the existing contaminants might have even more sinister effects. Future research in this perspective should also focus on risk assessment and guidance towards solving the issues as well.

5. Conclusion

Phthalate metabolites and bisphenols were detected in blood samples of vulture chicks. Among the phthalate metabolites mBP had the highest detection frequency (DF), and BPA had the highest DF value among the bisphenols. The three biological factors (weight of the birds, development days, and days of incubation) measured had no statistical influence over the concentration of the two plasticizers. In addition, there was no visible correlation between the two plasticizers as well. This indicates that different plastics comprise different levels of these two compound classes and that they might possibly have different biotransformation pathways. The absence of pattern was also observed between the compounds while measuring total levels of phthalate metabolites and bisphenols providing further evidence of the above mentioned statement that different plastics may contain different levels of plasticizers. The clear lack of studies regarding these two compounds as well as their combined influence in raptors piled up while providing a definite constitution. One objective does manage to get fulfilled by the present study and previous one conducted on the samples is that the phthalate metabolites are associated with the plastic waste present in the landfill surrounding the nest of the vultures. Direct comparison with previous studies was not possible as phthalate metabolites and bisphenol in vulture (or any other raptors) blood sample was scarce.

Further studies with a bigger sample size, involving different sample matrices may help to elucidate correlation between the biological factors and the chemicals. Also it will help to assess the consequences of phthalates and bisphenols caused by plastic waste on wildlife. Additionally, usage of BPA has been banned or restricted for but still its dominant detection frequency and high concentration is a matter of concern and should be looked further into.

6. References

Allen S.F., Ellis, F., Mitchell, C., Wang, X., Boogert, N.J., Lin, C.Y., Clokey, J., Thomas, K.V., and Blount, J.D. 2021. Phthalate diversity in eggs and associations with oxidative stress in the European herring gull (*Larus argentatus*), *Marine Pollution Bulletin*. 169, 112564.

Andevski, J., Tavares, J., Williams, N. P., Moreno-Opo, R., Botha, A. & Renell, J. (2017). *Flyway Action Plan for the Conservation of the Cinereous Vulture*. CMS Raptors MOU Technical Publication No. 6. Coordinating Unit of the CMS Raptors MOU, Abu Dhabi, United Arab Emirates.

Asai D., Tahara Y., Nakai M., Yakabe Y., Takatsuki M., Nose T. 2000. Structural essentials of xenoestrogen dialkyl phthalates to bind to the estrogen receptors. *Toxicol. Lett.* 118, 1–8.

Asimakopoulos, A. G., Xue, J. C., Decarvalho, B. P., Iyer, A., Abualnaja, K. O., Yaghmoor, S. S., Kumosani, T. A. and Kannan, K. 2016. Urinary biomarkers of exposure to 57 xenobiotics and its association with oxidative stress in a population in Jeddah, Saudi Arabia (vol 150, pg 573, 2016). *Environmental Research,* 153**,** 205-205.

Baini, M., Martellini, T., Cincinelli, A., Campani, T., Minutoli, R., Panti, C., et al. 2017. First detection of seven phthalate esters (PAEs) as plastic tracers in superficial neustonic/planktonic samples and cetacean blubber. Analytical Methods, 9(9), 1512–1520.

Benjamin, S.; Masai, E.; Kamimura, N.; Takahashi, K.; Anderson, R.C.; Faisal, P.A. Phthalates impact human health: Epidemiological evidences and plausible mechanism of action. *J. Hazard. Mater.* 2017, *340*, 360–383.

Berg, S., Halldin, K., Brunstrom, B., 2001. Effects of bisphenol A and tetrabromobisphenol A on sex organ development in quail and chicken embryos. *Environ. Toxicol. Chem.* 20, 2836 – 2840.

Blas, J., Bortolotti, G. R., Tella, J. L., Baos, R. and Marchant, T. A. 2007. Stress response during development predicts fitness in a wild, long lived vertebrate. Proceedings of the National Academy of Sciences of the United States of America, 104, 8880 – 8884.

Carlin, J., Craig, C., Little, S., Donnelly, M., Fox, D., Zhai, L., & Walters, L. 2020. Microplastic accumulation in the gastrointestinal tracts in birds of prey in central Florida, USA. *Environmental Pollution*, *264*, 114633.

Castro, G., Fourie, A. J., Marlin, D., Venkatraman, V., González, S. V., & Asimakopoulos, A. G. 2022. Occurrence of bisphenols and benzophenone UV filters in wild brown mussels (*Perna perna*) from Algoa Bay in South Africa. *The Science of the total environment*, *813*, 152571.

Clara M, Windhofer G, Hartl W, Braun K, Simon M, Gans O, et al. 2010. Occurrence of phthalates In surface runoff, untreated and treated wastewater and fate during wastewater treatment. *Chemosphere*. 78, 1078-1084.

Crain, D. A., Eriksen, M., Iguchi, T., Jobling, S., Laufer, H., LeBlanc, G.A., and Guillette, L. J., (2007). An ecological assessment of bisphenol-A: evidence from comparative biology. *Reprod. Toxicol*, 24, 225 - 239.

Crespi, E. J., Williams, T. D., Jessop, T. S. and Delehanty, B. 2013. Life history and the ecology of stress: how do glucocorticoid hormones influence life-history variation in animals? *Functional Ecology,* 27**,** 93 – 106.

del Hoyo J, Elliott A, Sargatal J. Handbook of the birds of the world, vol. 2. Barcelona: Lynx Edicions; 1994

Donázar, E. A. 1993. Los Buitres Ibéricos. Biología y conservación, Madrid.

Du, Z. H., Xia, J., Sun, X. C., Li, X. N., Zhang, C., Zhao, H. S., Zhu, S. Y., and Li, J. L. 2017. A novel nuclear xenobiotic receptors (AhR/PXR/CAR)-mediated mechanism of DEHP-induced toxicity in quails (*Coturnix japonica*) via disrupting CYP enzyme system homeostasis. *Environmental Pollution*, 2017, 1 – 9.

Duke, G., Vrezec, A., Walker, L., J. García-Fernández, A., Movalli, P., Wernham, C., & Krone, O. (2021, November). Vulture Guidance for Biomonitoring. *European Raptor Biomonitoring Facility*.

Dvořáková M., Kejlová K., Rucki M., Jírová D. Selected bisphenols and phthalates screened for estrogen and androgen disruption by in silico and in vitro methods. *Neuroendocrinol. Lett.* 39, 409–416.

Egwumah F.A., Egwumah P.O., Edet D.I. 2017. Paramount roles of wild birds as bioindicators of contamination. *Int J Avian & Wildlife Biol*. 2(1), 194 – 200.

Ekman D.R., Hartig P.C., Cardon M., Skelton D.M., Teng Q., Durhan E.J., Jensen K.M., Kahl M.D., VilleneuveD.L., Gray L.E. Jr., Collette T.W., Ankley G.T. 2012. Metabolite profiling and a transcriptional activation assay provide direct evidence of androgen receptor antagonism by bisphenol A in fish. *Environ Sci Technol.* 46, 9673-9680.

Eladak, S., Grisin, T., Moison, D., Guerquin, M.-J., N'Tumba-Byn, T., Pozzi-Gaudin, S., Benachi, A., Livera, G., Rouiller-Fabre, V., & Habert, R. (2015). A new chapter in the bisphenol A story: Bisphenol S and bisphenol F are not safe alternatives to this compound. *Fertility and Sterility*, *103*(1), 11–21.

Elliott, S. M., Route, W. T., DeCicco, L. A., VanderMeulen, D. D., Corsi, S. R., & Blackwell, B. R. 2019. Contaminants in bald eagles of the upper Midwestern U.S.: A framework for prioritizing future research based on in-vitro bioassays. *Environmental pollution (Barking, Essex : 1987)*, *244*, 861–870.

European Food Safety Authority. 2023. *Bisphenol A* [Online]. EFSA. Available: https://www.efsa.europa.eu/en/topics/topic/bisphenol [Accessed 2023].

European-Parliament, 2011. Commission directive 2011/8/EU. Off. J. Eur. Union 29–32.

Fäys, F., Hardy, E. M., Palazzi, P., Haan, S., Beausoleil, C., & Appenzeller, B. M. R. (2021). Biomonitoring of fast-elimination endocrine disruptors – Results from a 6-month follow up on human volunteers with repeated urine and hair collection. *Science of the Total Environment*, *778*, 146330.

Fergusson-Lees, J., and Christie, D. (2001). *Raptors of the world*. London, United Kingdom: Christopher Helm.

Ferreira, A. 2011. Assessment of heavy metals in Egretta thula: Case study: Coroa Grande mangrove, Sepetiba Bay, Rio de Janeiro, Brazil. *Brazilian Journal of Biology*, *71*(1), 77–82.

Fiege, H., Voges, H.-W., Hamamoto, T., Umemura, S., Iwata, T., Miki, H., Fujita, Y., Buysch, H.-J., Garbe, D., & Paulus, W. (2000). Phenol Derivatives. In Wiley-VCH Verlag GmbH & Co. KGaA (Ed.), *Ullmann's Encyclopedia of Industrial Chemistry* (p. a19_313). Wiley-VCH Verlag GmbH & Co. KGaA.

Fossi, M. C., Coppola, D., Baini, M., Giannetti, M., Guerranti, C., Marsili, L., et al. 2014. Large filter feeding marine organisms as indicators of microplastic in the pelagic environment: The case studies of the Mediterranean basking shark (*Cetorhinus maximus*) and fin whale (*Balaenoptera physalus*).*Marine Environmental Research*, 100, 17–24.

Fourgous, C., Chevreuil, M., Alliot, F., Amilhat, E., Faliex, E., Paris-Palacios, S., Teil, M.J., Goutte, A., 2016. Phthalate metabolites in the European eel (*Anguilla anguilla*) from Mediterranean coastal lagoons. *Sci. Total Environ*. 569–570, 1053–1059.

Frederiksen, H., Jorgensen, N., Andersson, A.M. 2010. Correlations between phthalate metabolites in urine, serum, and seminal plasma from young Danish men determined by isotope dilution liquid chromatography tandem mass spectrometry. *J. Anal. Toxicol*, *34*, 400–410

Gibert Y., Sassi-Messai S., Fini J.B., Bernard L., Zalko D., Cravedi J.P., Balaguer P., Andersson-Lendahl M., Demeneix B., Laudet V. 2011. Bisphenol A induces otolith malformations during vertebrate embryogenesis. *BMC Dev Biol*. 11:4.

González-Rubio, S., Ballesteros-Gómez, A., Asimakopoulos, A. G., & Jaspers, V. L. B. (2021). A review on contaminants of emerging concern in European raptors (2002-2020). *The Science of the total environment*, *760*, 143337.

Gonzalez-Rubio, S., Vike-Jonas, K., Gonzalez, S.V., Ballesteros-Gomez, A., Sonne, C., Dietz, R., Boertmann, D., Rasmussen, L.M., Jaspers, V.L.B., and Asimakopoulos, A.G. 2020. Bioaccumulation potential of bisphenols and benzophenone UV filters: A multi residue approach in raptor tissues. *Science of the Total Environment,* 741. 140330.

Goto Y, Kitamura S, Kashiwagi K, Oofusa K, Tooi O, Yoshizato Y, Sato J, Ohta S, Kashiwagi A. 2006. Suppression of amphibian metamorphosis by bisphenol A and related chemical substances. *J Health Sci.* 52, 160-168.

Hart, L.B., Beckingham, B., Wells, R.S., Alten Flagg, M., Wischusen, K., Moors, A., et al. 2018. Urinary phthalate metabolites in common bottlenose dolphins (*Tursiops truncatus*) from Sarasota Bay, FL, USA. *GeoHealth*, 2, 313–326.

Harvard-Health-Publishing. 2020. *Understanding the stress response* [Online]. Harvard Medocal School. Available: https://www.health.harvard.edu/stayinghealthy/understanding-the-stress-response. [Accessed 2023].

Haynes, W. M., Lide, D. R., & Bruno, T. J. (Eds.). (2017). *CRC handbook of chemistry and physics: A ready-reference book of chemical and physical data* (2016th–2017th, 97th edition ed.). CRC Press.

Houston, D. C., Mee, A., & McGrady, M. 2007. WHY DO CONDORS AND VULTURES EAT JUNK?: THE IMPLICATIONS FOR CONSERVATION. *Journal of Raptor Research*, *41*(3), 235–238.

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.

Hulak M., Gazo I., Shaliutina A., Linhartova P. 2013. *In vitro* effects of bisphenol A on the quality parameters, oxidative stress, DNA integrity and adenosine triphosphate content in sterlet (*Acipenser ruthenus*) spermatozoa. *Comp Biochem Physiol C Toxicol Pharmacol*. 158, 64-71.

Iwamuro S, Yamada M, Kato M, Kikuyama S. 2006. Effects of bisphenol A on thyroid hormone dependent up-regulation of thyroid hormone receptor alpha and beta and down-regulation of retinoid X receptor gamma in *Xenopus* tail culture. *Life Sci*. 79, 2165-2171.

Jessl, L., Scheider, J., and Oehlmann, J. 2018. The domestic fowl (*Gallus gallus domesticus*) embryo as an alternative for mammalian experiments - Validation of a test method for the detection of endocrine disrupting chemicals. *Chemosphere*, 196, 502 – 513.

Jobling S., Reynolds T., White R., Parker M. G., Sumpter J. P. 1995. A variety of environmentally persistent chemicals, including some phthalate plasticizers, are weakly estrogenic. *Environ. Health Perspect.* 103, 582–587.

Kang, J. H., Aasi, D., Katayama, Y., 2007. Bisphenol A in the aquatic environment and its endocrine-disruptive effects on aquatic organisms. *Crit. Rev. Toxicol*, 37, 607 – 625.

Kim E. J., Kim J. W., Lee S. K. 2002. Inhibition of oocyte development in Japanese medaka (*Oryzias latipes*) exposed to di-2-ethylhexyl phthalate. *Environ. Int.* 28, 359–365.

Kitaysky, A. S., Wingfield, J. C. and Piatt, J. F. 2001. Corticosterone facilitates begging and affects resource allocation in the black-legged kittiwake. *Behavioral Ecology,* 12**,** 619 – 625.

Kyunghee, J., Seongjin, H., Younglim K., and Kyungho, C. 2013. Effects of Bisphenol S Exposure on Endocrine Functions and Reproduction of Zebrafish. *Environmental Science & Technology*. *47* (15), 8793-8800

Lee S. K., Owens G. A., Veeramachaneni D. N. 2005. Exposure to low concentrations of di-*n*-butyl phthalate during embryogenesis reduces survivability and impairs development of *Xenopus laevis* frogs. *J. Toxicol. Environ. Health A* 68, 763–772.

Lyche, J. L., Gutleb, A. C., Bergman, A., Eriksen, G. S., Murk, A. J., Ropstad, E., Saunders, M., & Skaare, J. U. (2009). Reproductive and developmental toxicity of phthalates. *Journal of toxicology and environmental health. Part B, Critical reviews*, *12*(4), 225–249.

Malisch, J. L., Saltzman, W., Goems, F. R., Rezende, E. L., Jeske, D. R., and Garland, T. (2007). Baseline and stress-induced plasma corticosterone concentrations of mice selectively bred for high voluntary wheel running. *Physiological and Biochemical Zoology,* 80**,** 146 – 156.

Mathieu-Denoncourt, J., Wallace, S. J., de Solla, S. R., & Langlois, V. S. (2015). Plasticizer endocrine disruption: Highlighting developmental and reproductive effects in mammals and non-mammalian aquatic species. *General and comparative endocrinology*, *219*, 74–88.

Mentor A., Wänn M., Brunström B., Jönsson M., and Mattsson A. 2020. Bisphenol AF and bisphenol F induce similar feminizing effects in chicken embryo testis ad bisphenol A. *Toxicological Sciences,* 178(2), 239 – 250.

Munshi, A.B., Karim, N., Shaukat, S., Hashmi, D., Boardman, G.D., and 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.

Natsheh, M. A., Alawi, M., Fayyad, M., and Tarawneh, I. (2015). Simultaneous GC-MS determination of eight phthalates in total and migrated portions of [plasticized polymeric toys and childcare articles. *J. Chromatogr. B* 985(2015), 103 – 109.

Nilsen, S. E. S. 2021. *Plastic and Associated Chemicals in Cinereous Vulture (Aegypius monachus): Exposure and Effects* [Master's thesis, Norwegian University of Science and Technology]. NTNU Open. https://hdl.handle.net/11250/3016410

Oehlmann, J., Schulte-Oehlmann, U., Kloas, W., Jagnytsch, O., Lutz, I., Kusk, K.O., Wollenberger, L., Santos, E.M., Paull, G.C., Van Look, K.J., Tyler, C.R., 2009. A critical analysis of the biological impacts of plasticizers on wildlife. *Phil. Trans. R. Soc. B,* 364, 2047 - 2062.

Ogada, D.L., Keesing, F. and Virani, M.Z. 2012. Dropping dead: causes and consequences of vulture population declines worldwide. Annals of the New York Academy of Sciences, 1249, 57-71.

Ohore, O. E., & Zhang, S. (2019). Endocrine disrupting effects of bisphenol A exposure and recent advances on its removal by water treatment systems. A review. *Scientific African*, *5*, e00135.

Oró-Nolla, B., Lacorte, S., Vike-Jonas, K., Gonzalez, S.V., Nygård, T., Asimakopoulos, A.G., Jaspers V.L.B. 2021. Occurrence of Bisphenols and Benzophenone UV Filters in White-Tailed Eagles (*Haliaeetus albicilla*) from Smøla, Norway. *Toxics*. 9(2), 34.

Peijnenburg, W. J. G. M., Sven, E. J., Brian, F. 2008. Phthalates. In: Jørgensen SE, Fath BD, editors. Encyclopedia of Ecology. Oxford: Academic Press.

Philips, E. M., Jaddoe, V. W. V., Asimakopoulos, A. G., Kannan, K., Steegers, E. A. P., Santos, S., & Trasande, L. (2018). Bisphenol and phthalate concentrations and its determinants among pregnant women in a population-based cohort in the Netherlands, 2004-5. *Environmental research*, *161*, 562–572.

Pravosudov, V. V. and Kitaysky, A. J. 2006. Effects of nutritional restrictions during post- hatching development on adrenocortical function in western scrub-jays (Aphelocoma californica). General and Comparative Endocrinology, 145, 25–31

Provencher, J., Avery-Gomm, S., Braune, B., Letcher, R., Dey, C., and Mallory, M. 2019. Are phthalate ester contaminants in northern fulmar preen oil higher in birds that have ingested more plastic? *Marine Pollution Bulletin*. 150. 110679.

Przybylińska, P. A., and Wyszkowski, M. 2016. Environmental Contamination with Phthalates and Its Impact on Living Organisms. *Ecol. Chem. Eng. S.* 23(2), 347 – 356.

Rian, M., Vike-Jonas, K., Gonzalez, S.V., Venkatraman, V., Lindstrøm, U., & Jenssen, B.M., Asimakopoulos, A.G., and Ciesielski, T. 2020. Phthalate metabolites in harbor porpoises

(*Phocoena phocoena*) from Norwegian coastal waters. *Environment International*. 37, 105525.

Rideout, B. A., Stalis, I., Papendick, R., Pessier, A., Puschner, B., Finkelstein, M. E., Smith, D. R., Johnson, M., Mace, M., Stroud, R., Brandt, J., Burnett, J., Parish, C., Petterson, J., Witte, C., Stringfield, C., Orr, K., Zuba, J., Wallace, M., & Grantham, J. 2012. PATTERNS OF MORTALITY IN FREE-RANGING CALIFORNIA CONDORS (*GYMNOGYPS CALIFORNIANUS*). *Journal of Wildlife Diseases*, *48*(1), 95–112.

Riu, A., McCollum, C. W., Pinto, C. L., Grimaldi, M., Hillenweck, A., Perdu, E., Zalko, D., Bernard, L., Laudet, V., Balaguer, P., Bondesson, M., & Gustafsson, J. A. (2014). Halogenated bisphenol-A analogs act as obesogens in zebrafish larvae (Danio rerio). *Toxicological sciences: an official journal of the Society of Toxicology*, *139*(1), 48–58.

Rocha, B.A., Asimakopoulos, A.G., Barbosa Jr., F., Kannan, K., 2017. Urinary concentrations of 25 phthalate metabolites in Brazilian children and their association with oxidative DNA damage. *Sci. Total Environ.* 586, 152–162.

Salgueiro-González, N., Turnes-Carou, I., Viñas, L., Besada, V., Muniategui-Lorenzo, S., LópezMahía, P., Prada-Rodríguez, D., 2016. Occurrence of alkylphenols and bisphenol a in wild mussel samples from the spanish Atlantic coast and Bay of Biscay. *Mar. Pollut. Bull*. 106, 360–365.

Schiffer, C., Müller, A., Egeberg, D. L., Alvarez, L., Brenker, C., Rehfeld, A., Frederiksen, H., Wäschle, B., Kaupp, U. B., Balbach, M., Wachten, D., Skakkebaek, N. E., Almstrup, K., & Strünker, T. 2014. Direct action of endocrine disrupting chemicals on human sperm. *EMBO Reports*, *15*(7), 758–765.

Shareef, A., Angove, M. J., Wells, J. D., & Johnson, B. B. (2006). Aqueous Solubilities of Estrone, 17β-Estradiol, 17α-Ethynylestradiol, and Bisphenol A. *Journal of Chemical & Engineering Data*, *51*(3), 879–881.

Shriastava, A., and Gupta, V.B. 2011. Methods for the determination of limit of detection and limit of quantitation of the analytical methods. *Chronicles of young scientists*. 1(2), 21-25.

Sopinka, N. M., Patterson, L. D., Redfern, J. C., Pleizier, N. K., Belanger, C. B., Midwood, J. D., Crossin, G. T., and Cooke, S. J. (2015) Manipulating Glucocorticoids in Wild Animals: Basic and Applied Perspectives. *Conserv. Physiol,* 3(1), cov031.

Staniszewska, M., Falkowska, L., Grabowski, P., Kwaśniak, J., Mudrak-Cegiołka, S., Reindl, A. R., Sokołowski, A., Szumiło, E., & Zgrundo, A. 2014. Bisphenol A, 4-tert-octylphenol, and 4-nonylphenol in the Gulf of Gdańsk (Southern Baltic). *Archives of environmental contamination and toxicology*, *67*(3), 335–347.

Stier, K. S., Almasi, B., Gasparini, J., Piault, R., Roulin, A. and Jenni, L. 2009. Effects of corticosterone on innate and humoral immune functions and oxidative stress in barn owl nestlings. *Journal of Experimental Biology,* 212**,** 2084 – 2090.

Sun, Y., Shen, J., Zeng, L., Yang, D., Shao, S., Wang, J., Wei, J., Xiong, J., & Chen, J. 2018. Role of autophagy in di-2-ethylhexyl phthalate (DEHP)-induced apoptosis in mouse Leydig cells. *Environmental Pollution*, *243*, 563–572.

Suzuki T., Nakagawa Y., Takano I., Yaguchi K., Yasuda K. 2004. Environmental fate of bisphenol A and its biological metabolites in river water and their xeno-estrogenic activity. *Environ Sci Technol.* 38, 2389-2396.

Thoene, M., Dzika, E., Gonkowski, S., & Wojtkiewicz, J. 2020. Bisphenol S in Food Causes Hormonal and Obesogenic Effects Comparable to or Worse than Bisphenol A: A Literature Review. *Nutrients*, *12*(2), 532.

Ullah, A., Pirzada, M., Afsar, T., Razak, S., Almajwal, A., and Jahan, S. 2019. Effect of bisphenol F, an analog of bisphenol A, on the reproductive functions of male rats. *Environ Health Prev Med.* 24, 41.

Ventrice, P, Ventrice, D, Russo, E., and De Sarro, G. (2013) Phthalates: European regulation, chemistry, pharmacokinetic and related toxicity. *Environ Toxicol Pharmacol*, 36, 88 – 96.

Verma, R., Vinoda, K. S., Papireddy, M., and Gowda, A. N. S. (2016). Toxic Pollutants from Plastic Waste- A review. *Procedia Environmental Sciences,* 35 (2016), 701 – 708.

Wang, H., Wang, J., Zhang, J., Jin, S., & Li, H. 2017. Role of PI3K/AKT/mTOR signaling pathway in DBP-induced apoptosis of testicular sertoli cells in vitro. *Environmental Toxicology and Pharmacology*, *53*, 145–150.

Wang, L., Nabi, G., Yin, L., Wang, Y., Li, S., Hao, Z., & Li, D. 2021. Birds and plastic pollution: Recent advances. *Avian Research*, *12*(1), 59.

Waring, R. H., Harris, R. M., & Mitchell, S. C. 2018. Plastic contamination of the food chain: A threat to human health? *Maturitas*, *115*, 64–68.

Wingfield, J. C. 2003. Control of behavioral strategies for capricious environments. *Animal Behaviour,* 66**,** 807 – 815.

Yang, Y., Shi, M., Chen, B., Lin, J., Yang, S., Zhu, B., Zhuang. B., Jia, Y., Huang, Z., Chen, J., Liu, H., Chen, J., Su, Y., Ke, X., Zhao, B. 2015. Levels of phthalate internal exposure levels in pregnant women and influencing factors. *Zhonghua Yu Fang Yi Xue Za Zhi*. 49(11), 998-1004.

Zoeller, R. T., Brown, T. R., Doan, L. L., Gore, A. C., Skakkebaek, N. E., Soto, A. M., Woodruff, T. J., and Vom Saal, F. S. (2012). Endocrine-disrupting chemicals and public health protection: a statement of principles from The Endocrine Society. *Endocrinology,* 153**,** 4097-110.

Zorníková G, Jarošová A, Hřivna L. 2011. Distribution of phthalic acid esters in agricultural plants and soil. *Acta Univ Agric Silvic Mendel Brun*. 59(3). 233-238.

Web links:

Harald F. 2017. 8.3 billion tons plastics on earth. DW.
https://www.dw.com/en/there-are-83-billion-tons-of-plastic-in-the-world/a-39765670

UN Environment Programme (UNEP). 2022. https://www.unep.org/interactives/beat-plastic-pollution/

Appendix

Table A1: Phthalate metabolite concentrations found in blood samples

Sample ID	mono methyl phthalate	mono ethyl phthalate	mono iso butyl phthalate	mono butyl phthalate	mono-n-pentyl phthalate	mono iso pentyl phthalate	mono cyclo hexyl phthalate	mono-n-hexyl phthalate	mono benzyl phthalate	mono-n-heptyl phthalate	mono (2-ethyl-1-hexyl) phthalate	mono-n-octyl phthalate	mono (2-ethyl-5-oxyhexyl) phthalate	mono nonyl phthalate	mono (2-ethyl-5-hydroxyhexyl) phthalate	mono-n-decyl phthalate	DEHP	mono (3-carboxy propyl) phthalate
1	20.53289527	0	21.49669121	30.98073395	0.041884319	0	0	0	0.098813	0	0.1996937	0	0	0.2162384	0	0.1629287	0	0
2	0	0	32.90767572	24.28209013	0.010994319	0	0	31.96496	0	0.4162497	27.741765	0	0.0388987	0	0	0	12.09958	0.934576
3	0	0	44.17754467	47.45561696	0.017926771	0	0	33.89637	0.366568	0.0266727	8.6175877	0.212952864	0	0.104691	0	0	40.26824	0
4	0	0	18.1928197	27.42595526	0.00209733	0	0	2.7211756	0.24192	0	14.594282	0	0	0	0	0	0	0
5	8.843485516	0	33.83544391	52.60989514	0	0	0	0	0	0	1.6498779	0	0	1.0221289	0	0.0258721	63.50446	0
6	16.94438133	0	23.07065986	22.45976966	0.000715484	0	0	0	0	0	0.8074059	0	0	0.6219897	0	0.119599	0	0
7	0	0	27.14702285	19.42355071	0	0	0	7.975801	0.34372	0	1.5106799	0	0	0	0	0.4039172	0	0
8	0	661.6439809	29.54235436	18.2522223	0.016387227	0	0	6.2552854	0.105359	0	6.1632076	0	0	0.2104096	0	0	0	0
9	0	0	71.56481892	64.88919799	0.110296308	0	0	17.660079	0.038991	0	5.1466834	0	0	0	0	0.1803014	85.55391	8.984926
10	0	0	26.19451131	22.23223712	0.001436463	0	0	17.399555	0.28595	0	0.5293525	0.339928845	0	0.3573734	0	0.3486175	6.402729	0.646075
11	0	0	20.60569465	21.81523687	0.00612765	0	0	9.9167563	0.097893	0	7.1718132	0	0	0.721318	0	0	0	0
12	0	0	24.31588498	21.00955244	0	0	0	8.7815829	0.161073	0	4.9866958	0	0	0.5149021	0	0.2701491	6.359172	0
15	0	0	0	22.9863578	0	0	0	20.921793	0.013823	0	0.9733693	0	0	0.2545064	0	0	0	0
16	0	0	16.48889224	12.10124644	0	0	0	5.1803399	0.178599	0	1.9105763	0	0	0.1492245	0	0.2217367	0	0
17	0	0	26.51191139	34.64393269	0	0	0	0	0	0	1.3588353	0	0	0.2159294	0	0	0	0
18	0	0	14.20594376	18.16947456	0	0	0	16.420485	0	0	0.4697234	0	0	0.0340123	0	0	0	6.556461
19	0	0	0	17.0576075	0.020355977	0	0	9.1694999	0.354155	0	6.2833808	0.580393921	0	0.1492714	0	0	0	1.990606
20	0	0	6.398284061	31.54731552	0.007185488	0.113701	0	6.3611606	0.0997	0	0.9801171	0	0.1542497	1.592553	0	0	0	0
21	0	0	25.44273022	22.41303065	0	0	0	0	0.23527	0	5.8657071	0	0	0	0	0	0	0
22	0	0	32.24492574	41.56013176	0.004005133	0	0	9.9449747	0.080693	0	1.4330768	0	0	0.3845161	0	0.7843059	0	0.440782
23	0	0	27.57946006	37.27092404	0	0	0	23.345477	0.82713	0.2309315	14.12308	0.515973252	0	0.3716323	0	0	16.92963	2.141679
24	0	0	34.77653583	50.22858441	0.072366735	0	0	17.573643	0.325143	0.1589827	7.4833362	0.067151631	0	0	0	0.0695402	18.46219	32.52990
25	0	0	27.56586608	36.05730791	0.006944548	0.6441732	0	66.201435	0	0	3.3705709	0	0	0	0	0	109.5140	8.537077
26	0	0	29.23765336	39.42160871	0	0	0	5.4978964	0	0	1.2819134	0	0	0.1369572	0	0.6477629	3.988212	2.404752
28	1.025855161	0	45.0839447	56.50452415	0	0	0	33.015467	0.230364	0	1.2377907	0	0	0	0	0	0	56.24845
29	0	0	52.19475813	56.21826498	0.021437	0	0	17.928109	0.099338	0	9.6197551	0	0	0.0560335	0	0	6.095067	6.808385
30	0	0	74.79437162	72.43329276	0.000420045	0	0	27.28291	0.018614	0	30.65685	0	1.628911	0.0868424	7.771849	0.1262037	118.6193	0
31	0	0	52.10835433	49.43942448	0.053394579	0	0	25.239011	0.001372	0	9.1035967	0	0	0	0	0	1.157930	0
32	0	344.395738	33.71446905	39.3000077	0	0	0	25.371308	0	0	23.087849	0	0.010426	0.1941965	0	0	10.68799	5.718858
33	0	0	36.03925924	40.57216951	0	0	0	7.0867748	0.033443	0	5.7773035	0.654957853	0	0.1151226	0	0	28.96741	0.478561
34	0	0	25.03749921	33.81306882	0	0	0	12.91127	0	0	6.2310113	0	0	0	0	0	0	4.531018
35	0	0	51.39932823	49.32770078	0	0	0	21.125206	0.084772	0	9.1508953	0	0	0	0	0.8098165	0	0
36	0	614.8260391	61.41241465	57.83352006	0.002405483	0	0	31.858643	0.347961	0	18.437343	0	0	0	0	0	0	0
37	12.85816638	0	0	57.89289145	0.058182818	0	0	12.25985	0.016878	0	4.4386607	0	0.1018394	0	0	0.0469297	21.15423	0
38	0	0	47.16806905	58.95738871	0	0	0	28.991313	0	0	13.878192	0	0	0	0	0.2827623	0	1.227633
39	0	825.8799359	36.56644491	36.15176758	0	0	0	32.951762	0.04813	0	12.132856	0	0.0332058	0	0.211913	0.3243364	5.696119	0
40	0	0	24.59056779	29.10684387	0	0.3326281	0	21.607998	0	0	5.4558024	0	0	0.0593845	0	0.0766143	0	0
41	0	0	30.44554189	38.53921372	0.015577722	0	0	8.0753612	0	0	4.2227934	0	0	0.5433553	0	0.1509286	11.93104	0
42	0	0	38.4335872	34.92842855	0	0	0	7.8854375	0	0	7.7111387	0	0	0.4879988	0	0.1674853	0	0
43	0	0	37.02113596	45.47035684	0.030179391	0	0	5.8694708	0	0	3.1794727	0	0	1.7627645	0	0.1075314	0	0
44	0	0	33.9835543	35.36737972	0.059786713	0	0	4.1182741	0.035693	0.1018742	2.6007572	0	0.142754	1.0014367	0.269257	1.45782	8.338972	0

Table A2: Descriptive statistics for biological variables. Table includes mean, standard deviation, median, and range (minimum and maximum)

Variable	Mean	Standard deviation	Median	Min	Max
Weight of birds (g)	5303.66	1100.14	5400	2800	7400
Days of incubation	60.15	3.65	59	51	74
Development days	113.63	4.77	114	100	128

Table A3: Spearman's correlation coefficient among different phthalate metabolites

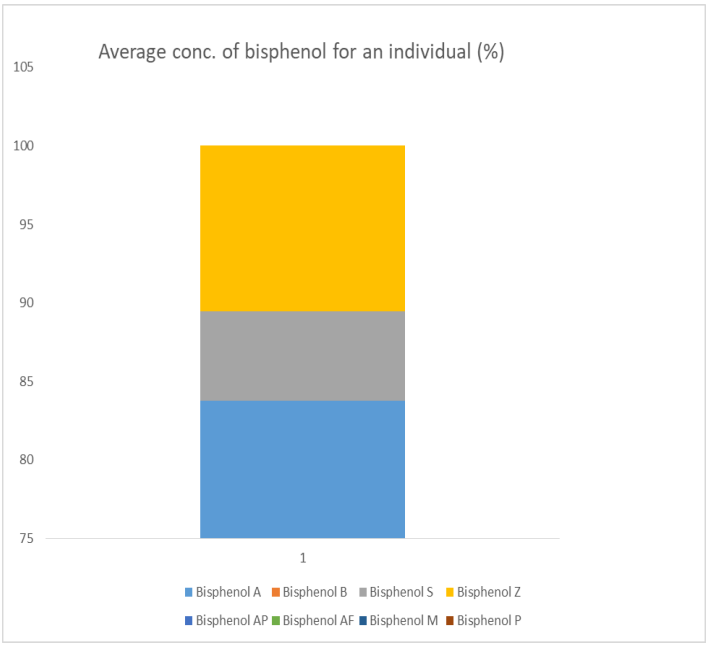
Variables		Spearman's coefficient	T-Value	P-Value
Mono methyl phthalate	**Mono ethyl phthalate**	-0.122	-0.756	0.454
Mono isobutyl phthalate	**Mono butyl phthalate**	0.749	3.099	0.004

Table A4: Calculated concentrations (ng g^{-1}) for each vulture (n=40)

Sample ID	Bisphenol A	Bisphenol B	Bisphenol S	Bisphenol Z	Bisphenol AP	Bisphenol AF	Bisphenol M	Bisphenol P
1	41.29299436	0.0023742	0.387874016	1.632151345	0.007808096	0.00135428	0	0
2	32.2544854	0	0.267872174	1.089951793	0.002812726	0	0	0
3	28.79738313	0	0.249594606	0.87932823	0	0	0	0
4	20.54373645	0	0.207847724	0.663430616	0	0	0	0
5	33.16966288	0	0.276970882	1.165651691	0.004135047	0	0	0
6	23.06443345	0	0.24846941	0.863961003	0	0	0	0
7	18.60902438	0	0.196003626	0.606958045	0	0	0	0
8	52.18177826	0.033475795	0.890332646	2.714834448	0.02026743	0.04120006	0	0
9	46.34986684	0.010662036	0.593014895	2.058516964	0.010348803	0.009134141	0	0
10	38.76020937	0	0.334029643	1.415147731	0.007481196	0	0	0
11	37.72366219	0	0.305437454	1.308677899	0.006213855	0	0	0
12	0	0	0.025465718	0	0	0	0	0
15	29.72174992	0	0.254090008	0.942955682	0.000205278	0	0	0
16	43.30124512	0.003053614	0.392184436	1.763491457	0.008316927	0.002215132	0	0
17	39.05989079	0.001545521	0.366266971	1.619380032	0.007512982	0	0	0
18	22.13215623	0	0.213703868	0.698453681	0	0	0	0
19	0	0	0.016676773	0	0	0	0	0
20	0.125880835	0	0.167546115	0.189554577	0	0	0	0
21	30.60685115	0	0.265676116	1.023858112	0.001056058	0	0	0
22	44.74806971	0.005863215	0.456978802	1.81449926	0.00851117	0.002826152	0	0
23	39.05439246	0	0.346799646	1.570379321	0.00750286	0	0	0
24	102.0606519	0.045712846	9.13057813	3.973072311	0.026786471	0.099418858	0	0
25	17.49738068	0	0.178940019	0.605146113	0	0	0	0
26	125.0754088	0.049654613	10.02385286	9.114827578	0.030651141	0.114961411	0	0
28	0.34037282	0	0.170070836	0.261957567	0	0	0	0
29	0.018658798	0	0.110296001	0.151739391	0	0	0	0
30	59.88025793	0.033544028	0.925550322	3.56747712	0.023459572	0.048351736	0	0
31	38.0828628	0	0.307527511	1.311505697	0.007335217	0	0	0
32	19.94157045	0	0.198192466	0.652076947	0	0	0	0
33	0.132806697	0	0.16999913	0.247341394	0	0	0	0
35	4.055565615	0	0.170559184	0.480166467	0	0	0	0
36	46.61826882	0.020409843	0.619311147	2.240019789	0.011997977	0.018540856	0	0
37	45.75939978	0.009528238	0.490043767	1.979007269	0.008676062	0.004109971	0	0
38	32.98078862	0	0.268265987	1.151248332	0.003248611	0	0	0
39	36.44590673	0	0.301886315	1.25728742	0.004178849	0	0	0
40	47.03104157	0.022981072	0.641510973	2.339204917	0.016386772	0.018836335	0	0
41	47.50343411	0.029048647	0.669233244	2.476074712	0.016692507	0.029200642	0	0
42	22.30228751	0	0.244249035	0.789940234	0	0	0	0
43	51.48674713	0.031181464	0.825504615	2.600580797	0.020094974	0.031834315	0	0
44	101.2859245	0.039119944	3.352281223	3.754509171	0.02436518	0.062050391	0	0

Table A5: Spearman correlation coefficients between different variables (three bisphenols). All concentrations for individuals (n=40) are measured in ng g^{-1}

Compound name	Factor	Spearman's co-efficient	T-Value	P-Value
Bisphenol Z	Days of incubation	0.41	2.31	0.03
	Development days	-0.03	-0.21	0.83
	Weight of birds (g)	-0.16	-0.98	0.33
Bisphenol S	Days of incubation	0.13	0.79	0.43
	Development days	0.02	0.11	0.91
	Weight of birds (g)	-0.06	-0.37	0.71
Bisphenol A	Days of incubation	0.29	1.71	0.09
	Development days	-0.28	-1.64	0.11
	Weight of birds (g)	-0.03	-0.18	0.86
Total BPs	Days of incubation	0.29	1.71	0.09
	Development days	-0.28	-1.64	0.11
	Weight of birds (g)	-0.03	-0.19	0.85



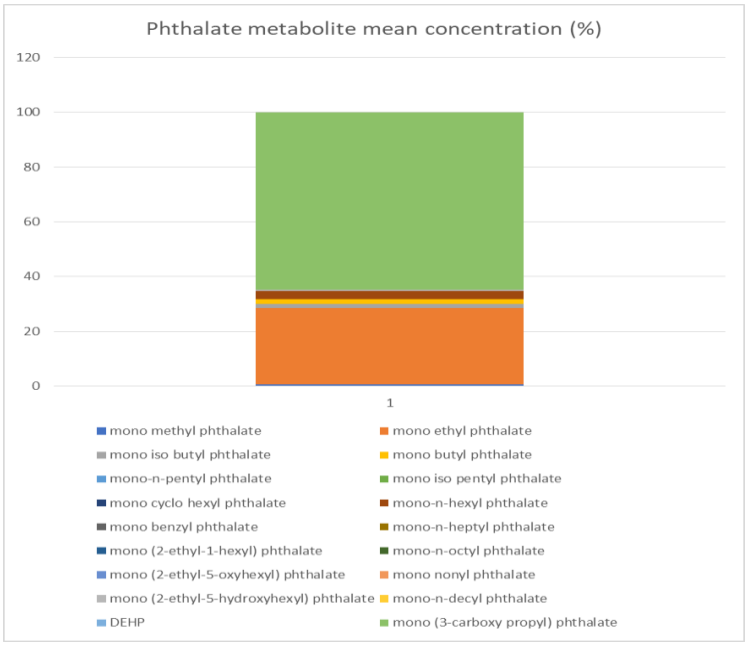


Figure A1: Mean bisphenols (left) and phthalate metabolite (right) concentrations in percentage.

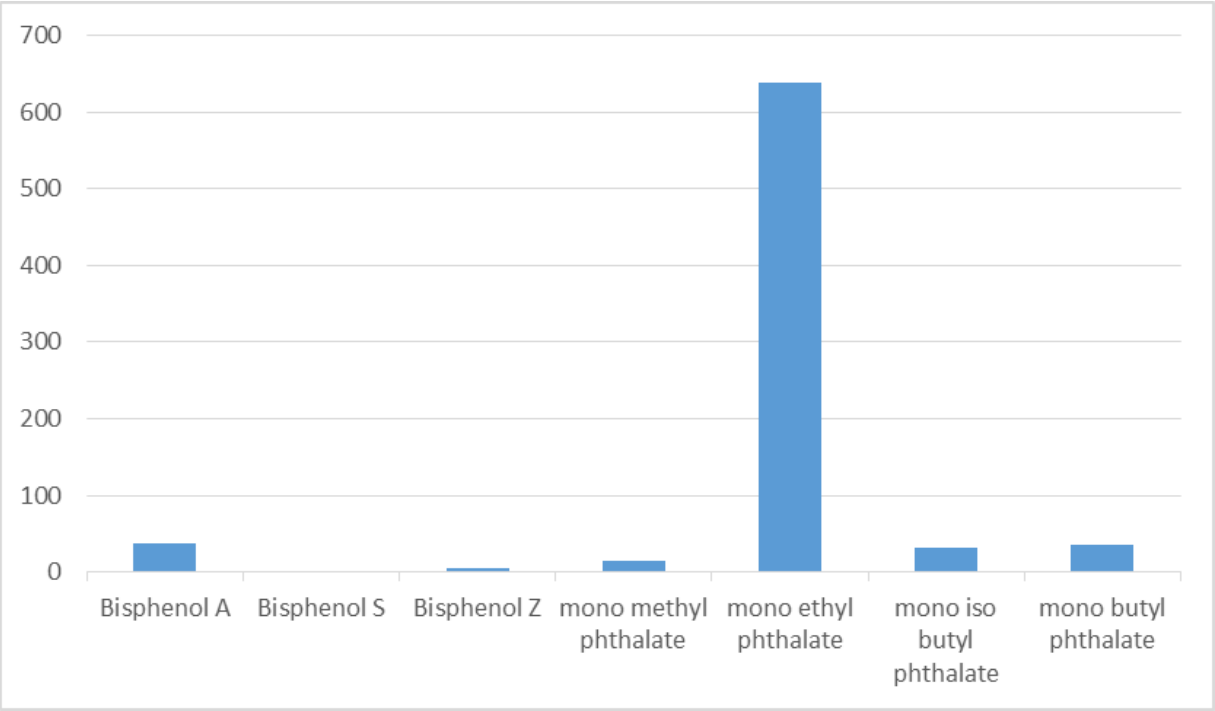


Figure A2: Median concentrations of Phthalate metabolites and BPs found in vulture chicks

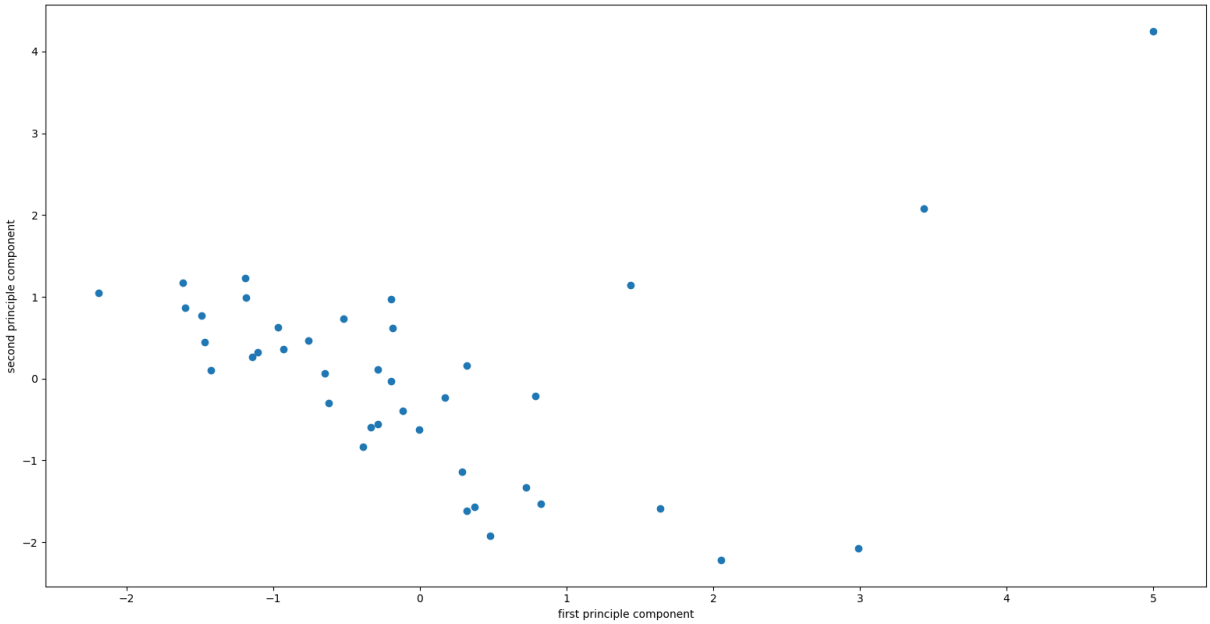


Figure A3: PCA scores plot between mMP, mBP, mIBP, BPS and BPZ.

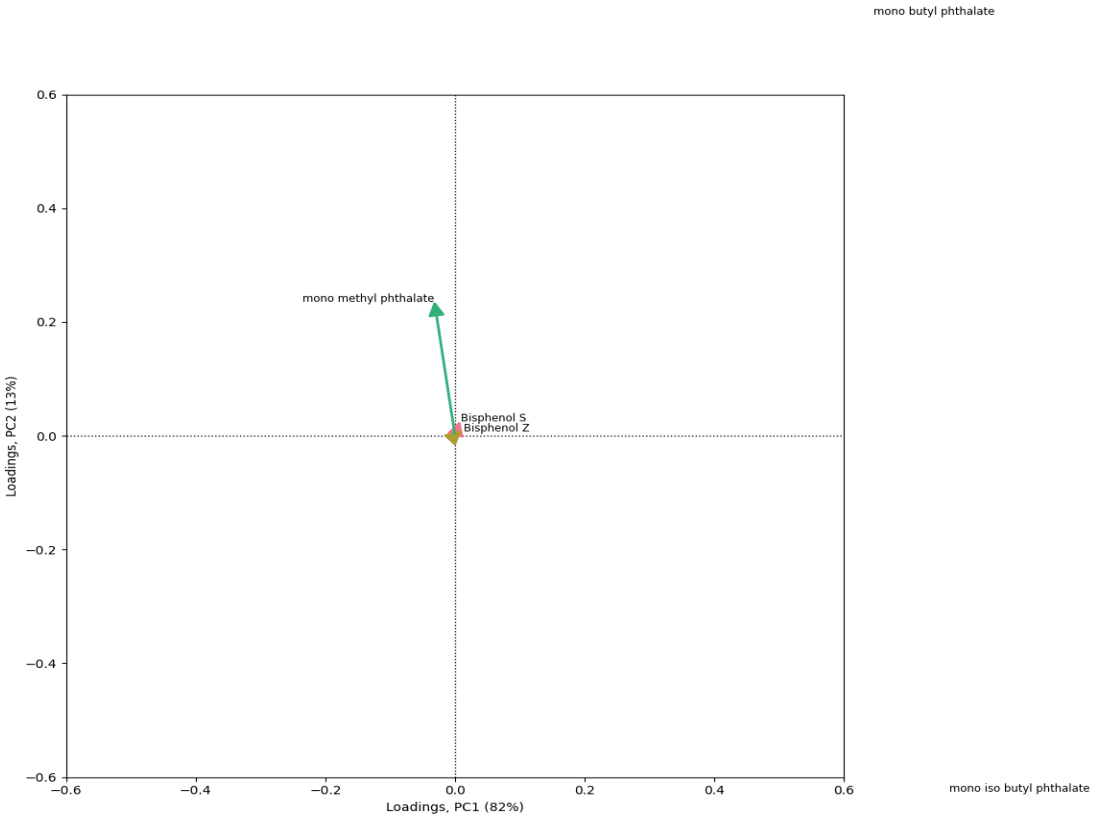
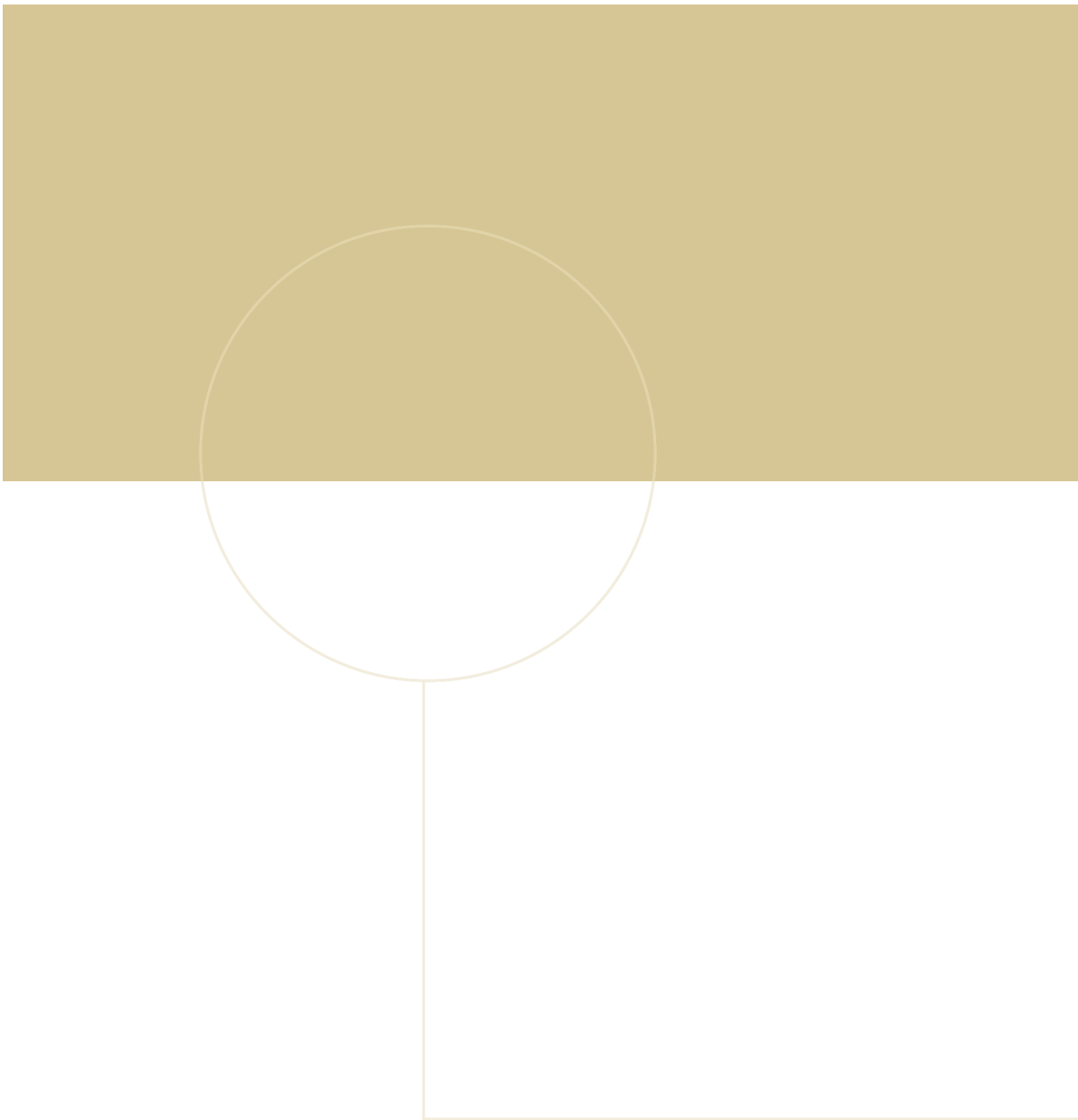


Figure A4: PCA loadings plot between mMP, mBP, mIBP, BPS and BPZ.



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