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Regurgitated pellets as a reliable tool for White stork (*Ciconia ciconia*) food selection and plastic ingestion determination.

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ABSTRACT

Plastic pollution is a global problem which impacts not only on the marine environment but also on the terrestrial one. Few studies have considered how plastic is being ingested by land birds whilst they are being affected both physically and in their endocrine system. By collecting regurgitated pellets from a white stork (*Ciconia ciconia*) colony in the Community of Madrid, Spain, we tried to assess, with non-invasive techniques, the number of plastics ingested, their chemical composition and their relation to the use of an open landfill. A 2.58% of the pellet was formed by plastic, being polyethylene and polystyrene the most abundant polymers, identified by Fourier Transform Infrared Attenuated Total Reflectance Spectroscopy (FTIR-ATR). We observed a slight tendency where the total amount of ingested plastics increased with the use of the landfill in the same way that it decreased with the age of the individuals, meaning older individuals (expert breeders) ingested less plastic than younger ones. The inexperience in young breeders leads to a higher plastic consumption than the older individuals, due to a less efficient trophic selection.

KEY WORDS

Land birds, landfill use, plastic pollution, trophic efficiency, young breeders.

RESUMEN

La contaminación por plástico es un problema global que altera no solo los hábitats marinos sino también los terrestres. Muy pocos estudios consideran la ingesta de plásticos por aves terrestres mientras que estas se están viendo afectadas tanto físicamente como a nivel del sistema endocrino. Se recogieron egagrópilas de una colonia de Cigüeña blanca (*Ciconia ciconia*) en la Comunidad de Madrid, España, para intentar evaluar con técnicas no invasivas las cantidades de plástico ingeridas, así como su composición química y su relación con el uso de vertedero a cielo abierto. Las egagrópilas estaban formadas por un 2,58% de plástico, siendo los polímeros más abundantes el polietileno y el

poliestireno, identificado por Espectroscopia Infrarroja Transformada de Fourier con Reflectancia Total Atenuada (FTIR-ATR). Se observó una ligera tendencia donde la cantidad total de plásticos ingeridos aumentaba con el uso del vertedero, de la misma forma que descendía con la edad de los individuos; lo que significa que aquellos de mayor edad (reproductores expertos) ingerían menos plástico que los más jóvenes. La inexperiencia en los reproductores jóvenes provoca un consumo más alto de plásticos que en los individuos más longevos, debido a una menor eficiencia trófica.

PALABRAS CLAVE

Aves terrestres, contaminación por plástico, reproductores jóvenes, uso de vertedero, eficiencia trófica.

INTRODUCTION

The global impact that human activities have on the ecosystems is one of the main characteristics of the Anthropocene (Lewis & Maslin, 2015). Currently, several factors threaten biodiversity such as climate change or global pollution. Among many synthetic products that profoundly affect the environment, plastic is one of the main ones. Some negative consequences of these contaminants are due to the fact that they practically do not degrade or do so very slowly, depending on their composition (Chamas et al., 2020). Additionally, there are currently no recycling methods effective enough to remove these materials in the quantities in which they are produced. It was estimated that there will be around 1200 millions of tons of plastic in the environment by 2050 (Welden, 2020).

Plastic has an impact on lots of animal species in different ways: a) new colonisations, as sessile fauna could travel through the ocean in plastic waste (Barnes & Milner, 2005); b) wounds, entanglements, or movement limitations that do not allow proper progress of the individual's life (Jepsen & de Bruyn, 2019); or c) ingestion by similarities between plastics and real food (Santos et al., 2021).

Not only are there physical aspects that affect the individuals but also chemical and hormonal ones, such as hepatic stress, impaired development, or bioaccumulation (Alabi et al., 2019). Nonetheless, some authors state that plastic ingestion might not be so harmful as it is usually claimed (Roman et al., 2019).

There are several types of plastic polymers, which can even be mixed to synthesize new materials. Most used ones are polypropylene (PP), polystyrene (PS), high-density polyethylene (HDPE), low-density polyethylene (LDPE), polyvinyl chloride (PVC), polyethylene terephthalate (PET), etc. Previous research shows that many of these polymers might affect animals and their conditions by causing endocrine disruption, growth alteration, or even mortality (Lithner et al., 2012; Rochman et al., 2014; Au et al., 2015; Horn et al., 2020; Estrela et al., 2021). In addition to the compounds used in the manufacture of plastic polymers (monomer, crosslinker, etc.), they may contain derivatives and additives, known as plasticizers, which are chemical substances added to plastics to improve some characteristics such as lowering the processing temperature, improving ductility, or to act as lubricants (Bialecka-Florjaczyka E. & Florjanczyk Z., 2007). An example is organophosphorus ester (OPE) which can be easily found in the environment and it is identified as carcinogenic or neurotoxic (Wang et al., 2020). These plasticizers could be released while they are synthesized, processed, used and displaced (Bialecka-Florjaczyka E. & Florjanczyk Z., 2007).

Once plastic gets to the environment, it might split into smaller fragments: microplastics and nanoplastics, with a size of 0.1 – 100 µm and 1 – 100 nm, respectively. These particles might also get into the system as a residue from the cosmetics industry, pharmaceuticals industry, or some other type of sector (Evode et al., 2021).

Most research about plastic pollution and its impact on fauna is based on the marine environment (Reusch, 2014; Jagiello et al., 2019; Peng et al., 2020; Santos et al., 2021). Up until this day, studies on land are scarce, however, the effects on fauna should be addressed since large quantities of plastics, microplastics and their derivatives end up on the soil (Bläsing & Amelung, 2018). Some bird species have evolved to a close contact with humans. Therefore, they can be used as bioindicators to estimate the environmental pollution (Furness &

Camphuysen Furness, 1997; Burger & Gochfeld, 2004). On an urban landscape, generalist species benefit from opportunities created by humans; for instance, feeding from organic waste or supplementary food, nesting in urban infrastructures, the lack of natural predators or the heat island effect (Anderies et al., 2007; Clavel et al., 2011; Seress & Liker, 2015; Tryjanowski et al., 2015; Katlam et al., 2018).

The white stork (*Ciconia ciconia*) is a migratory bird which had an increase on its populations due to the use of open landfills as a feeding source, agricultural methods and reintroduction and conservation projects (Tortosa et al., 2002; Martí, 2003; Molina & del Moral, 2005). Growth in the population numbers would not have been possible without open solid waste landfills, since they provide nourishment in an abundant and predictable way for many bird species (López-García & Aguirre, 2023). However, the landfill usage might lead to an ingestion of plastics, which could then interfere with the animal system.

Regurgitated pellets are indigestible residues of food formed in the bird's gizzard that many species eliminate some hours after the ingestion. It is composed of bones, exoskeletons of insects, and other organic and inorganic materials (Winkler et al., 2020). Thanks to the analysis of the chemical composition, the presence of plastics and their types of polymers can be verified, as well as other parameters such as the amount of mineral or organic matter that make up the pellet. Pellets might be a good sampling method of immediate food intake of a particular individual. Nevertheless, being able to access to the whole digestive system could give a more exact view of the plastic consumption (Provencher et al., 2019). As animal welfare should always take precedence, non-invasive testing is essential, this way the individual can carry on their vital activities with no harm. So, the use of regurgitated pellets is an interesting choice for living white storks.

The aim of the present study is to assess the presence of plastics and microplastics in regurgitated pellets of a bioindicator species, such as the white stork, and to relate it to the landfill use of each individual. An attempt will also be made to assess whether the size and composition of the pellets are determined by the dietary preferences of particular individuals.

Due to the plastic debris that can be found in high densities in landfills (Zhou et al., 2014), we predict that the collected samples will contain fragments of plastic and other anthropogenic materials. In the same way, we predict that those individuals that use the landfill more often will produce pellets with a higher plastic content.

MATERIALS AND METHODS

Study area

Our study area was established in a white stork colony located in Soto del Real (40.74º N, 3.89º W), Community of Madrid, Spain; 12 km away from an open landfill (Colmenar Viejo, 40.66° N, 3.72º W), where white storks regularly feed. Since 1999, stork fledglings at the colony have been marked with PVC rings. In addition, weekly visits to the nearby landfill have been performed allowing identifying rings from feeding individuals inside of it. This way, it is possible to establish a landfill use index (LUI) for each individual, as a coefficient between the number of sightings of one particular bird and the number of total visits from a specific period of time (López-García et al., 2021).

To identify individuals breeding at each nest of the colony by their PVC rings, camera traps were used. In total, we collected 125 adults' regurgitated pellet samples (which 23 of them had their composition analysed) from 71 nests. Since this species shows assortative mating behaviour (Barbraud & Barbraud, 1999; Jagiello et al., 2018), we assumed both members of the breeding pair would make similar exploitation of the feeding habitat and, therefore, will have a similar LUI.

Procedure for sample analysis

Pellet samples (n=125) were collected in the surface of the nest during the period prior to egg hatching to ensure they belonged solely to breeding individuals. They were immediately frozen at -18 ºC until analysis. Once defrosted they were dried at 50 ºC for 24 hours before processing, following the protocol of Provencher et al. (2019). Once they were all dried up, we weighed and measured them to obtain a volume value (height*width*length in mm^3). Then, we disaggregated them manually with metal tweezers. Large plastics and identifiable organic and inorganic materials such as arthropod exoskeletons, bones, glass or pebbles were separated. These larger plastics were stored in Petri dishes for further analysis and identification.

To isolate possible smaller plastics and microplastics masked by organic matter, a digestion study was carried out in different media: basic, acidic and oxidizing, capable of destroying organic matter without destroying or damaging the plastics. In order to do this, the samples, already free of large particles, underwent different digestions, hot and with constant orbital agitation, using the following attacking mixtures described in the literature: a) Fenton's reagent (0.05M $FeSO_4$ and H_2O_2); b) 20% potassium hydroxide (KOH) and 30% hydrogen peroxide (H_2O_2); and c) 30% KOH, 30% H_2O_2, (Prata et al., 2019; Provencher et al., 2019).

Given the diversity of the samples under study (some contained a large amount of vegetal and animal material while others did not), none of the attacking reactive mixtures was effective in the total removal of organic matter for all the samples studied. Subsequently, a 30% nitric acid (HNO_3) solution was used, which markedly improved digestion, but not enough. Continuing with the optimization, the best digestion was based in the use of 20% HNO_3 and 30% H_2O_2 mixture as the attacking reagent, heating to 45 ºC and with orbital shaking at 120 rpm for 15 hours. This digestion allowed to eliminate the organic matter without damaging the plastics and thus to be able to isolate the possible plastic materials present in the samples.

To ensure that this reaction did not interfere with the plastic composition, we carried out a control digestion in identical conditions of several polymers such as polyvinyl chloride, PVC; polypropylene, PP; high-density polyethylene, HDPE; low-density polyethylene, LDPE; and polystyrene, PS. We weighed two samples per polymer before the process and did it again afterwards remaining constant, which confirmed the polymers were not affected by the attacking reagent.

The samples, already digested, were filtered on filter paper with a Büchner funnel and a Kitasato flask. The retained part on the filter was dried and the plastic materials were extracted and deposited in a Petri dish. Before carrying out the analysis, the plastics and microplastics that had been found were cleaned with methanol (CH_3OH) in an ultrasonic bath for 20 minutes.

Visual identification of plastics and microplastics was carried out using a stereoscopic microscope (Motic SMZ-171) with a camera (Moticam S6) to increase its visualization and to take measurements and photographs of the plastic particles. Height and width of each plastic were measured to obtain a total area value (total sum of height*width in mm^2) for each pellet sample.

For the identification of the types of polymer that make up the plastics and microplastics found in the samples, Fourier transform infrared spectroscopy with attenuated total reflectance (FTIR-ATR) (Jasco FT/IR-4100) was used. The obtained infrared spectra from the possible plastic and microplastic particles were compared with a library of reference spectra, created by own measurements in the FTIR-ATR with standards. It is shown in Figure 1, as an example, the comparison of the sample and standard infrared (IR) spectra.

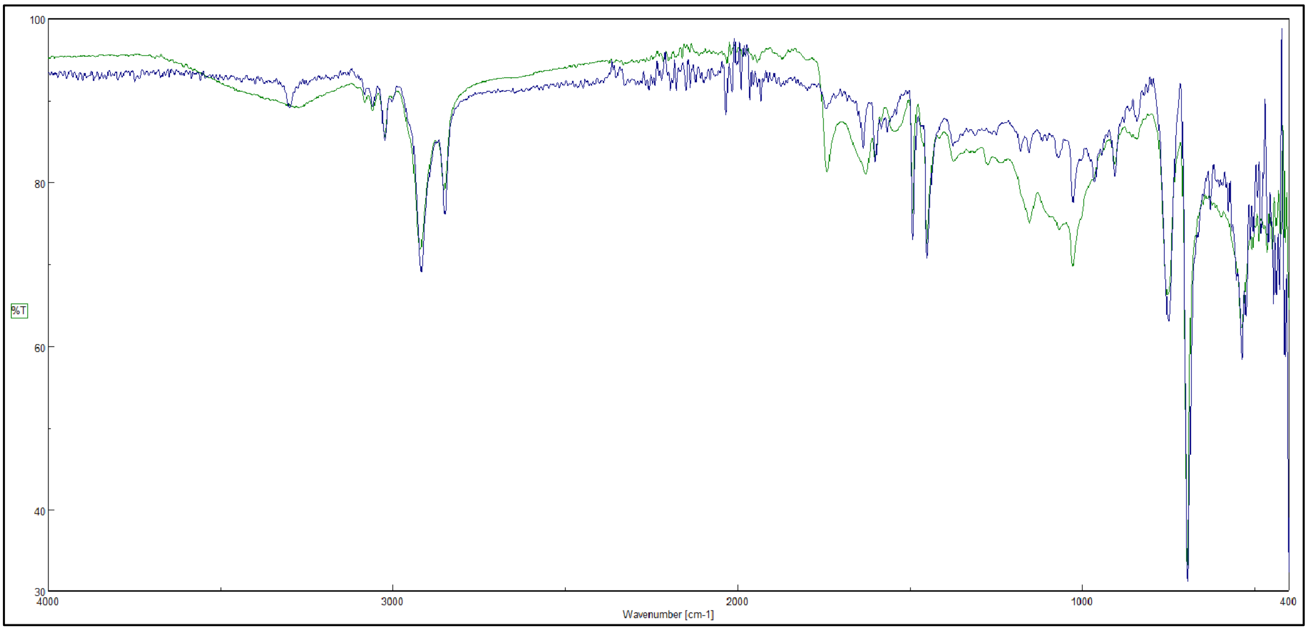

Figure 1. Spectrum of polystyrene standard IR spectra (blue line) and sample 11 (green line). Overlay confirms the same composition.

Statistical analysis

To characterize pellet size, we constructed a General Linear Model, GLM, with weight of the regurgitated pellet as a response variable and the volume of the pellet as an explanatory one. To determine if the use of the landfill or the individual's age had an influenced in the pellet's weight, another GLM was performed with the weight as a response variable and the age and LUI (landfill use index) were used as covariates. In this model we classified the age of each individual in three categories: 1) young breeders, YB, from 2 to 6 years old; 2) mature breeders, MB, from 7 to 11 years old; and 3) expert breeders, EB, from 12 to 24 years old.

A GLM was also performed to establish if landfill usage varies with age. To do so, the LUI was used as a response variable and the age of the marked individuals as an independent one. Finally, a generalized linear mixed model (GLIMMIX) was performed to determine differences in the total area of the consumed plastics, which was used as the response variable. The age and the LUI were used as covariates and the individual as a random factor since there were cases where there was more than 1 sample per individual. The most accurate model following the Akaike Information Criterion (AIC) was chosen by performing a manual backward stepwise procedure to ensure the lowest AIC. RStudio (version 4.2.1) was used to perform these analyses.

RESULTS

Regurgitated pellet characteristics

A total of 125 pellets were measured and weighed resulting in the data of table 1. It was observed that more voluminous pellets had higher weights ($F = 94.4$; $p < 0.0000$), as it is shown in figure 2.

Table 1. Pellet size and volume characteristics

N = 125	Weight (g)	Length (mm)	Height (mm)	Width (mm)	Volume (mm^3)
Mean	11.49	43.06	30.78	23.14	31455.53
Interval (max – min)	25.47 – 3.71	74.99 – 27.74	45.21 – 16.60	38.93 – 11.28	66073.52 – 9723.02

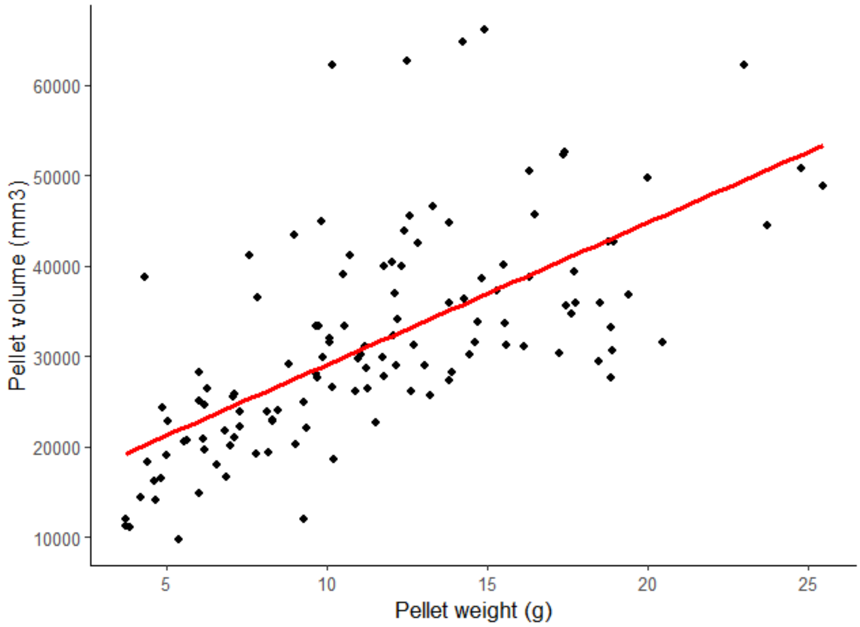

Figure 2. Graph between the weight and volume of the regurgitated pellets.

Regarding pellet composition, 23 samples were analysed. Mineral and organic matter were the majority constituents of the regurgitated pellet. Nonetheless, there was a 2.58% of plastic materials inside them (Table 2). The plastic fraction was mostly constituted by polyethylene (34%), polystyrene (31%) and polypropylene (16%) (Figure 3). In these 23 pellets, 683 pieces of plastic and microplastic were found, measured and analysed to determine their polymeric composition. Figure 4 and 5 show images of plastic elements collected after the digestion process.

Table 2. Pellet composition. Percentage of the mineral fraction, the organic matter fraction and plastic fraction.

N = 23	Mineral	Organic matter	Plastic
Mean	50.94	46.48	2.58
Interval (max – min)	81.01 – 24.50	71.80 – 18.93	13.47 – 0.06

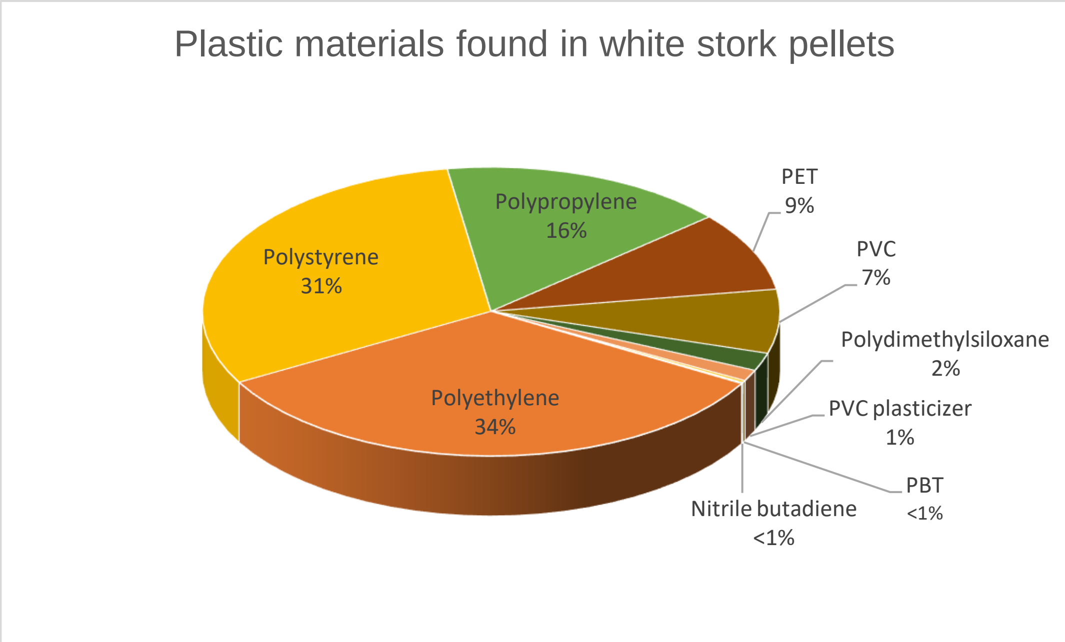

Figure 3. Percentages of the plastic materials found in white stork pellets (N = 23).

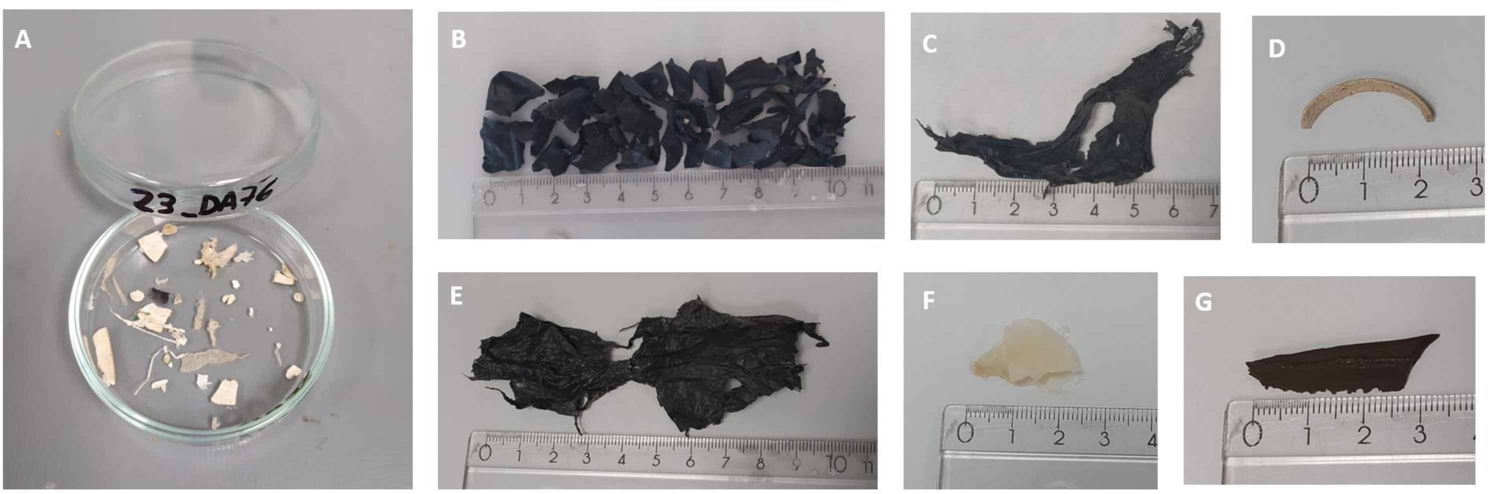

Figure 4. Plastic elements collected from regurgitated pellets after the digestion process. Scale in centimeters. A: Plastics in sample 23. B: Nitrile butadiene. C: Polyethylene. D: Polyethylene. E: Polyethylene. F: Polydimethylsiloxane. G: Polydimethylsiloxane. Note that the same plastic polymer can show different presentations (C, D & E; F & G).

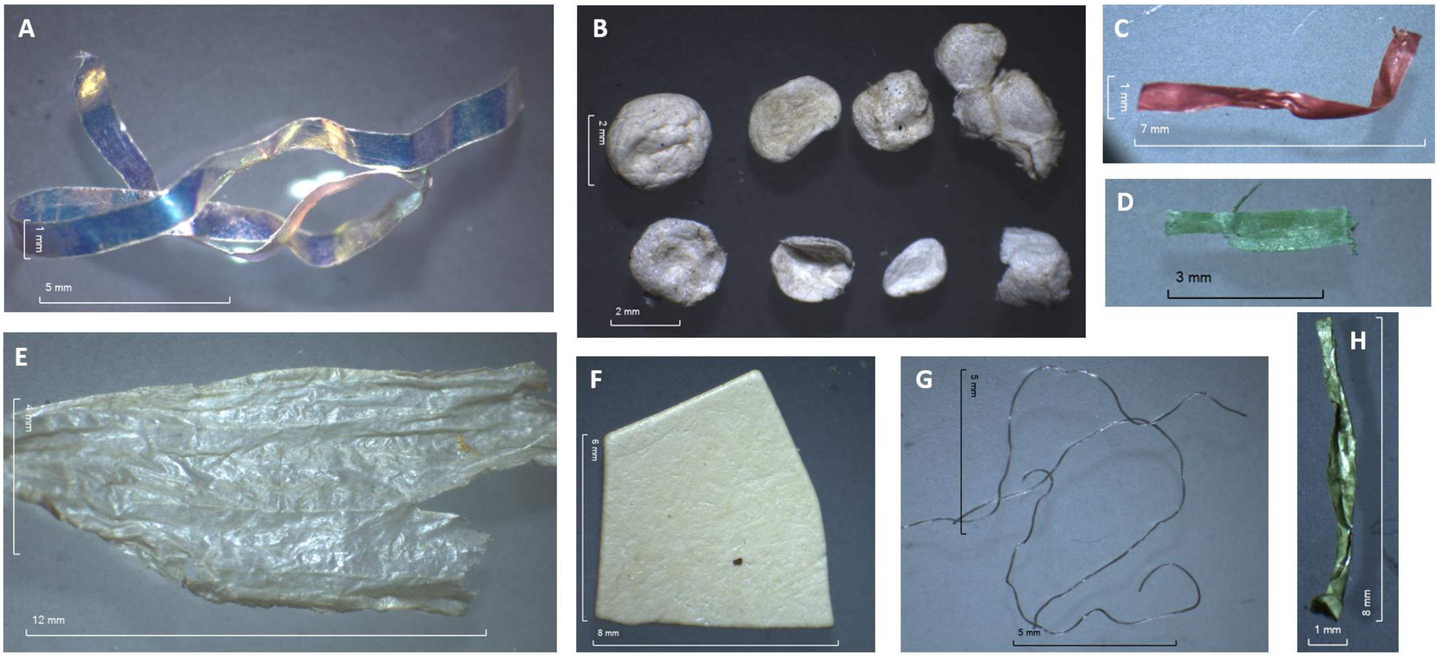

Figure 5. Plastic elements collected from regurgitated pellets after the digestion process. Scale in millimeters. A: PET. B: Polystyrene. C: Polyethylene. D: Polypropylene. E: PVC. F: Polystyrene. G: Polypropylene. H: PVC.

Statistical results

Significant differences in pellets size were found between the individual's ages. A Tukey test showed that larger pellets were produced by older individuals, that is, expert breeders (12 to 24 years old) ($F = 6.0623$; $p = 0.0036$; Tukey test: expert breeders differed from young breeders significantly at $p = 0.0445$ and from mature breeders at $p = 0.0179$), (figure 6). However, the LUI had no effect on the pellet's weight ($F = 0.0077$; $p = 0.9301$).

There were significant differences between the LUI and the ages of the individuals: mature breeders (7 to 11 years old) and expert breeders (12 to 24 years old) showed higher indices than younger breeders (2 to 6 years old), (MB: t value = 2.382, $p = 0.0196$; EB: t value = 2.189; $p = 0.0315$) (Figure 7).

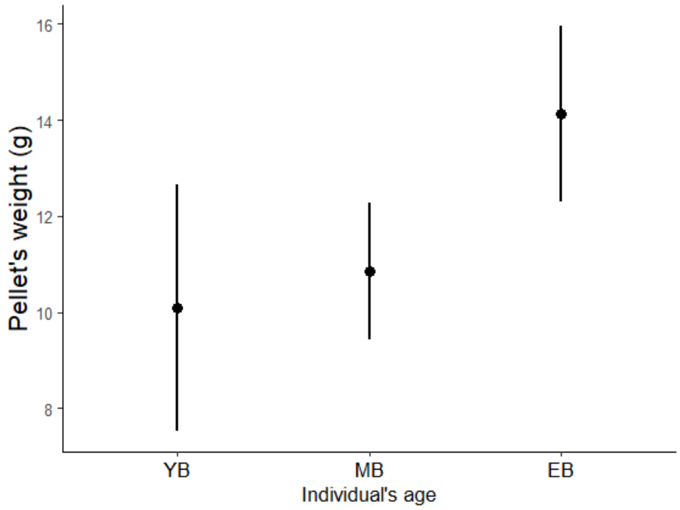

Figure 6. Graph that shows how expert breeders, EB, regurgitate heavier pellets than mature breeders, MB, and younger breeders, YB.

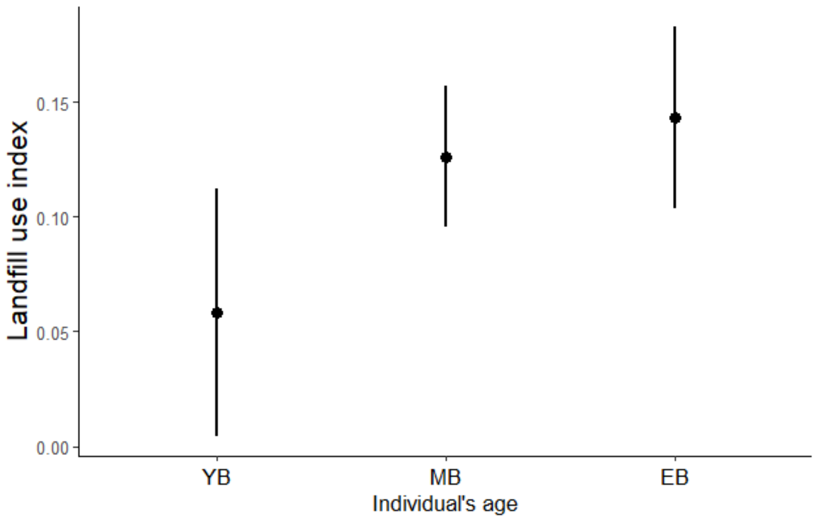

Figure 7. Graph that shows how older individuals (expert breeders, EB, and mature breeders, MB) use the landfill more frequently than younger breeders, YB.

We got no significant differences between the total plastic area consumed and the age ($p = 0.1309$) or LUI ($p = 0.1853$) with the individual as a random factor. Nevertheless, there was a trend where the older the individuals are, they ingest less plastics according to the total plastic consumption area. In the same way, a more frequent use of the landfill translates into more plastics consumed (Figure 8).

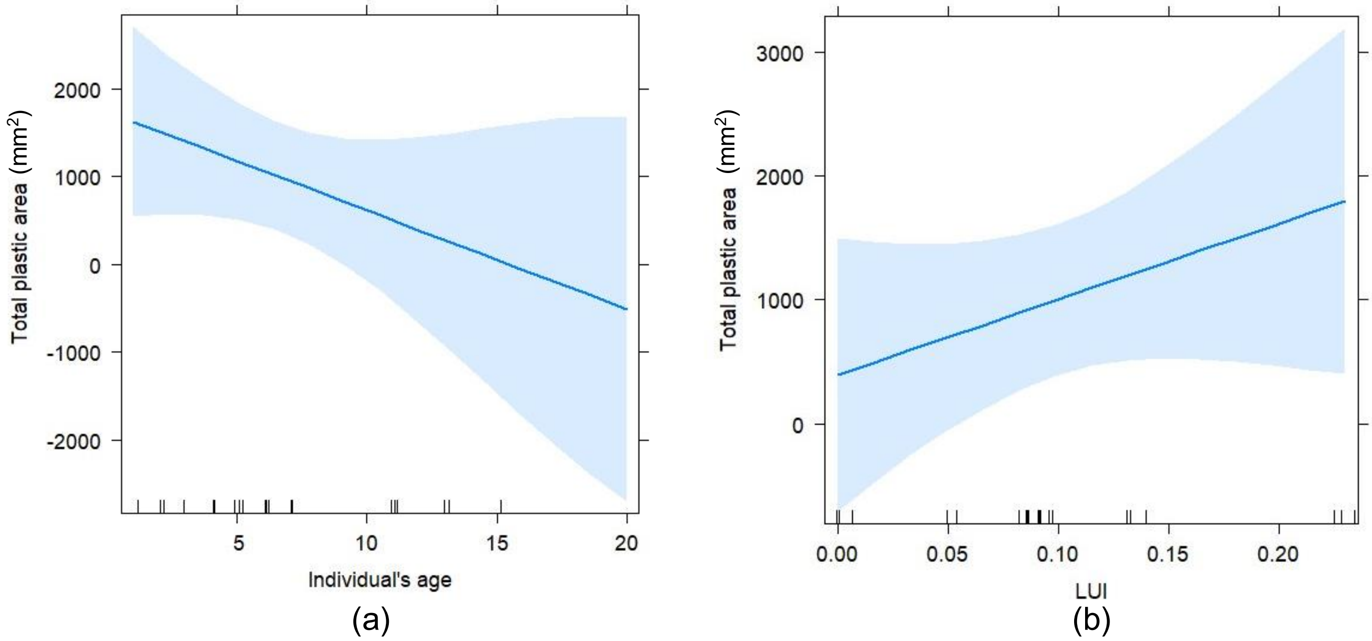

Figure 8. Graphs where it is observed there is a tendency for older individuals to ingest less plastic (a) and another tendency of a higher plastic consumption for those individuals that have a higher usage of the landfill (b).

DISCUSSION

This research shows that pellets can be a reliable tool to determine food preferences in white storks and potential risks associated with plastics in the environment. The amount of plastic materials, as well as their polymer composition, that white storks ingest in their diet can be assessed. Pellets are stable in their size/volume ratio and expert breeders usually form larger ones than mature and young breeders. The data obtained in this work show that older storks have a higher frequency of visits to landfills whilst young breeders do not visit the landfill so often. Besides, the plastic found in the regurgitated pellet samples does not seem to relate to the usage of landfills, even though we found a soft trend.

Pellet samples weight increased with the volume of the sample and the results show that expert individuals regurgitate larger and heavier pellets than younger and mature ones. Larger pellets might mean more foraging time and greater amount of food returning to the nest, which could imply a longer time spent defending their nest or incubating their eggs. This way they would be reducing the energy cost by going out less frequently. Storks usually have a higher food intake when there is greater food availability, these results would follow an energy-maximizing strategy (Alonso et al., 1991; Zurell et al., 2015). There is no relation to landfill usage so stork pellet's size seems to not be influenced by the type of food individuals can get in it.

Regarding pellet's composition, there are considerable differences in the percentages of plastic within them (max.:13.50%; min.: 0.06%). However, content of plastic material was found in every sample, mainly polyethylene, polystyrene and polypropylene. These types of polymers may generate biochemical changes in the individual's organs leading to complications as seen in different bird species as *Coturnix japonica* with polystyrene ingestion (de Souza et al., 2022), or with polyethylene as well as polystyrene in *Coragyps atratus,* where body conditions remain constant even though biochemical alterations occur (Cunha et al., 2022). Besides, plastics are a potential means of transport for contaminants and chemicals due to their size and to their absorption characteristics (Thompson et al., 2009), which can affect wildlife by releasing those inside the animals' system. White storks' plastic consumption may be causing some disruption in their body conditions still unknown. Moreover, it can lead to the individual's death as seen by Henry et al. (2011) in white storks or by Pierce et al. (2004) in guillemots, as a result of the plastic ingestion. As it was mentioned above, not only the chemical components that make up the plastic or the contaminants are a problem for the organism, but also the shape (sharp, splintered, etc.) and the hardness of the polymer can cause obstruction and other damage to the animal's digestive system.

After the study was carried out, no significant differences were found between the total area of the plastics ingested and the age of the individuals. However, there was a marked tendency where older white storks consumed less plastics, which Peris had already noticed in 2003. This trophic efficiency could be explained since younger individuals may have more trouble than experimented ones when it comes to distinguishing between edible and non-edible matter, as Day reported in 1980 relating it to a less efficient foraging strategy due to inexperience. In a long living species, there is a process of learning and optimizing the determination of which elements are acceptable as food (naïve consumers, as reflected in Acampora et al. (2014)), when they are inexperienced there may be a less effective feeding as it has also been noticed in other species (Stalmaster et al., 1984).

Additionally, although not entirely significant, it was observed that there was a trend that the higher the landfill usage is, the greater amount of plastic is consumed. Plastic debris in landfills is highly common (Zhou et al., 2014), which might facilitate plastic consumption if the individual cannot easily differentiate between elements, as if the food is wrapped in plastic films or if it has small plastic items stuck to it. For instance, once the white stork colony has a greater distance to the landfill than the typical foraging area, fewer plastic materials are found in the stomach of the individuals (Henry et al., 2011). This can be related to the trend that the data show. Nonetheless, further research should be needed to confirm this tendency. In case this hypothesis is not proven, it would mean storks are still feeding on plastic even though there is not a clear plastic source such as the open landfill.

On the contrary of some authors (Sanz-Aguilar et al., 2015), our data show that expert and mature breeders have a higher frequency of visiting the landfill than younger ones. The greater feeding efficiency of older breeders is shown by the fact that, despite more frequent visits to the landfill (a potentially hazardous area for plastic ingestion), the total plastic area per pellet is lower than in younger, less experienced breeders. Usually, young breeders tend to feed more often in these places due to the constant supply of food and the easiness of it (Peris, 2003; Pineda-Pampliega et al., 2021; López-García & Aguirre, 2023), whilst older individuals are more likely to forage in wet meadows or pastures, the optimal place for white storks (Johst et al., 2001; Nowakowski, 2003). The year the study was carried out (2023) there was an intense drought on the study period months (March, April and May) (Agencia Estatal de Meteorología, 2023a,b,c) which could have altered, in part, the data obtained. The lack of resources for white storks might have forced adults to feed on the landfill, modifying our results.

It is essential to have non-invasive tools that allow the research of plastics without needing the animal's corpse, like it is needed to study the whole digestive system. By using regurgitated pellets and keeping track of the plastics or some other found materials like construction debris, metal elements, clothes, etc., it is possible to assess the anthropogenic impact. Not only the general effect on birds but also on the ecosystem, and therefore on wildlife, as the presence of these components in pellets imply an availability on the environment. These elements could be gathered from synthetic sources (such as open landfills) or from natural landscapes, meaning they are polluted and in need of protection. It is an interesting starting point for future research since plastic on terrestrial birds is not so studied as on the marine environment.

CONCLUSION

This research remarks the importance of regurgitated pellets as a means of plastic pollution study. Synthetic polymers such as polystyrene or polyethylene, which were the most abundant elements in the collected samples, have been reported to have a negative impact on birds by altering organs or biochemical reactions.

Whilst being non-significant, we found a tendency where older individuals (more experimented ones) consumed less plastic than younger ones, which could be related to their experience in foraging edible matter. In relation to the landfill usage, there is a trend where there is a larger consumption of plastic when individuals have a higher landfill frequency. Nonetheless, this result is not significant. If white storks are feeding on plastic regardless of their LUI, it would mean that many natural sources are highly polluted with plastics, up to the point where they easily feed on it. Another possibility is a change in behaviour where white storks are purposefully ingesting plastics. In order to clarify this result, further research should be carried out.

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