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Feeding in the garbage: physiological and microbiological consequences of using landfills as food resource in the white stork (Ciconia ciconia)

Comer en la basura: consecuencias fisiológicas y microbiológicas de usar los vertederos como fuente de alimento en la cigüeña blanca (Ciconia ciconia)

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

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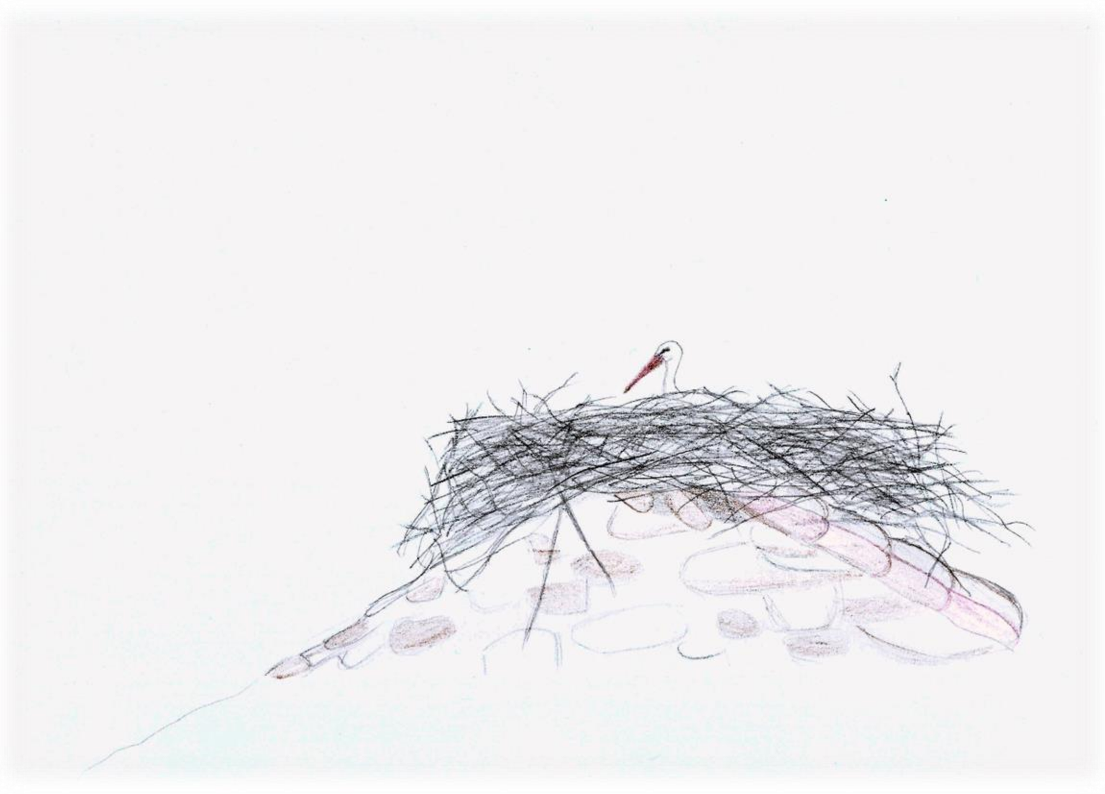

Incluso la noche más oscura terminará con la salida del Sol
Victor Hugo (1802 – 1885)

Aquellas personas que piensan que lo saben todo, son una gran molestia para aquellos de nosotros que lo estamos intentando.
Isaac Asimov (1920 – 1992)

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He hablado del laboratorio, pero claro, las muestras no vienen solas. Muchas gracias a todos los que me habéis ayudado en el campo. Siempre he podido contar con vosotros, me habéis ayudado con una sonrisa, y claro, ¡he conseguido las muestras! Además, nunca olvidaré el primer día "de buitres": cuestas infernales, caminos de cabras, acabar el día corriendo en mitad de la sierra porque había tormenta... He de confesar que más de una vez pensé "no vuelvo más, bueno, si sobrevivo, no vuelvo más", ¡pero al día siguiente estaba allí otra vez! Y otro, y otro…, y seguiré volviendo, porque es fantástico.

Creo que una parte esencial de la tesis son las estancias. El tiempo que pasé en Groningen me ha permitido ver el mundo (académico y personal) con otros ojos, enseñándome la importancia de viajar, no solo de vacaciones, sino a vivir otra cultura y forma de vida, y me ha permitido conocer a amigos que espero que sean para toda la vida. Gracias por abrirme las puertas de vuestro laboratorio, vuestro grupo, e incluso vuestras casas; ¡si hasta he celebrado el día de Navidad con vosotros! Muchas gracias a todos los que me ayudasteis allí, dentro y fuera del laboratorio, ya fuese evitando que me afectase la radioactividad, explicándome lo que decía la radio en neerlandés, enseñándome como hacer gráficas, recordándome como se monta en bici, remarcando lo bonitos que son los mirlos o haciéndome sentir como en casa a cientos de kilómetros de la mía. A la "familia", ¡espero volver a dormir en vuestra nueva casa, que no hay que romper las tradiciones! *In my opinion, an essential part of the PhD is the stays. The time I spent in Groningen has allowed me to see the world (academic and personal) from a different point of view, discovering me the importance of travelling, not only on vacation, but to live another culture and way of life, and has allowed me to meet friends who I hope they are for life. Thank you for opening the doors of your laboratory, your group, and even your houses. I have even celebrated Christmas with you! Thank you very much to all who helped me there, inside and outside the laboratory: by preventing radioactivity from affecting me, by explaining what the Dutch radio said, teaching me how to make good graphs, reminding me how to ride a bike, pointing out the beauty of blackbirds or making me feel at home hundreds of miles from mine. To the "family", I hope to stay again in your new home, we have to continue the tradition!*

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Abstract

This PhD thesis aimed to establish if the use of landfills as a food resource by white storks, which a priori, judged by the increasing population appears positive, could have any effects on the health status of nestlings. For this purpose, initially, we evaluated the status of individuals which were fed different proportions of food from landfills, using a multidisciplinary approach based on the evaluation of nutritional status, liver and renal function, oxidative stress balance and the presence of pathogens. This way we identified that, in fact, the higher the proportion of food from landfills the better the nutritional status, indicating higher food intake. Hepatic and/or renal function were not affected. Also, oxidative stress balance, which is more sensitive to damage by the potentially present pollutants in the landfill, is suggestive of a hormetic response; i. e. an increase in defences that allows to cope with limited damage without compromising the organism. Landfill foraging storks also have a higher prevalence of antibiotic resistant *E.coli*, which could be a problem not only for the health of the storks, also for the transmission of these microorganisms to other wildlife, livestock and humans.

After obtaining a broad overview of the effect of this use, we tried to confirm previous results at a much larger scale, increasing the number of colonies of study, and more importantly, to verify if the benefits hold, regardless of the year of study. In the second chapter, we confirmed that the benefits on nutritional status of nestlings which were fed with food from landfills are constant between years, as well as the apparent lack of affectation on hepatic and/or renal systems. However, the response of the oxidative stress balance was not always the same, because of dietary antioxidants and the necessity of natural food during the first period of life. Even so, the use of landfill

foraged food never produced a disbalance in the oxidative stress balance, which indicates that, despite the year, the potential damage could be perfectly counteracted by the organisms of the nestlings.

To make the monitoring of these colonies in the future easier it is necessary to find some external measures which could be related to the use of landfills, making the collection of data less invasive and less challenging in terms of cost and time. We found that both body condition and the redness of legs are affected by the use of landfills but with an opposite trend, as body condition increases with the use, due to the higher amount of food, redness of legs decreases, by the lower amount of carotenoids, responsible of the redness. These results could be useful to continue the monitoring of the studied colonies with less expenditure of resources, time, and money.

These results revealed at least the short-term benefits of the use of landfills as a food resource. However, we did not know if this behaviour would have carry-over effects, as early-development conditions can influence different life-history traits later in life. As the monitoring of these individuals during their entire life is practically impossible, as a proxy to this effect we chose to measure telomeres, as their length has been suggested to be responsible for the link between early-life conditions and potential lifespan, and because their rate of attrition could be altered by external factors which act through oxidative stress balance. We approached this question experimentally, and our results, showed that the administration of antioxidants in free-living individuals maintains telomeres longer, suggesting that possibly these individuals suffer a lack of dietary antioxidants, which in turn could affect their potential lifespan.

The use of these multidisciplinary approaches to the study of the status of certain species, such as the white stork, is becoming increasingly necessary. This is mainly due to progressive globalization, which will lead to a greater number of interactions between different species and humans.

Resumen

El objetivo de esta tesis era establecer si el uso de vertederos como fuente de alimento por las cigüeñas blancas, el cual a priori es beneficioso, tal y como demuestra el aumento en la población, podría tener efectos en la salud de los pollos. Con este propósito, evaluamos el estado de individuos alimentados con distintas proporciones de comida proveniente de vertederos, utilizando una aproximación múltiple basada en la evaluación del estado nutricional, de la funcionalidad hepática y renal, del balance del estrés oxidativo y de la presencia de patógenos. Este primer paso nos permitió identificar que, efectivamente, según aumentaba la proporción de comida proveniente de vertederos, mejor era el estado nutricional de los pollos de cigüeña, lo que indicaba una mayor ingesta de comida. Además, no encontramos efectos negativos en el organismo, al no verse afectada ni la función hepática ni la renal. El balance del estrés oxidativo, que es mucho más sensible al daño por los posibles contaminantes de los vertederos, muestra los primeros pasos de una respuesta hormética; aumento de las defensas por un daño, pero que se soluciona sin comprometer al organismo. Respecto a la presencia de patógenos, la presencia de *E. coli* resistente a antibióticos es mayor en individuos que utilizan vertederos, lo cual puede ser un problema no solo para su propia salud, también por la transmisión de estos microorganismos a los animales de vida libre, al ganado y a humanos.

Tras obtener una visión amplia de los efectos de este uso, tratamos de confirmar los resultados previos a mayor escala, aumentando el número de colonias de estudio, y principalmente, verificar si los beneficios se mantienen independientemente del año de estudio. En el segundo capítulo, confirmamos que los beneficios en el estado nutricional en los pollos que se alimentan en vertedero son constantes entre años, así como la falta de afectación hepática o renal. Sin embargo, la respuesta del balance del estrés oxidativo no es siempre la misma, debido a la importancia de los antioxidantes en la dieta y la necesidad de comida natural durante el primer periodo de la vida. Sin embargo, el uso de este tipo de alimento nunca produce

un desorden en el balance del estrés oxidativo, lo que indica que, sin importar el año, el daño potencial está siendo contrarrestado.

Para facilitar el seguimiento de estas colonias en el futuro es necesario encontrar alguna medida externa que varíe en relación con el uso de vertederos, haciéndolo menos invasivo y costoso en términos de tiempo y dinero. Encontramos que tanto la condición corporal como el color rojo de las patas se han visto afectadas por el uso de vertederos, pero en sentido opuesto, ya que la condición corporal aumenta con el uso, debido a la mayor cantidad de comida, pero el color rojo de las patas disminuye, debido a la menor cantidad de carotenoides, responsables del color rojizo. Estos resultados pueden ser útiles para continuar el seguimiento de las colonias de estudio de forma menos invasiva y con menor gasto económico.

Estos resultados revelaron el beneficio a corto plazo del uso de los vertederos como fuente de alimento. Sin embargo, no sabemos si este comportamiento podría tener efectos a largo plazo, ya que las condiciones durante el desarrollo pueden influir en rasgos que aparecen más tarde en la vida. Como el control de estos individuos durante toda su vida es prácticamente imposible, y como aproximación a este efecto hemos elegido medir los telómeros. A estos elementos se les sugiere como responsables de unir las condiciones durante la juventud y la esperanza de vida potencial, y cuya tasa de acortamiento puede verse alterada por factores externos que actúan a través del balance del estrés oxidativo. Nuestros resultados, que muestran que la administración de antioxidantes en individuos de vida libra mantiene sus telómeros más largos, podrían sugerir la posibilidad de que estos individuos padezcan una falta de antioxidantes en su dieta, lo que podría potencialmente afectar a su esperanza de vida.

El uso de estas aproximaciones multidisciplinares al estudio del estado de determinadas especies, como es el caso de la cigüeña blanca, se está haciendo cada vez más necesario. Esto se debe principalmente a la progresiva globalización, lo que conllevará un mayor número de interaciones entre distintas especies y el ser humano.

General introduction

Human activities have been one of the major drivers for modification of our planet for centuries (Plaza & Lambertucci, 2017). In particular, land-use activities, which have transformed the landscape across the world through the clearing of forests, the extension of the agriculture, the intensifying of farmland production and the expansion of urban areas (Foley et al., 2005). Despite the differences in land-use practices across the world, the outcome is generally the same: the acquisition of natural resources for immediate human needs, at the expense of a degrading environment (Foley et al., 2005). In fact, it is well known that these processes have transformed the Earth´s surface, seriously affecting ecosystems (Tauler-Ametller et al., 2017 and 2019).

Due to the transformation of natural ecosystems, croplands and pastures have become some of the most widespread biomes on the planet, not only by their extension, but also by the disappearance of forest areas, partly due to transformation into agricultural land and timber extraction (Foley et al., 2005). This phenomenon, along with the increase in the number and size of urban areas (Foley et al., 2005) entails a drastic reduction in the availability of food resources for wildlife (Tauler-Ametller et al., 2017).

However, the opposite scenario, in other words, the improvement of food availability due to the provision of subsidies (food remains produced by humans and exploited by other species) is also quite important to wildlife (Oro et al., 2013). This phenomenon is not new; the first hunter-gatherer societies of humans left food remains to be exploited by other scavenging opportunistic species, and those subsidies have increased substantially since the appearance of Neolithic societies (Oro et al., 2013). This provision of food from humans is now a global phenomenon, which is intensified in parrelel with human population growth and development (Cereghetti, 2019). The term PAFS (Predictable Anthropogenic Food Subsidies) refers to all the food resources that humans offer to animals, intentionally or not, and whose appearance in space and/or time is predictable (Oro et al., 2013). There are different PAFS (i.e. bird feeders, middens, restaurants, etc.), however, the three main PAFS, in terms of distribution, volume of residuals and availability, are crop residuals, fishing discards and urban household waste landfills (landfills onwards) (Oro et al., 2013).

In Spain, before 1984, garbage was disposed of by each town by dumping it in pits, where it was burned; however, in the middle of 1980s, the “open rubbish dumps” concept was introduced. Very extensive áreas were acconditioned by isolating pits to prevent residuals from leaking into the ground by alternating layers of packed residuals and inert materials such as gravel or sand. Although such areas were isolated by fences preventing mammals from accessing the residuals, many bird species could still access the remains left behind in the process of being covered (Tortosa et al., 2002). This kind of landfills are distributed worldwide, proliferating especially in the urbanized world, with a relatively large amount of food discards, which is even bigger in industrialised countries (Oro et al., 2013). To get an idea of the dimension of the situation, the edible food waste per capita by consumers in Europe ranges from 95 to 115 kg/year (Oro et al., 2013). This large amount of organic matter, together with its predictability (due to the daily disposal), makes them a productive feeding patch having the capacity of sustain a large number of animals, with significant impact on the populations of numerous species (Plaza & Lambertucci, 2017). Different species of mammals, reptiles and amphibians are present on landfills, but the best represented group is birds (Plaza & Lambertucci, 2017), possibly as a consequence of the use of fences as the main deterrent tool to avoid the presence of wild animals in landfills, which are not a dissuasive element for avian species. From the different bird species associated to landfills across the world in which the effect of the landfill on their populations has been studied, e.g. several species of gulls: the yellow-legged (*Larus michahellis*), glaucous (*Larus hyperboreus*), herring (*Larus argentatus*), great blackbacked (*Larus marinus*) and iceland gulls (*Larus glaucoides*); rooks (*Corvus frugilegus*), black kites (*Milvus migrans*) or bald eagles (*Haliaeetus leucocephalus*) (Duhem, et al., 2003; Giacomo et al., 2008; Mazumdar et al., 2016; Olea and Balgione, 2008; Seif, et al., 2018, Turrin et al., 2015; Weiser, et al., 2010), there is one bird species that stands out. The white stork (*Ciconia Ciconia*).

The white stork has been associated with anthropogenic habitats for centuries, and even today most of the individual nests are near human settlements (Ciach and Kruszyk, 2010). This species is an opportunistic forager, with a natural diet composed of earthworms, insects such as beetles and locusts, fish, amphibians, and small mammals, especially voles (Ciach and Kruszyk, 2010). Meadows and grasslands near the water represent their natural feeding areas (Ciach and Kruszyk, 2010). The transformation of these areas in agricultural lands produced a sharp decline after 1945, leading to a severe threat of disappearance (Carrascal 1993, Hilgartner et al., 2014). For example, breeding white storks disappeared in 1950 in Belgium and Switzerland and in 1955 in Sweden, and remaining only 11 pairs in 1974 in France, 5 pairs in 1984 in Netherlands and 6 pairs in 1996 in Denmark (Hilgartner et al., 2014). However, these populations in central and northern Europe have been partially restored by conservation actions like reintroduction projects, supplementary feeding, and habitat improvement (Hilgartner et al., 2014).

In general terms, this disappearance halted in later decades and most of the European population is recovering or maintaining stabile. In the later decades landfills have become a typical foraging habitat for white storks, and in combination with invasive Red Swamp Crayfish (*Procambarus clarkii*) (Gilbert et al., 2016) have been essential to the recovery of the species (Ciach and Kruszyk, 2010; Tortosa et al., 2002; Gilbert et al., 2016). In Spain, the number of breeding pairs ranged from 14,050 (Bernis, 1959) to 16,643 in 1948 (Schulz, 1999); however, in the last national census of 2004, the number increased up to 33,217 breeding pairs (Molina and del Moral, 2005; BirdLife International, 2015), more than double the population of fifty years ago. Unfortunately, no data are available for 2014 in Spain since the country did not participate in the world census. The nature of landfills as a food resource; predictable, abundant, and renewed daily, which makes it virtually unlimited (Plaza and Lambertucci, 2017), has profoundly impacted the ecology of white storks. On the one hand, the use of landfills has been shown to modify the migratory pattern of the species, shortening their migration distance, causing some of the individuals to winter in Europe instead of Africa (Archaux et al., 2004). An early return to breeding areas (or the permanent residency in these areas) allows the occupation of the better nesting locations, thus enabling breeding earlier in the year, which is related to larger clutches and higher breeding success (Vergara 2007, Gilbert et al., 2016). On the other hand, the high concentration of organic matter, along with their constant renewal and easy access makes the food intake by parents, and consequently by nestlings, higher, which is again reflected in larger clutch sizes (Vergara 2007, Djerdali et al., 2008) as well as a higher breeding success (Djerdali et al., 2016).

Overall, the use of landfills as food resource appears to have a positive impact on several species of wildlife, and, particularly, on white storks. However, the negative consequences of this behaviour have also been described.

Although the amount of food is considerable, its nutritional quality does not match the proper requirements. The food obtained from landfills could also be deficient by some important dietary components as minerals and vitamins, especially organic compounds that are sensitive to environmental conditions, suffering degradation during their time on the landfill, as it is the case with vitamin E (Tauler-Ametller et al., 2019).

The most obvious hazard from foraging on landfills is physical damage that can result from the presence of metals, glass, wire, and plastic (Plaza & Lambertucci, 2017, Jagiello et al., 2020). In particular, the ingestion of rubber bands mistaken for earthworms can affect the absorption of nutrients, artificially induce satiety and, in the last term, produce intestinal obstruction (Henry et al., 2011).

In addition, indirect damage may be produced by the presence of different pollutants on landfills, which are in the case of white storks, the most important source of toxic substances (Plaza & Lambertucci, 2017). As an example of organic pollutants, bromated flame retardants have been reported in eggs from white storks feeding on landfills. These originate from their use in many products to reduce flammability and are transferred to organic matter in the landfills (Muñoz-Arnanz et al., 2011). Even more frequent in landfills are heavy metals, the levels of which have increased in landfills due to the increase of electronic waste (Kidee et al., 2013). Some examples are lead (Pb), which is used in circuit boards, mercury (Hg) which is essential for lamps, and cadmium (Cd), a key component of rechargeable batteries (Kidee et al., 2013). The transference of these novice elements to storks foraging on landfills has been demonstrated, as nestlings near landfills have higher values of heavy metals in their blood (De la Casa-Resino et al., 2014 and 2015).

Avian species that forage at landfills have been found to acquire a range of pathogens (Ciach and Kruszyk, 2010). These animals also could potentially disperse these pathogens to domestic animals and humans, especially by species associated to humanized landscapes such as the white stork (Gomez et al., 2015; Migura-García et al., 2019; Höfle et al., 2020). An additional problem is the rise in the incidence of antimicrobial resistance (AMR) that results in a reduction in the efficacy of different antimicrobial treatments, causing a problem in health care expenditure and morbidity and mortality due to infections (Borquaye, 2019). The inadequate disposal of unused and expired antibiotics leads to their presence on landfills, which promotes the presence and acquisition of resistant genes (Borquaye, L. S., 2019). The presence of AMR bacteria is becoming one of the major threats for human health, and their presence in landfills could be particularly harmful to both humans and livestock due to the dispersal potential by bird species during local and migratory movements (Migura-García, L., et al., 2019).

To summarise, man has transformed the natural habitat of white storks to agricultural land, which caused a general population decline (Carrascal 1993). However, another result of human activities, the creation of landfills, has allowed the recovery of the population (Molina and del Moral, 2005; Vergara 2007, Djerdali et al., 2008; Djerdali et al., 2016), as it is an abundant and predictable food resource (Plaza and Lambertucci, 2017).

The different effects of the use of landfills have been studied mainly at population level, notably the higher breeding success (Djerdali et al., 2016), and the consequent increase in the number of individuals (Ciach and Kruszyk, 2010; Tortosa et al., 2002; Gilbert et al., 2016). However, individual effects have been ignored, with very few studies about changes at a physiological level. The advantage of using physiological parameters is the higher accuracy to describe the condition of an individual, reflecting, for example, nutritional status (Tauler-Ametller et al., 2019) or the functionality of different organs. Within these physiological approaches, the evaluation of oxidative stress has become more and more important in ecological studies (Costantini, 2008; Beaulieu and Costantini, 2014). In aerobic organisms, free radicals are generated as a by-product of metabolism. These can damage biomolecules because of their high reactivity. Radicals are counteracted by both endogenous (enzymatic and non-enzymatic) and exogenous (i.e. dietary) antioxidants. However, when these defences are not able to neutralize the free radicals, it is defined as oxidative stress. This pro-oxidant condition causes further oxidative reactions damaging different biomolecules; accumulation of which leads to cellular dysfunction or even apoptosis (Halliwell, 2007). In the context of this PhD the importance of the evaluation of oxidative stress lies in the fact that different pollutants which are potentially present in landfills (e.g. heavy metals) exert their harmful effect by increasing oxidative stress (Isaksson, 2010; Koivula and Eeva, 2010), meaning that oxidative stress balance would be the first to be affected if landfill foraged food causes an indivuduals’ health decline.

This PhD comprehensively addresses for the first time the impact that landfill foraging has at an individual level on white stork nestlings, using for this purpose a multidisciplinary approach, based on different measurements of current and potential long-term health status.

The generalized use of this food resource could be explained by two hypotheses. Firstly, its use produces an individual negative effect, balanced by higher productivity (i.e., good populational effect). Secondly, the non-negative effect of its use on the health status of nestlings. To confirm these hypotheses, in chapter one we obtained a general pattern of the effect of the use of landfills as a food resource by a multidisciplinary approach, concluding that the use of this food resource implies a good nutritional status without a disbalance in oxidative stress balance, but a higher prevalence of antibiotic-resistant phenotypes of commensal *E. coli*. We studied nestlings from four different colonies that differed in their degree of food intake from landfills (measured as by its proxy distance to the landfill). We analyzed a wide range of biochemical variables in their blood which indicate nutritional status and the functionality of the liver and kidney, both of which are involved in the detoxification of the organism. Furthermore, we studied oxidative stress balance (both damage and defence) due to its higher sensibility to the presence of pollutants (Isaksson, 2010) which have been shown to be present in landfills (Smith, 2009). Finally, the presence of pathogens was also tested, including the presence of enterobacteria carrying antimicrobial resistance genes that could negatively impact the intestinal microbiota of storks and, if spread, disperse to humans and domestic animals.

Despite the importance of the landfills as a food resource, how individuals respond to its use depends on yearly environmental conditions. In chapter two, we showed that the pattern found in chapter one is not very consistant. Even though we found good nutritional status, oxidative stress balance was dependent of the year of study. We obtained these results after replicating the study of chapter one and increasing the number of colonies from four to ten in addition to do in two consecutive years.

Different external traits vary regarding external factors, as the use of a food resource (Djerdali et al., 2008, Djerdali et al., 2016). The relation between the changes in externally measured traits and the use of landfills as a food resource could be used as a practical tool to follow the evolution of white stork population previously studied, with reduced manipulation time, less invasive techniques and reduced cost. In chapter three we showed that the measurement of the redness of the legs and the body condition (measured as SMI) of nestlings varies with the use of landfills, suggesting that future control of these colonies could be done using these parameters.

Adressing the current effect of landfills as a food resource on health status is very important; however, long-term effects are also a key point, as the early-life conditions during nest-period may have carry-over effects in adult life (Monaghan, 2008; Monaghan and Haussmann; 2015). The fact that white storks are a long-lived species (up to 25 years of age (Prieto, 2002) complicates long term studies. Thus, we used telomere length and its attrition as proxy indicators of potential lifespan and quality (Monaghan and Haussmann, 2006) from an experimental point of view, to confirm potential future problems due to the use of landfill foraged food. To nestlings from one of our study colonies, we administered a dose of antioxidants, as these are supposed to be deficient in landfill derived food (Tauler-Ametller et al., 2019), and studied if this supplement had a positive effect on their telomeres. In this experiment, included in the fourth chapter of this Thesis we demonstrate, for the first time in a field situation, that antioxidant treatment can slow telomere shortening, which would imply a potential long-term negative effect of this food resource on nestlings, possibly due to the lack of antioxidants in this food.

Human pressure initially produced a decrease in white stork numbers. Though later a new food resource was created which allowed the species' recovery. Despite the populational effect of the use of landfills as a food resource described in European countries, little is known about the individual effect, and what this would imply now and in the future. This thesis aims to evaluate landfill use at a wide scale, to try and disentangle the different effects onnestlings of this emblematic species.

General objectives

- Evaluate the potential individual effects the use of landfills as a food resource could have in white storks.
- Evaluate whether the abundance and quality of food on landfills is reflected in the health status of the individuals and study potential short-term negative effects.
  - Does the easy access to food on landfills increase foraging and feeding and does it lead to a better nutritional status?
  - Does the use of this food resource affect the oxidative stress balance of nestlings?
  - Does frequent use of landfills affect blood chemistry?
  - What is the exposition to pathogenic, zoonotic, or antibiotic-resistant enterobacteria in these nestlings through food from landfills?
  - Are differences in nutritional status, haematological variables, blood chemistry and oxidative stress balance consistent over time?
- Determine whether the use of landfills could produce long-term effects.
  - Is it possible to determine telomere length and attrition in white storks during the nestling period?
  - Is there a relation between telomere dynamics and oxidative stress *in vivo*?
- Investigate measurable non-invasive variables that change in association with the use of landfills as a food resource, to use them as a tool for monitoring studied populations.

Chapter 1. A multidisciplinary approach evaluation of the effects of foraging on landfills on white stork nestlings

This chapter is a transcription of the manuscript: **Javier Pineda-Pampliega**, Yolanda Ramiro, Amparo Herrera-Dueñas, Mónica Martínez-Haro, José Manuel Hernández, José I. Aguirre, Ursula Höfle. A multidisciplinary approach evaluation of the effects of foraging in landfills on white stork nestlings. *In preparation*

Abstract

The use of landfills as foraging areas by white storks is a well-known behaviour. Several studies have highlighted its positive effects at a populational level. However, the presence of pollutants and pathogens and the low quality of the food there suggests that the use of landfills could pose a health risk for individuals. The objective of this study was to obtain a general pattern of the effect of the use of landfill as a food resource on white stork nestlings, by a multidisciplinary approach based on the evaluation nutritional status, body condition, renal and hepatic function, oxidative stress balance and the presence of pathogens.

Results showed better body condition in individuals associated with landfills in relation to the ones feeding on natural resources, as well as better nutritional status, as indicated by higher levels of albumin, cholesterol, glucose and triglycerides in plasma. The use of landfills did not lead to an increase of ALP, ALT, AST, CK and creatinine in plasma. As many pollutants have a pro-oxidant effect, we evaluated oxidative stress balance, with no differences in the indicators of damage except for methaemoglobin (metHb), with significantly higher in nestlings associated with landfill-origin food. The antioxidant biomarkers analysed showed the same trend, both endogenous (GSH in red blood cells) and exogenous (tocopherol), were higher in nestlings associated to landfills, which indicates a hormetic response induced potentially by the presence of pollutants in waste. Nestlings fed food from landfills also had a higher presence of Escherichia coli with a multiresistant phenotype to antibiotics.

In conclusion, our results indicated that the nestlings fed with a higher proportion of food from landfills were apparently healthier and even in better condition than those fed with a higher proportion of natural diet. Nestlings exposed to landfill foraged food showed a hormetic antioxidant response, being the only indicatives of a negative effect the higher percentage of metHb and the higher presence of antibiotic resistant E. coli.

Introduction

The “optimal foraging theory” (Stephens and Krebs, 1986) proposes that animals optimize resource acquisition by selecting the more productive patches. The urban household waste landfills are one of the most important PAFS (Predictable Anthropogenic Food Subsidies), due to the large amount of organic material present, which makes them both predictable and virtually unlimited on food resources (Oro et al., 2013). Therefore, landfills are highly productive feeding patches, with the potential to sustain many individuals, having an impact at a population level of numerous species (Plaza and Lambertucci, 2017), with the white stork (*Ciconia ciconia*) as one of the most representives.

White storks are a well-studied species, primarily due to their association with anthropized areas (Ciach and Kruszyk 2010; Flack et al., 2016). In the 20th century, this species suffered a sharp decline in all Europe, primarily due to habitat destruction by agriculture intensification, as storks are opportunist foragers using large irrigated meadows and grasslands (Carrascal et al., 1993). However, since the end of the ’80s, the population steadily increased (BirdLife International, 2015). One of the main thrives for this increase has been related to the adaptation to the use of landfills as a food resource (Tortosa et al., 2002; Gilbert et al., 2016).

Feeding in landfills improves body condition, reproductive fitness, survival and population levels of different species (Oro et al., 2013; Plaza and Lambertucci, 2017). In white storks, feeding on landfills has been associated with a significantly higher breeding success in populations in the North of Africa, Central and Southern Europe (Djerdali et al., 2016). In addition, the use of this feeding resource has changed the migratory pattern of some individuals and age groups within the species, leading to shorter migration distance and wintering in Europe as an alternative to Africa (Archaux et al., 2004) or even complete loss of migratory behaviour in some other individuals, thus decreasing both daily energy expenditure (Flack et al., 2016) and the risk of mortality (Rotics et al., 2016). Furthermore, residency allows for the occupation of the better nesting locations, thus providing an earlier onset of breeding phenology, which is related to larger clutches and higher breeding success (Vergara et al., 2007; Gilbert et al., 2016).

While overall the use of landfills appears to have had a positive impact on the species, negative aspects have also been described at both individual and colony level. On one hand, potential physical damage could result from the presence of glass, metal, wire and plastic (Plaza and Lambertucci, 2017, Jagliello et al. 2020). Physical damage by ingestion of rubber bands, confused with earthworms, can affect the absorption of nutrients, may artificially induce satiety and their accumulation coudl also produce intestinal obstruction (Henry et al., 2011). Exposure to chemical pollutants, such as heavy metals and organic compounds, can result from the presence of these in waste, mixed with organic material, because of their use in industrial products; i.e. lead in plastics and crystal glass; mercury in tubes and lamps and cadmium in batteries (Smith, 2009). Organic compounds such as bromated flame retardants have already been reported in eggs from storks feeding in landfills (Muñoz-Arnanz et al., 2011). In addition, food obtained from landfills could be deficient in some important dietary components especially organic compounds that rapidly degrade such as vitamins (Tauler-Ametller et al., 2019).

Furthermore, in addition to physical damage and the exposure to toxic substances, the acquisition of pathogenic bacteria by birds feeding on waste has also been documented in the last years (Plaza and Lambertucci, 2017, Höfle et al., 2020). This represents a concern both because of the potential of pathogen dispersal by birds during local and migratory movements and due to the potential effects on chick survival (Ciach et al., 2010).

This study had the objective of obtaining a general pattern of the effect of the use of landfills as a food resource on the health status of nestlings using a multidisciplinary approach, based on the evaluation of nutritional status, blood chemistry, oxidative stress balance and the presence of pathogens.

Material and Methods

Study area

Our study area was in the province of Ciudad Real in the south-central Iberian Peninsula, with a mean altitude of 629 m.a.s.l. (Figure 1).

We conducted our study on four white stork colonies along a gradient of exposure to landfill foraged food (Figure 1). Colony “Natural” (“N”) (384579 X, 4349217 Y) is inside a National Park; colony “Semi-Natural” (“SN”) (375153 X, 4306733 Y) is in an open oak forest with extensive sheep farming; colony “Semi-Landfill” (“SL”) (396688 X, 4287548 Y) is located at a landfill site that was sealed in 2007; and colony “Landfill” (“L”) (480827 X, 4362491 Y) is directly associated to an open active landfill site. Satellite tracking data from two adults captured and tagged at colonies “N”, “SN” and “SL”, in addition to ring identification data of adults in all colonies confirmed the intensity of use of each colony. Furthermore, it is established that the distance to the landfills is associated with the intensity of the use of this food resource (Djerdali et al., 2016; Gilbert et al., 2016), being the colony “N” 64.91 km from the nearest landfill; “SN” 60.95 km; “SL” 50.58 km and “L” less than 1 km of their nearest landfills.

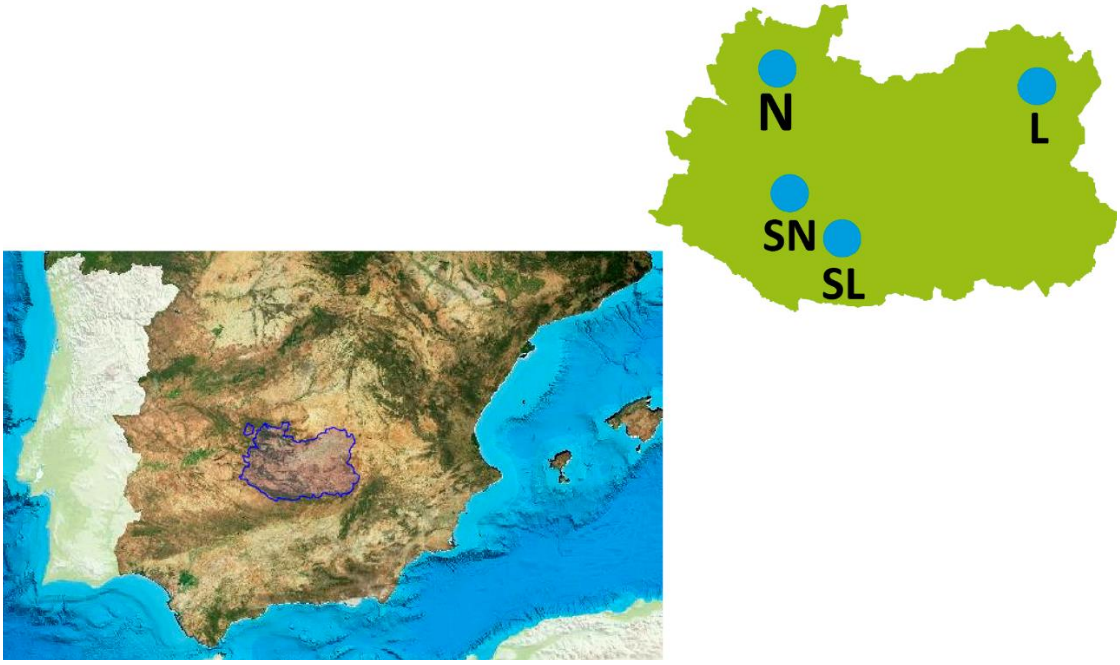


Figure 1: Geographic location of the study colonies in the Iberian Peninsula (Autonomous Community of Castilla-La Mancha, Province of Ciudad Real).

Field procedures

In July 2011, at the end of the breeding season, 103 white stork nestling (42,6 ± 6,8 days old) were sampled and equipped with a metal ring and a long-distance reading ring (PVC rings). Each bird was weighed (to the nearest 5 g), and tarsus and beak length measured using an electronic calliper (to the nearest 1mm). The use of nestlings as sample units ensures through parental food provisioning a representation of feeding resources from the specific habitat within the colony stands.

Using sterile needles and syringes blood samples (3ml) were collected via venipuncture of the brachial vein. Immediately after extraction, two blood smears were made, air-dried and kept at room temperature. Approximately 0.01ml of the sample was transferred to 1ml absolute alcohol and the rest of the blood was transferred to sterile containers containing Lithium-heparine as an anticoagulant. With sterile cotton swabs, we collected cloacal swabs in AMIES transport medium (Deltalab, Barcelona, España) for the isolation of enterobacteria. Blood and swabs were refrigerated directly after extraction and were maintained at 4-8°C until arrival at the laboratory in less than 12 hours.

Laboratory analysis

Upon arrival at the laboratory, 50 µl of the anticoagulated whole blood was separated into cryovials and frozen immediately in liquid nitrogen for metHb analysis. The remaining sample was centrifuged at 10080 G for 10min to separate the plasma and blood cells. The plasma was divided into several aliquots for blood chemistry and carotenoid determination and frozen at -80º C. The red blood cells (RBC) were washed three times in ice-cold physiologic (0.9%) sodium chloride solution and stored at -80º C until further analysis. One of the blood smears was stained with a commercial Papanicolaou type stain (Hemaquick, Biochemical Sciences, Swedesboro, The Netherlands).

Age, sex and body condition

We used head-bill length to determine an approximate age index for the nestlings using the formula developed by Cabodevilla and Aguirre (2019). We calculated body condition according to the scaled mass index proposed by Peig and Green (2009). For sex determination in the nestlings, DNA was extracted from blood samples in absolute ethanol by ammonium acetate technique and used in the PCR described by Fridolfsson and Ellegren (1999).

Haematology and Blood Chemistry

For each nestling, we obtained the hematocrit. In the blood smears an estimated total white cell (TWCC) and differential count was obtained using a light microscope at 400x (TWCC) and 1000x oil immersion (differential count) magnification. The heterophil/lymphocyte ratio (H/L Ratio) was calculated to obtain a measure of first-line constitutive immunity.

We obtained a plasma chemistry profile that included albumin, alanine aminotransferase (ALT), alkaline phosphatase (ALP), aspartate aminotransferase (AST) calcium (Ca), bilirubin, cholesterol, creatine kinase (CK), creatinine, ferritin, glucose, magnesium (Mg), phosphorous (P), proteins, transferrin, triglycerides and uric acid. All analyses were carried using an A25 BioSystems spectrophotometer autoanalyser (BioSystems S.A., Barcelona, Spain), using the corresponding reaction kit for each determination (BioSystems, Barcelona, Spain) according to the manufacturer's instructions.

Oxidative stress balance

Using plasma, we evaluated its antioxidant capacity (BAP test, Diacron international, Grossetto, Italy), correcting the value with the uric acid value (Costantini,

2011) Also in plasma, we evaluate the presence of reactive oxygen metabolites (ROMs; d-ROM kit, Diacron international), and lactate dehydrogenase (LDH). We also determined the plasmatic levels of dietary antioxidants such as α-tocopherol (aka Vitamin E), retinol (aka Vitamin A), and the carotenoids lutein and zeaxanthin by high-performance liquid chromatography (HPLC, Agilent Technologies 1100 Series) coupled to a photodiode detector and a fluorescence detector (Rodríguez-Estival et al., 2010).

Both in plasma and RBC homogenates, we measured malondialdehyde (MDA), commonly used as an indicator of lipid peroxidation, by high-performance liquid chromatography (HPLC, Agilent Technologies 1100 Series) coupled to a fluorescence detector (Romero-Haro and Alonso-Alvarez, 2014).

Only in RBC homogenates, total (GSH) and oxidized (GSSG) glutathione levels were measured with an A25 BioSystems spectrophotometer autoanalyser (BioSystems S.A., Barcelona, Spain). For antioxidant enzymes, we used Ransod and Ransel kits (Randox Laboratories, Cornella de Llobregat, Spain) to measure the activities for the superoxide dismutase (SOD, EC 1.15.1.1) and glutathione peroxidase (GPX, EC 1.11.1.9) in RBC homogenates. Their activities were calculated relative to protein (mg), using the Bradford method to quantify total proteins (Bradford, 1976).

Finally, we used whole blood to evaluate the amount of metHb using spectrophotometric biochemistry analyser (A25 Random Access Analyser, BioSystems; Martinez-Haro and Mateo, 2008). This oxidized form of the haemoglobin shows a lack of function as effective oxygen-transporting protein, and its proportion in the blood is used as a biomarker of exposure to oxidising toxicants.

Pathogen detection and serology

Cloacal swab samples in AMIES transport medium were cultured for the detection of *Salmonella* spp. and *Escherichia coli* (*E.coli*) as described previously in Camacho et al. (2016), including testing of *E.coli* strains for the presence of five genes characteristic of avian pathogenic *E. coli* (APEC) (Johnson et al., 2008).

The phenotypic pattern of antimicrobial resistance of *E. coli* strains was assessed by culture in MacConkey media supplemented with gentamicin, cefotaxime or enrofloxacin respectively; and the same procedure was employed for *Salmonella* spp. isolates replacing MacConkey agar by XLD agar (detailed in Camacho et al. (2016)).

The choice of different antimicrobials was based on the frequency of use in Spain. Gentamicin and Enrofloxacin are used in livestock and pets. Cefotaxime and similar cephalosporins are administered to humans. The concentrations used are those recommended by the National Antimicrobial Resistance Monitoring System (Camacho et al., 2016).

Statistical Analysis

We used general linear mixed models fitted with REML (Restricted Maximum Likelihood) to analyze the data.

To assess how the degree of association of the colony to landfills affects the different dependent variables, we constructed 29 different models with normal distributed dependent variables (Table 1, Table 2) and 4 models with binomial distributed dependent variables with logit function (Table 3, Table 4). *TWCC* and *H/L* ratio were log-transformed; In all models, *Colonies* (“N”, ”SN”, “SL” and “L”) and *Sex* (Male or Female) were included as factors, and Nestlings (number of siblings) is included as a covariate. *Nest* was included as a random factor in all models to avoid pseudo-replication. A detailed description of each variable is included in Table S3.

All models were validated by visual inspection of the residual graphs to verify the assumptions of normality of the residuals and homogeneity of the variances. We used a backward stepwise method to create a final model only with significative variables, but always keeping *Colonies* in the final model due to the objective of the study is the evaluation of the differences between colonies. When the factor Colonies showed significant differences, pairwise comparison with p adjust method "Bonferroni" was done to establish which colonies were different between them. All analyses were performed in R 3.6.2 (R Core Team, 2019) using the R packages 'lme4' (1.1-21), 'lmerTest' (3.1-1), 'car' (3.0-5) and 'REdaS' (0.9.3) (Bates et al., 2015; Kuznetsova et al., 2017; Fox and Weisberg S, 2019; Maier, 2015). Significance was set at $p \leq 0.05$ for all analysis.

Results

Our results in four white stork colonies along a gradient of exposure to landfill foraged food showed a better nutritional status and body condition, hormetic antioxidant response, increased metHb and acquisition of *E.coli* with a multiresistant phenotype in stork nestlings fed a landfill-foraged diet.

The plasmatic concentration of albumin ($F_{34.68} = 5.162$, $p < 0.01$), glucose ($F_{38.51} = 7.37$, $p < 0.01$), cholesterol ($F_{39.2} = 3.107$, $p = 0.037$) and triglycerides ($F_{37.53} = 5.078$, $p < 0.02$) was lowest in nestlings from the N colony (Table 1 and 2, Figure 2). Magnesium concentration was significantly lower in nestling from colony L ($F_{34.04} = 6.329$, $p < 0.01$) (Table 1 and 2, Figure 2).

Body condition, expressed as Scaled Mass Index (SMI), was higher in nestlings from colony L, followed by the ones in colony SL, being lowest in individuals from colonies SN and N ($F_{38.39}$, = 14.48p < 0.01) (Table 1 and 2, Figure 2).

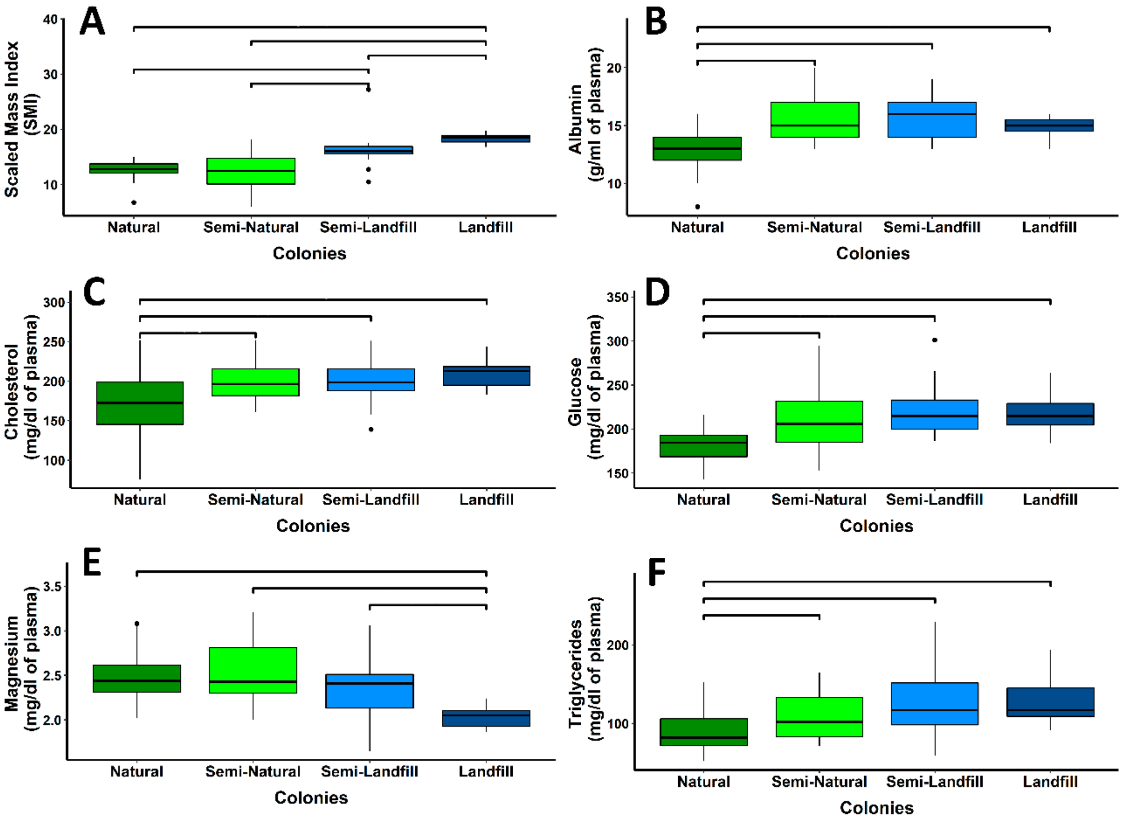


Figure 2: A) SMI (Scaled Mass Index)*;* concentration of **B) albumin***,* **C) cholesterol***,* **D) glucose** and **E) magnesium** and **F) triglycerides** in plasma of white stork nestlings in a gradient of use of landfill as a food resource. Box plots show the median, upper quartiles, maximum and minimum values, and outliers. Keys indicate differences between groups.

Aspartate ($F_{35.34}$ = 11.86, p < 0.01) and alanine aminotransaminase ($F_{35.71}$ = 9.343, p < 0.01) (AST and ALT) activity present the lowest values L colony nestlings, and, despite the higher values appears in the individuals of “SN” colony, the values looks to increase as the degree of exposure to landfill foraged food decreases (Table 1 and 2, Figure 3). Bilirubin concentration was significantly lower in nestlings from colony L ($F_{31.61}$ = 7.72, p < 0.01) (Table 1 and 2, Figure 3).

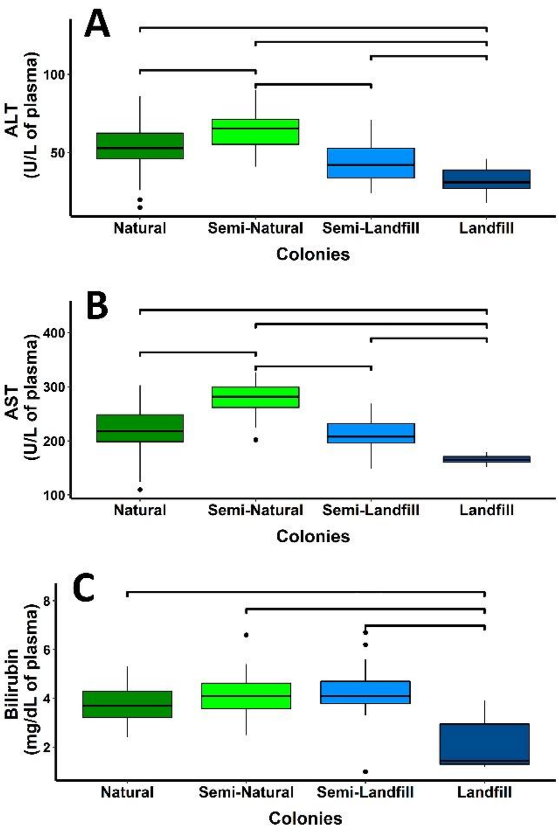


Figure 3: Plasmatic activities of **A) ALT** (Alanine transaminase), **B) AST** (Aspartate transaminase) and plasmatic concentration of **C) Bilirubin** in in plasma of white stork nestlings in a gradient of use of landfill as a food resource. Box plots show the median, upper quartiles, maximum and minimum values, and outliers. Keys indicate significant differences between groups.

Lactate dehydrogenase (LDH) activity was significantly lower in nestlings from colony L ($F_{30.08} = 5.11$, $p < 0.01$) (Table 1 and 2, Figure 4). The same happens with ROMs, which showed the lowest concentration in the plasma of nestlings from colony L, followed by individuals in colony SL ($F_{25.97} = 7.878$, $p < 0.01$) (Table 1 and 2, Figure 4). However, metHb was significantly higher in the blood of nestlings from colony L ($F_{30.22} = 22.07$, < 0.01) (Table 1 and 2, Figure 4).

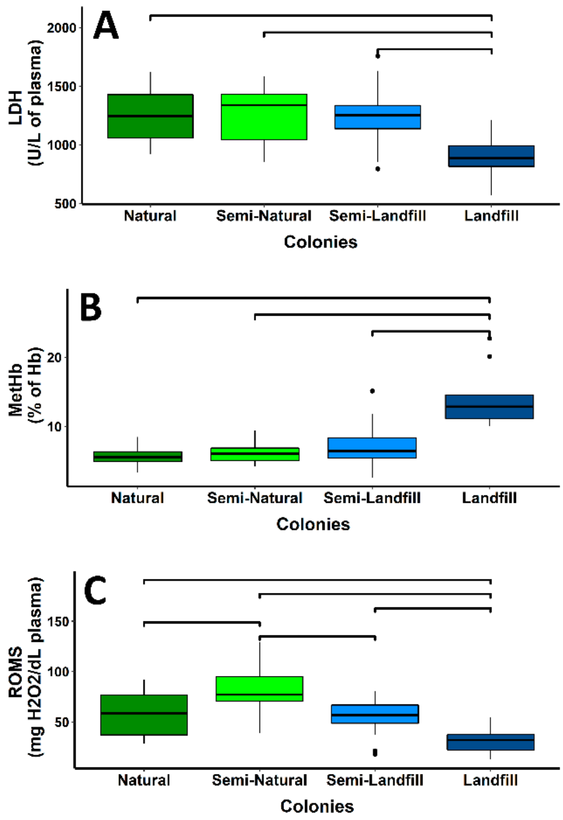


Figure 4: Plasmatic activity of **A) LDH** (Lactate dehydrogenase), percentage of **B) MetHb** (Methaemoglobin) in blood and of **C) ROMs** (Reactive oxygen metabolites) in plasma of white stork nestlings in a gradient of use of landfill as a food resource and concentration. Box plots show the median, upper quartiles, maximum and minimum values, and outliers. Keys indicates significant differences between groups.

GSH concentration was significantly higher in RBC of nestlings from colonies L and SL ($F_{34.08}$ = 9.325, $p < 0.01$) (Table 1 and 2, Figure 5). The same pattern was found regarding the plasmatic concentration of retinol ($F_{32.26}$ = 5.757, $p < 0.01$) (Table 1 and 2, Figure 5). The concentration of transferrin was higher in plasma of nestlings from colony SL than in the nestlings from colonies SN and N ($F_{17.82}$ = 7.465, $p < 0.01$) (Table 1 and 2, Figure 5).

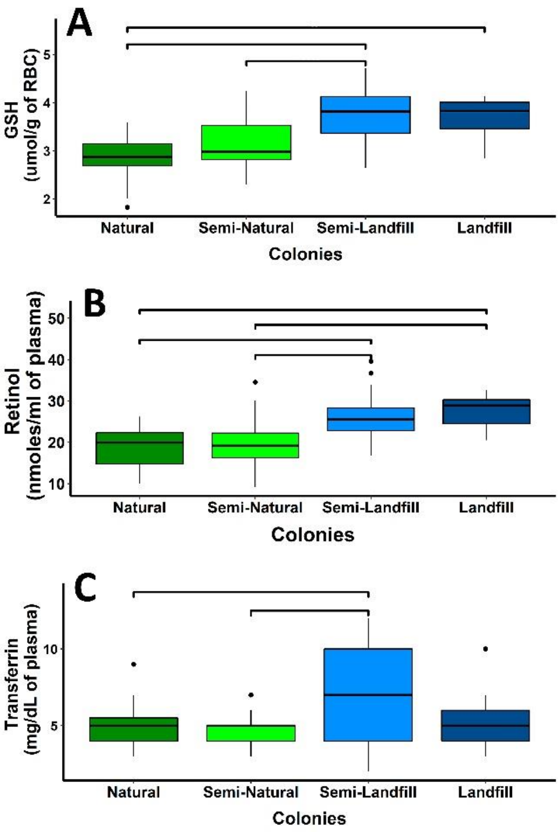


Figure 5: Concentration of **A) GSH** (Glutathione) in RBC (Red Blood Cells) and **B) retinol** and **C) transferrin** in plasma of white stork nestlings in a gradient of use of landfill as a food resource. Box plots show the median, upper quartiles, maximum and minimum values, and outliers. Keys indicates significant differences between groups.

Higher TWCC was observed in nestlings sampled in the colony N in comparison with the ones sampled in the colonies SN and SL ($F_{36.03} = 3.298$, $p = 0.031$). In addition, nestlings from the colony N had a significantly higher H/L ratio than those from the other three colonies ($F_{37.8} = 18.1$, $p < 0.01$) (Table 1 and 2, Figure 6).

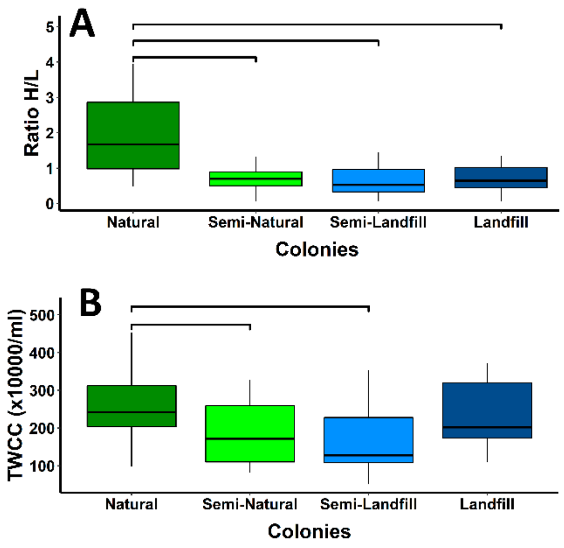


Figure 6: Total White Cell Counts (TWCC) and **Ratio H/L** (Heterophils / Lymphocytes ratio) of white stork nestlings. Box plots show the median, upper quartiles, maximum and minimum values, and outliers. Keys indicates differences between groups.

Table 1: Results of the final models performed to assess nutritional status, health status, oxidative damage, antioxidant defence and immune system status on nestling white stork in a gradient of use of landfill as a food resource. Significant factors (p ≤ 0.05) have been highlighted in **bold**. Random term values are in *italic*.

	Fixed terms	Rejected terms	F (d.f.)	p	Random terms	Variance
			Nutritional status			
Albumin	**Colonies**	Sex, Nestlings	**5.162 (34.68)**	**<0.01**	*Nest*	*1.994*
					Residual	*1.308*
Calcium	Colonies	Nestlings	2.459 (37.47)	0.078	*Nest*	*0.598*
	Sex		**20.44 (67.22)**	**<0.01**	*Residual*	*0.207*
Cholesterol	**Colonies**	Nestlings	**3.107 (39.2)**	**0.037**	*Nest*	*736*
	Sex		**17.51 (65.82)**	**<0.01**	*Residual*	*229*
Glucose	**Colonies**	Sex, Nestlings	**7.37 (38.51)**	**<0.01**	*Nest*	*270*
					Residual	*347*
Magnesium	**Colonies**	Nestlings	**6.329 (34.04)**	**<0.01**	*Nest*	*0.038*
	Sex		**7.712 (82.61)**	**<0.01**	*Residual*	*0.045*
Phosphorus	Colonies	Sex, Nestlings	2.13 (34.48)	0.114	*Nest*	*0.735*
					Residual	*0.345*
Triglycerides	**Colonies**	Sex, Nestlings	**5.078 (37.53)**	**<0.01**	*Nest*	*508.8*
					Residual	*473.7*
			Health status			
ALP	Colonies	Sex	2.678 (35.62)	0.062	*Nest*	*20946*
	Nestlings		**6.94 (36.66)**	**0.012**	*Residual*	*7998*
ALT	**Colonies**	Nestlings	**9.343 (35.71)**	**<0.01**	*Nest*	*104*
	Sex		**5.712 (73.63)**	**0.019**	*Residual*	*69.13*

AST	**Colonies**	Sex	**11.86 (35.34)**	**<0.01**	*Nest*	*1063*
	Nestlings		**4.229 (36.16)**	**0.047**	*Residual*	*298*
Bilirubin	**Colonies**	Sex, Nestlings	**7.72 (31.61)**	**<0.01**	*Nest*	*0.582*
					Residual	*0.388*
CK	Colonies	Sex, Nestlings	2.034 (36.26)	0.126	*Nest*	*34778*
					Residual	*16449*
Creatinine	Colonies	Sex, Nestlings	1.928 (28.91)	0.147	*Nest*	*0.002*
					Residual	*0.005*
SMI	**Colonies**	Sex, Nestlings	**14.48 (38.39)**	**<0.01**	*Nest*	*123233*
					Residual	*17113*
	Oxidative damage					
LDH	**Colonies**	Sex, Nestlings	**5.11 (30.08)**	**<0.01**	*Nest*	*21472*
					Residual	*28192*
MDA (plasmatic)	Colonies	Sex, Nestlings	1.752 (34.59)	0.175	*Nest*	*12.3*
					Residual	*9.869*
MDA (RBCs)	Colonies	Sex, Nestlings	2.128 (30.11)	0.117	*Nest*	*1302*
					Residual	*2690*
MetHb	**Colonies**	Sex, Nestlings	**22.07 (30.22)**	**<0.01**	*Nest*	*2.485*
					Residual	*3.362*
ROMs	**Colonies**	Sex, Nestlings	**7.878 (25.97)**	**<0.01**	*Nest*	*242*
					Residual	*158*
	Antioxidant defence					
Ferritin	Colonies	Sex, Nestlings	0.48 (82)	0.697	*Nest*	*0*
					Residual	*2.67*
GPx	Colonies	Sex, Nestlings	0.623 (38.76)	0.605	*Nest*	*0.014*
					Residual	*0.003*
GSH	**Colonies**	Sex, Nestlings	**9.325 (34.08)**	**<0.01**	*Nest*	*0.115*
					Residual	*0.133*
GSSG	Colonies	Nestlings	1.145 (36.84)	0.344	*Nest*	*0.187*
	Sex		**4.177 (74.22)**	**0.044**	*Residual*	*0.075*
Lutein	Colonies	Nestlings	2.854 (36.71)	0.05	*Nest*	*2.428*
	Sex		**7.725 (54.23)**	**<0.01**	*Residual*	*0.27*
Retinol (Vitamin A)	**Colonies**	Sex, Nestlings	**5.757 (32.26)**	**<0.01**	*Nest*	*20.66*
					Residual	*10.42*
SOD	Colonies	Sex, Nestlings	0.273 (36.06)	0.845	*Nest*	*0.154*
					Residual	*0.067*
TAC	Colonies	Sex, Nestlings	1.344 (24.18)	0.284	*Nest*	*27278*
					Residual	*10271*
Tocopherol (Vitamin E)	Colonies	Sex, Nestlings	1.78 (37.42)	0.168	*Nest*	*12.14*
					Residual	*4.39*
Transferrin	**Colonies**		**7.465 (17.82)**	**<0.01**	*Nest*	*0.182*
	Sex		**9.536 (69.02)**	**<0.01**	*Residual*	*3.177*
	Nestlings		**5.898 (17.98)**	**0.026**		
Zeaxanthin	Colonies	Nestlings	2.396 (38.36)	0.083	*Nest*	*3.105*
	Sex		**11.33 (60.92)**	**<0.01**	*Residual*	*0.705*
	Immune system					
Ratio H/L	**Colonies**	Sex, Nestlings	**18.1 (37.8)**	**<0.01**	*Nest*	*0.083*
					Residual	*0.332*
TWCC	**Colonies**	Sex, Nestlings	**3.298 (36.03)**	**0.031**	*Nest*	*3664*
					Residual	*4030*

ALP: Alkaline phosphatase; AST: Aspartate transaminase; ALT: Alanine transaminase; CK: Creatine Kinase; SMI: Scaled Mass Index; LDH: Lactate dehydrogenase; MDA: Malondialdehyde; MetHb: Methaemoglobin; ROMs: Radical Oxygen Metabolites; GPx: Glutathione peroxidase; GSH: Reduced glutathione; GSSG: Oxidized glutathione; SOD: Superoxide dismutase; TAC: Total Antioxidant Capacity (BAP corrected by uric acid concentration); TWCC: Total White Cell Count.

Table 2: Estimates and standard error of each significant model (the values are in comparison with N colony). Significant factors (p ≤ 0.05) have been highlighted in **bold**. Random terms values are in *italic*. Arrow indicates the direction of increase of each parameter. SN: Semi-Natural; SL: Semi-Landfill; L: Landfill.

	Estimate (standard error)		
	Colony SN	**Colony SL**	**Colony L**
	Nutritional status		
Albumin	2.317 (0.685)	2.293 (0.654)	1.936 (0.945)
		→	
Cholesterol	29.26 (12.04)	30.96 (11.61)	36.08 (17.05)
		→	
Glucose	26.17 (8.825)	37.61 (8.444)	37.5 (12.08)
		→	
Magnesium	0.037 (0.101)	-0.163 (0.098)	-0.524 (0.142)
		←	
Triglycerides	23.92 (11.17)	40.33 (10.81)	38.87 (15.65)
		→	
	Health status		
ALT	8.557 (4.948)	-9.984 (4.718)	-22.3 (6.867)
		←	
AST	56.83 (14.71)	3.288 (14.45)	-52.59 (20.28)
		←	
Bilirubin	0.369 (0.36)	0.429 (0.348)	-1.854 (0.51)
		←	
SMI	-191 (152)	487 (144)	949 (212)
		→	
	Oxidative damage		
LDH	2.424 (79)	-14.42 (75.34)	-383.8 (107.9)
		←	
MetHb	0.299 (0.857)	1.078 (0.845)	9.107 (1.203)
		→	
ROMs	22.74 (8.69)	-2.369 (8.444)	-26.47 (10.95)
		←	
	Antioxidant defence		
GSH	0.327 (0.172)	0.831 (0.167)	0.759 (0.251)
		→	
Retinol (Vitamin A)	1.83 (2.102)	7.215 (2.058)	8.249 (2.95)
		→	
Transferrin	-0.003 (0.536)	2.316 (0.567)	0.268 (0.744)
		→	
	Immune system		
Ratio H/L	-1.205 (0.199)	-1.25 (0.194)	-1.23 (0.272)
		←	
TWCC	-63.12 (30.62)	-89.28 (29.86)	-22.1 (43.11)
		←	

ALT: Alanine transaminase; AST: Aspartate transaminase; SMI: Scaled Mass Index; LDH: Lactate dehydrogenase; MetHb: Methaemoglobin; ROMs: Radical Oxygen Metabolites; GSH: Reduced glutathione; TWCC: Total White Cells Count.

Three nestlings tested positive for Salmonella sp., while *E.coli* was isolated from nearly all nestlings. Phenotypic resistance of these isolates against Enrofloxacin (Chi = 15.5, p < 0.01), Gentamicin (Chi = 10.65, p = 0.014), or both together (Chi = 10.35, p = 0.016) were more frequent in isolates from nestlings from colonies L and SL (Table 3 and 4). There were no differences found in resistance against cefotaxime (Table 3).

Table 3: Values of the models of each dependent variable with binomial distribution. Significant factors (p ≤ 0.05) have been highlighted in **bold**. Random terms values are in *italic*.

	Fixed terms	Rejected terms	Chi square (d.f.)	p
APEC	Colonies	Sex, Nestlings	4.87 (3)	0.182
Resistance to Enrofloxacin	**Colonies**	**Sex, Nestlings**	**15.5 (3)**	**<0.01**
Resistance to Gentamicin	**Colonies**	**Sex, Nestlings**	**10.65 (3)**	**0.014**
Resistance to Enro/Genta	**Colonies**	**Sex, Nestlings**	**10.35 (3)**	**0.016**
Resistance to Cefotaxime	Colonies	Sex, Nestlings	5.033 (3)	0.169

APEC: Avian pathogenic *Escherichia coli*.

Table 4: Estimate and standard error of each significant model with a dependent variable with binomial distribution (the values are in comparison with Colony N). Significant factors (p ≤ 0.05) have been highlighted in **bold**. Arrow indicates the direction of increase of each parameter. SN: Semi-Natural; SL: Semi-Landfill; L: Landfill.

	Estimate (standard error)		
	Colony SN	**Colony SL**	**Colony L**
Enrofloxacin resistant E. coli	1.828 (5.665)	19.29 (6.57)	21.6 (8.29)
		→	
Gentamicin resistant E. coli	3.269 (1.302)	4.391 (1.593)	6.995 (2.299)
		→	
Resitance to both Enro/Genta	1.749 (4.606)	16.58 (6.48)	19.37 (7.05)
		→	

Discussion

We observed that the use of landfills as food resource had a positive effect on nutritional status of white stork nestlings, without effects on health status and an hormetic response of the oxidative stress balance. However, the higher prevalence of *E.*

coli with multiresistant phenotypes suggested an impact on the level of resistance of the bacterial microbiota.

White storks have associated for centuries with anthropized habitats, due to the preference of both species to establish near water bodies (Kruszyk and Ciach, 2010, Ciach and Kruszyk 2010). Changes in agricultural practices have dramatically reduced foraging areas for white storks in all of Europe. However, this trend has been reversed in the recent past, primarily due to the adaptation of storks to using landfills as a continuous predictable food resource (Tortosa et al., 2002; Ciach and Kruszyk, 2010; Gilbert et al., 2016).

Landfills are at the moment important foraging areas during the non-breeding season leading to reduced migration time-lapse and distance and thus reduced mortality and favouring early occupation of breeding sites (Tortosa et al. 2002, Archaux et al. 2004, Vergara et al 2007). Studies have also shown that the proximity of landfills near wintering and breeding areas has a positive effect on clutch size, egg volume, hatching and breeding success (Djerdali et al. 2016). Such effects are likely to be mediated by a higher food intake by the parents (with the consequent higher spare energy to spend in the care of nestlings) and in consequence a higher and more constant feeding rate by nestlings, as reflects their higher concentration of glucose in blood. The better body condition and significantly higher concentrations of glucose, albumin, cholesterol and triglycerides in the blood of stork nestlings from landfill-associated colonies found in our results, seems to confirm this assumption. Higher body weight and seemingly increased body condition have also been documented in other species feeding on landfills such as egyptian vulture (*Neophron percnopterus*; Tauler-Ametller et al., 2019) and american black vulture (*Coragyps atratus*; Plaza and Lambertucci, 2018). However, in contrast to natural prey, food resources foraged at landfills could be expected to lack some essential elements such as minerals or vitamins. We have found no differences in the concentration of phosphorus or calcium, but nestlings from landfill associated colonies had significantly less magnesium in their blood than nestlings from natural habitats. Egyptian vultures, Tauler-Ametller et al. (2019) have shown a negative effect on vitamin and carotenoid concentrations in the blood of nestlings fed on a diet-related to landfills. However, we did not detect any difference in tocopherol concentration in the blood of stork nestlings between colonies; furthermore, retinol was present in higher concentrations in plasma of nestlings from the SL and L colonies.

Regarding the potential negative effects of the use of this food resource, very few studies have evaluated the impact of exposure to chemical pollutants on the physiology of the exposed animals (Plaza and Lambertucci, 2017; Tauler-Amettller et al., 2019). Previous studies have demonstrated that the use of landfills by white storks produce higher concentrations of brominated flame retardants (BFRs) in addled eggs Muñoz-Arnanz et al. (2011); and higher blood concentrations of different heavy metals such as lead, arsenic and mercury (De la Casa-Resino et al., 2015).

We evaluated those effects using a combination of blood chemistry and oxidative stress parameters to evaluate the physiologic function of stork nestlings. Regarding blood chemistry, our values were consistent with parameters reported previously from individuals of the same species (Puerta et al., 1989). Blood chemistry parameters associated to liver functionality (ALT and AST) and the increase of which could indicate liver damage, were significantly lower in nestlings more exposed to food foraged on landfills, with no differences in parameters associated to renal functionality or damage (CK and creatinine).

Many pollutants are active pro-oxidants, or in other cases could inhibit gene expression of antioxidants (Isaksson, 2010). In contrast to studies on white stork nestlings exposed to heavy metal pollution from mining activities (Kaminsky et al., 2009, Tkachenko and Kurhaluk, 2012, 2013) we did not find any increase in oxidative damage indicators in nestlings of colonies associated to landfills, with no differences neither in the concentration of MDA (as an indicator of lipid peroxidation) nor in red blood cells or plasma. In addition, we found a lower presence of ROS and lower activity of LDH in the plasma of these nestlings. A potential explanation could be the higher degree of pollution to which nestlings from other the studies were exposed as these derived from mining activities and copper manufacture (Kaminsky et al., 2009, Tkachenko and Kurhaluk, 2012, 2013).

In terms of antioxidant defences, retinol concentration, which acts as a dietary antioxidant, was increased in nestlings associated with landfills. GSH, the main soluble endogenous antioxidant inside the cells, shows the same pattern, with higher values in nestlings from colonies SL and L. In accordance, Total Antioxidant Capacity (TAC) of plasma, although without significant difference, shows the same trend, with increasing values as the use of landfill as food resource increases. These results suggest that some of the substances contained in the waste were landfill-associated storks forage triggers an effect over oxidative balance, increasing antioxidant defence in a hormetic response (Mattson, 2008; Rattan, 2008). This represents a dose-response phenomenon characterised by a low dose stimulation, high dose inhibition; in which the consideration of a dose as low or high depends on the species and the parameter of study (Mattson, 2008; Rattan, 2008). This phenomenon is a characteristic response to the presence of metals, which have been demonstrated to be present in higher concentrations in white stork individuals that use landfills as a food resource (de la Casa-Resino et al., 2015). Our results agree with previous studies in white storks, where the use of landfills increases the concentration of metals and GSH, without any variation of MDA (de la Casa-Resino et al., 2015). Something similar happens in other studies where the origin of the metal contamination was mining activities (Tkachenko and Kurhaluk, 2012 and 2013), having the individuals from the colony near a polluted area higher lipid peroxidation and GPx activity, bur lower activity of SOD. These results illustrate that in our case we could likely be observing a hormetic response.

The only parameter associated with oxidative damage that increased in nestlings from landfill associated colonies is metHb. MetHb is haemoglobin in which hematic iron is oxidised to iron (III) and therefore cannot function as effective oxygen-transporting protein (Patton et al., 2016). The percentage of metHb in blood is used as a biomarker of exposure to oxidising toxicants such as nitrates, nitrites and N-nitroso compounds (Martínez-Haro and Mateo, 2008). Nitrite and nitrate are commonly used food preservatives in meat products (e.g. E249 and E252) that stabilise red meat colour, delay oxidative rancidity, inhibit some anaerobic microorganisms' growth and may contribute to product flavour characterisation (Iammarino and Di Taranto, 2012). These preservatives cannot be considered totally innocuous for humans (Iammarino and Di Taranto, 2012), and possibly also for birds, as in our study, other than the increase in the concentration of metHb that potentially indicates exposure to such oxidising compounds, we were not able to characterize any additional negative impact on the stork's oxidative balance, has happens in most cases in humans (Mansouri and Lurie, 1993).

Pathogen exposure due to the consumption of waste or refuse has been documented (Plaza and Lambertucci, 2017; Migura-Garcia et al., 2019) but its degree and impact are still largely unknown. Exposure of white stork nestlings to *Salmonella* or APEC in our study seemed not to be related to the use of landfills. However, the higher prevalence of *E. coli* with multiresistant phenotypes in nestlings more exposed to food from landfills suggests an impact on the level of resistance of the intestinal microbiota of these birds. Acquisition of multiresistant bacteria while feeding on waste had been previously reported (Gómez et al., 2016; Barbara et al., 2017; Plaza and Lambertucci, 2017; Migura-García et al., 2019) and could be mediated by the acquisition of resistant strains or resistance genes through contaminated food items or inter and intra-species aggregation, but also due to selection of more resistant phenotypes in the intestinal microbiota by exposure to antibiotic residues (Borquaye, 2019). The presence of antibiotics in landfills could be due to their use in the treatment of bacterial infections, where its overuse and uncontrolled disposal lead to their presence in the landfills (Wang et al., 2019), as long as due to the disposal of the sludge from wastewater treatment facilities (Ahlstrom et al., 2019). The presence of antibiotics in landfills has produced an increase in the prevalence of ABR (Borquaye et al., 2019), to the point to be identified as a one of the primary global health threats and research need (Chung et al., 2018). Another mechanism of adquisition is the co-selection of genes in bacterias exposed to heavy metals, due to the development of resistance against these metals (Xiangyang Li et al., 2019), which could be a potential situation in landfills due to the higher presence of heavy metals. The increase in ABR is a threat to public health, and their relation with avian species is particularly dangerous, due to the role of birds in their dissemination, in particular in the case of white stork due to their close relation with humanized and natural habitat (Gómez et al., 2016).

In turn, the presence of pathogens/or a different potentially more aggressive microbiota could lead to an increased antioxidative response of the organism as the one observed here, also suggested in the case of egyptian vultures (Tauler-Ametller et al., 2019). Dietary deficiencies or pollutant and pathogen exposure through the exploitation of landfills as a food resource could also, both directly and indirectly, impact the immune function of individuals and more specifically nestlings that are in the course of fully maturing their immune capacity. Here we show a lower H/L ratio and TWCC for nestlings exposed to resources from landfills, suggesting such a tendency, however, this needs more exploration using more specific techniques to conclude the mechanisms involved in this context.

In conclusion, our results show that the nestlings fed with food foraged from landfills were apparently healthy and even in better body condition than those fed a diet foraged in a natural environment. Oxidative stress disbalance is very sensitive to the potentially present pollutants in landfill food. However, nestlings exposed to food from landfills present a hormetic response with an increase in their antioxidant defence, being the only indication of a negative effect the higher proportion of metHb, which may be due to the elevated use of nitrite and nitrate in the meat products. In addition, exposure to food foraged in landfills significantly increases antibiotic-resistant phenotypes in commensal *E. coli* meaning that birds using landfills could participate in the spread of antimicrobial resistance. The evaluation of nutritional status, oxidative stress balance and the presence of pathogens have seemed to be effective to provide a general pattern of the effects of the use of this food resource.

Supplementary Material

Table S1. Mean values and standard deviation of each variable with normal distribution. N: Natural; SN: Semi-Natural; SL: Semi-Landfill; L: Landfill.

	Colony N	Colony SN	Colony SL	Colony L
	Mean (standard deviation)			
	Nutritional status			
Albumin (g/ml plasma)	13.04 (1.837)	15.32 (1.827)	15.41 (1.803)	14.91 (0.94)
Calcium (mg/dl plasma)	10.2 (1.068)	11.06 (0.893)	10.9 (0.762)	10.43 (0.47)
Cholesterol (mg/dl plasma)	166.6 (43.1)	197.9 (22.78)	200.1 (25)	210.5 (19)
Glucose (mg/dl plasma)	181.8 (20.02)	204.6 (28.72)	219.7 (25.48)	219.5 (22.17)
Magnesium (mg/dl plasma)	2.478 (0.236)	2.53 (0.348)	2.352 (0.322)	2.03 (0.121)
Phosphorus (mg/dl plasma)	5.558 (1.151)	5.966 (1.127)	6.402 (0.71)	6.648 (0.548)
Triglycerides (mg/dl plasma)	88.1 (23.49)	108.6 (26.98)	127.6 (38.84)	128.5 (33.22)
	Health status			
ALP (U/l plasma)	501 (189.1)	746.7 (181.2)	576.4 (166.3)	599.5 (90.31)
ALT (U/l plasma)	52.67 (16.66)	64.32 (11.91)	43.46 (12.19)	32.18 (8.471)
AST (U/l plasma)	217.6 (47.22)	276.5 (30.79)	212.3 (31.78)	165.4 (7.685)
Bilirubin (mg/dl plasma)	3.76 (0.77)	4.111 (0.844)	4.238 (1.03)	2.06 (1.121)
CK (U/l plasma)	675.1 (212.5)	804.5 (238)	807 (215.1)	717.4 (193.9)
Creatinine (mg/dl plasma)	0.405 (0.074)	0.443 (0.071)	0.448 (0.101)	0.36 (0.079)
SMI	2350 (207)	2226 (630.3)	2863 (259)	3297 (166)
	Oxidative damage			
LDH (U/l plasma)	1246 (216)	1253 (220.1)	1252 (218.6)	909.5 (191.1)
MDA (plasmatic) (nmol/ml plasma)	12.41 (3.516)	15.83 (5.207)	13.95 (5.297)	15.38 (2.398)
MDA (RBCs) (nmol/g of RBC)	158.2 (50.79)	178.2 (63.1)	196.6 (73.17)	140.1 (47.32)
MetHb (% of Hb)	5.767 (1.361)	6.155 (1.308)	7.087 (2.954)	14.15 (4.445)
ROMs (mg H_2O_2/dl plasma)	57.98 (21.22)	80.9 (21.5)	57.76 (16.92)	31.03 (11.99)
	Antioxidant defence			
Ferritin (µg/l plasma)	3.42 (1.714)	3.452 (1.606)	2.958 (1.467)	3.149 (1.833)
GPx (U/mg protein)	0.275 (0.01)	0.315 (0.104)	0.293 (0.146)	0.325 (0.151)

GSH (umol/g of RBC)	2.869 (0.394)	3.167 (0.537)	3.726 (0.516)	3.676 (0.434)
GSSG (umol/g of RBC)	1.434 (0.311)	1.641 (0.466)	1.765 (0.551)	1.462 (0.665)
Lutein (nmol/ml plasma)	1.971 (1.03)	1.685 (0.842)	3.439 (2.239)	2.085 (0.617)
Retinol (vitamin A) (nmol/ml plasma)	19.2 (4.529)	19.98 (5.76)	25.99 (5.383)	27.49 (3.948)
SOD (U/mg protein)	1.801 (0.355)	1.888 (0.459)	1.72 (0.485)	2.022 (0.467)
TAC (BAP corrected)	-60.35 (189.8)	-33.09 (187.5)	42.49 (155.9)	131.9 (103.1)
Tocopherol (vitamin E) (nmol/ml plasma)	12.9 (4.81)	13.58 (4.084)	13.2 (2.857)	17.46 (2.678)
Transferrin (mg/dl plasma)	4.926 (1.385)	4.962 (1.038)	6.913 (3.059)	5.444 (2.068)
Zeaxanthin (nmol/ml plasma)	3.173 (1.591)	2.918 (1.066)	4.613 (2.73)	2.805 (0.864)
	Immune system			
H/L ratio	1.886 (1.037)	0.666 (0.299)	0.65 (0.394)	0.702 (0.387)
TWCC (x10000/ml)	253.9 (88.62)	186.3 (80.67)	167.4 (85.26)	232.4 (95.06)

ALP: Alkaline phosphatase; ALT: Alanine transaminase; AST: Aspartate transaminase; CK: Creatine Kinase; SMI: Scaled Mass Index; LDH: Lactate dehydrogenase; MDA: Malondialdehyde; MetHb: Methaemoglobin; ROMs: Radical Oxygen Metabolites; GPx: Glutathione pexosidase; GSH: Reduced glutathione; GSSG: Oxidized glutathione; SOD: Superoxide dismutase; TAC: Total Antioxidant Capacity (BAP corrected by uric acid concentration); TWCC: Total White Cells Count.

Table S2: Frequency and confidence interval of the presence of *E. coli* showing phenotypic resistance to different antibiotics and carrying virulence genes designating them as avian pathogenic *E.coli* (APEC). N: Natural; SN: Semi-Natural; SL: Semi-Landfill; L: Landfill.

	Colony N	Colony SN	Colony SL	Colony L
APEC	0.032 (-0.034 : 0.098)	0.286 (0.107 : 0.464)	0.25 (0.079 : 0.421)	0 (0 : 0)
Resistat to Enrofloxacin	0.032 (-0.034 : 0.098)	0.25 (0.079 : 0.421)	0.536 (0.339 : 0.733)	0.909 (0.707 : 1.112)
Resistat to Gentamicin	0.065 (-0.027 : 0.156)	0.5 (0.303 : 0.697)	0.571 (0.376 : 0.769)	0.909 (0.707 : 1.112)
Resistat to Enro/Genta	0.032 (-0.034 : 0.098)	0.25 (0.079 : 0.421)	0.5 (0.303 : 0.697)	0.818 (0.546 : 1.09)
Resistat to Cefotaxime	0.032 (-0.034 : 0.098)	0.143 (0.005 : 0.281)	0.321 (0.137 : 0.506)	0.455 (0.104 : 0.805)

APEC: Avian pathogenic *Escherichia coli*.

Table S3: Description of each variable included in the study.

Variable	Definition
Albumin	The most abundant blood plasma protein. It regulates blood volume by maintaining osmotic pressure and serves as a carrier for molecules of low water solubility. Its concentration is influenced by hydration status and it is useful to infer liver function and it is affected by liver, kidney, and gastrointestinal disease.
ALP	Alkaline phosphatase is an enzyme with the role of dephosphorylating different compounds. It is a parameter useful to infer liver function and to infer different bone pathologies (e.g., trauma, osteomyelitis). ALP increases in growth periods.
ALT	Alanine transaminase catalyses the two parts of the alanine cycle. This hepatic enzyme is useful to diagnose hepatic damage and to diagnose muscle damage (e.g. trauma, capture myopathy).
APEC	Avian pathogenic *E. coli.* strains containing five virulence genes characteristic for potential pathogenicity for birds.
AST	Aspartate transaminase is an important enzyme in amino acid metabolism. This hepatic enzyme is useful to diagnose hepatic damage.
Bilirubin	Compound that occurs in the normal catabolic pathway that breaks down haeme group in vertebrates after the destruction of aged or altered red blood cells. This parameter is useful to infer liver function.
Calcium	Essential element which participates in signal transduction, protein synthesis and bone formation. It is an important parameter to infer nutritional status (e.g., nutritional disorders). It is also affected by different pathologic states.
Cholesterol	Lipid with an essential role as a structural component of animal cell membranes. It is also a precursor of steroid hormones and vitamin D. This parameter is useful to infer nutritional status (e.g. decrease in starvation).
CK	Creatine kinase creates phosphocreatine, an energy reservoir for the regeneration of ATP and is a biomarker of acute kidney and muscle injury.
Creatinine	Breakdown product of creatine phosphate in muscle tissue, a biomarker of renal health, but with a limited diagnosis value in the detection of renal function in birds.
Ferritin	An intracellular protein that stores iron and releases it when is necessary. It also is a biomarker of the total amount of iron stored in the body.
Glucose	The most abundant monosaccharide, being the most important source of energy. It’s a metabolic parameter influenced by diet and by stress, being an indicator of fasting periods.
GPx	Glutathione peroxidase is the name of an enzyme family with peroxidase activity whose biological role is to protect the organism from oxidative damage. This parameter is useful to infer the oxidative stress balance.
GSH	Glutathione is the most abundant thiol in animal cells, being the main non-enzymatic endogenous antioxidant. This parameter is useful to infer the oxidative stress balance.
GSSG	Glutathione disulfide is the oxidized version of GSH. This parameter is useful to infer the oxidative stress balance.
H:L ratio	The ratio between heterophils and lymphocytes in thin blood smears. Useful to infer stress in birds.
LDH	Lactate dehydrogenase catalyses the conversion of lactate to pyruvate and back, and it converts NAD+ to NADH and back. It is a biomarker of common injuries and oxidative damage. Elevated values are common with liver disease in birds, and also occur with heart of muscle damage.

Lutein	A carotenoid that can only be obtained through the diet. An isomer of zeaxanthin. In some species it could be associated with immune system function.
Magnesium	Essential element to all cells and some 300 enzymes.
MDA	Malondialdehyde is an organic compound which is indicative of oxidative damage to lipids. This parameter is useful to infer the oxidative stress balance.
MetHb	Methaemoglobin is a haemoglobin in which the iron in the heme group is in ferric instead of ferrous state. Methemoglobin cannot bind oxygen..
Phosphorus	Essential element due to its structural role in DNA, RNA, ATP and phospholipids.
Resistant to Enrofloxacin	*E. coli* showing phenotypic resistance to the antibiotic enrofloxacin.
Resistant to Cefotaxime	*E. coli* showing phenotypic resistance to the antibiotic cefotaxime.
Resistant to Enro/Genta	*E. coli* showing phenotypic resistance to enrofloxacin and gentamicin.
Resistant to Gentamicin	*E. coli* showing phenotypic resistance to the antibiotic gentamicin.
Retinol	Known as vitamin A, essential for eye function and the integrity of skin and mucosal barrier in the respiratory and digestive tract. This parameter is useful to infer the oxidative stress balance.
ROMs	Reactive Oxygen Metabolites produced by the attack of free radicals of oxygen to organic molecules. High values are indicative of oxidative stress. This parameter is useful to infer the oxidative stress balance.
SMI	Scaled Mass Index
SOD	Superoxide dismutase is an enzyme that catalyses the partitioning of the superoxide radical, which is a by-product of oxygen metabolism and could produce oxidative damage. This parameter is useful to infer the oxidative stress balance.
TAC	Total Antioxidant Capacity of plasma. This parameter is useful to infer the oxidative stress balance.
Tocopherol	Known as Vitamin E, fat-soluble antioxidant. This parameter is useful to infer the oxidative stress balance.
Transferrin	An iron-binding glycoprotein that controls the level of free iron. A biomarker to evaluate iron deficiency and iron overload disorders.
TWCC	Estimated Total White Cell Count from stained thin blood smears.
Zeaxanthin	A carotenoid that can only be obtained by diet. An isomer of lutein. In some species it could be associated with immune system function.

Chapter 2. Inter-year consistence in the effects of foraging on landfills for the white stork nestlings

This chapter is a transcription of the manuscript: **Javier Pineda-Pampliega**, José I. Aguirre, Ursula Höfle. Evaluation of the inter-year consistence of the effects of foraging in landfills by white storks. *In preparation*

Abstract

The use of landfills as a food resource by white storks (*Ciconia ciconia*) it is one of the main reasons for the recovery of the populations of this species in different European countries. However, the pollutants in solid urban waste could produce a health-risk for nestlings exposed to them. Previously we showed better nutritional status and adaptive antioxidant response in landfill foraging storks in relation to the ones feeding on natural resources. We hypothesize that we should obtain a similar result studying a larger number of colonies, and, that the continuous availability of landfill origin food should mitigate differences in weather conditions and food availability between reproductive seasons. Thus, here we compared different indicators of the condition in nestlings along a gradient of exposure to food from landfills, and between two different breeding seasons in consecutive years. The closer the landfill, the higher the concentrations of cholesterol, triglycerides and HDL in plasma, indicators of higher feeding rates. The opposite trend was found in relation to tocopherol, with lower plasmatic concentration in nestlings close to a landfill. Other traits related to oxidative stress balance showed significant differences only when correcting results by study year, as TAC and MDA, which were positively and negatively related to landfills respectively only in 2013. In addition, some nutritional (the plasmatic concentration of albumin, cholesterol, glucose and magnesium) and oxidative stress-related variables (the plasmatic concentration of Lutein, Retinol and Tocopherol and the activity of LDH) showed yearly differences. This result, together with the different environmental conditions highlight the importance of the weather regardless of the use of landfills, possibly due to the need for natural prey from nestlings during their first period of life.

Our study shows that the use of landfills as a food resource has a consistently positive effect on nutritional status of white stork nestlings, with no effect on health status. The oxidative stress-balance response is more affected by yearly variations; however, the variations in no case indicate a potentially harmful effect of the use of this resource on the individuals. Our results support the idea that the use of landfills as a food resource could be considered positive at least for the development and early rearing period for white stork nestlings.

Introduction

One of the main effects that humans have produced in global ecosystems is the change in availability and use of food resources (Oro et al., 2013). Humans misuse vast amounts of food which end up in urban household waste landfills. From the wildlife point of view, landfills can be considered as "predictable anthropogenic food subsides" (Plaza and Lambertucci, 2017). The "Optimal foraging theory" (Stephens and Krebs, 1986) indicates that animals optimize their resource acquisition by selecting more productive areas. Landfills can be considered as highly predictable in space and time, with large amounts of food that are renewed daily, and with easy access for wild avifauna as compared to natural sources (Oro et al., 2013; Plaza and Lambertucci, 2017). There are many bird species with documented use of this food resource (e.g. different species of gulls such as the yellow-legged (*Larus michahellis*), glaucous (*Larus hyperboreus*), herring (*Larus argentatus*), great black-backed (*Larus* marinus) and iceland gulls (*Larus glaucoides*), and other species such as rooks (*Corvus frugilegus*), black kites (*Milvus migrans*) or bald eagles (*Haliaeetus leucocephalus*) (Duhem, et al., 2003; Giacomo et al., 2008; Mazumdar et al., 2016; Olea and Balgione, 2008; Seif, et al., 2018, Turrin et al., 2015; Weiser, et al., 2010)). Among these, we chose white stork (*Ciconia Ciconia*) as a model to explore the effects of this behaviour.

The white stork has been associated with anthropized habitats throughout the centuries (Ciach and Kruszyk, 2010). The alteration of their natural foraging areas produced a sharp decrease in their populations over the 20th century in entire Europe, but the use of landfills as a new food resource was one of the resons for the recovery of populations (Tortosa et al., 2002; Ciach and Kruszyk, 2010; Gilbert et al., 2016; BirdLife International, 2015). This use produced an increase in productivity, with earlier recruitment age (Tortosa et al., 2002), larger clutch sizes (Djerdali et al., 2008) and higher breeding success (Djerdali, et al., 2016) throughout Europe (Archaux, et al., 2004; Arizaga, et al., 2018; Ciach and Kruszyk, 2010).

Evidence exists on the presence of pollutants in eggs (Muñoz-Arnanz et al., 2011) and nestlings fed from landfills (de la Casa-Resino et al., 2014; de la Casa-Resino et al., 2015). However, in a previous study, we observed a positive effect on nutritional status of the use of this food resource, along with no negative impact either in health status or oxidative stress balance of nestlings (Pineda-Pampliega et al., *In preparation*). In this new study, we aimed to confirm our earlier findings at a much larger scale (increasing the number of colonies from four to ten) and to verify if the benefits hold regardless of the year weather conditions by including two years of study.

Material and methods

Study area

For our study, we sampled ten different colonies located in Spain. The colonies *Alcalá de Henares* (469188 X, 4481300 Y), *Perales del Rio* (447279 X, 4462731 Y), *Prado*

Herrero (430921 X, 4510381 Y) and *Pinilla del Valle* (431288 X, 4531359 Y) were in the Comunidad of Madrid, in the centre of the peninsula. The colonies *Alcázar de San Juan* (480827 X, 4362491 Y), *Almodóvar* (396688 X, 4287548 Y), *Abenójar* (375153 X, 4306733 Y) and *Cabañeros* (384579 X, 4349217 Y) were in the province of Ciudad Real, in the south centre of the peninsula. The colony *Malpartida de Cáceres* (714926 X, 4368737 Y) was in the province of Cáceres (in the west-centre of the country) and the colony *León* (305065 X, 4730865 Y) was in the province of León, in the north-west (Figure 1). As the distance to landfills has been shown to be associated with the intensity of the use of this food resource (Djerdali et al., 2016; Gilbert et al., 2016), the distance of each colony to the nearest landfill is included in Table 1.

Figure 1. Geographic location of the ten study colonies in the Iberian Peninsula.

Table 1. Distance (in km) to each colony to the nearest urban household waste landfills.

Colony (Name)	Colony (Number)	Distance to the landfill (km)
Alcázar de San Juan	1	0,771
Alcalá de Henares	2	2,609
Perales del Rio	3	6,343
Malpartida de Cáceres	4	6,547
Prado Herrero	5	11,424
Pinilla del Valle	6	30,417
León	7	38,28
Almodóvar	8	50,584
Abenójar	9	60,95
Cabañeros	10	64,914

Field procedures

Between May and June 2013, 212 nestlings were sampled in all colonies except in *Perales del Rio* and *Alcalá de Henares*; and in the same months of the year 2014, 184 nestlings were sampled in all colonies except in *León* and *Malpartida de Cáceres*. Each bird was fitted with a metal ring, in combination with a long-distance reading ring (PVC rings). In addition, weight (to the nearest 5 g, tarsus (mm) and beak length (mm) (using an electronic calliper for both) from each nestling were recorded at ringing.

Blood samples (3ml) were collected from the brachial vein using sterile needles and syringes and were transferred to sterile containers containing Lithium-heparine as an anticoagulant. Blood was refrigerated directly after extraction and maintained at 4-8°C until arrival at the laboratory, always within the 5 hours after extraction.

Laboratory analysis

In the laboratory, blood was centrifuged at 300 G for 10 minutes to separate plasma from the blood cells. Plasma was divided into three aliquots and frozen at -80º

C for posterior analysis of biochemical variables, the concentration of dietary antioxidants (carotenoids and vitamins) and the total antioxidant capacity of the plasma (TAC). The blood cells were washed three times with a cold physiologic sodium chlorine solution (0.9%) and stored at -80 ºC until further analysis.

Body condition

To calculate a proxy of body condition, we used the scaled mass index (SMI) proposed by Peig and Green in 2009.

Chemistry of plasma

To obtain a nutritional and health status profile of the nestlings we conducted biochemical tests on plasma which include: concentration of albumin, bilirubin, calcium, cholesterol, cholesterol HDL, creatinine, glucose, magnesium, phosphorus, triglycerides, proteins, urea and uric acid; and the activities of the enzymes ALP (alkaline phosphatase), ALT (alanine aminotransferase), AST (aspartate aminotransferase), CK (creatine kinase) and LDH (lactate dehydrogenase). All analyses were carried with the corresponding reaction kit for each determination provided by BioSystems (Barcelona, Spain), using an A25 BioSystems spectrophotometer autoanalyzer of BioSystems S.A. (Barcelona, Spain) according to the manufacturer’s instructions.

Oxidative stress balance

MDA (malondialdehyde) concentration is commonly used as an indicator of lipid peroxidation. For its measurement in red blood cells (RBC), we used high-performance liquid chromatography (HPLC, Agilent Technologies 1100 Series) coupled to a fluorescence detector (Romero-Haro and Alonso-Alvarez, 2014). We also used high-performance liquid chromatography, in this case, coupled to photodiode detector and fluorescence detector, to determine the plasmatic levels of dietary antioxidants such as the carotenoids lutein and zeaxanthin, retinol (aka vitamin A) and α-tocopherol (aka vitamin E) (Rodríguez-Estival et al., 2010).

Total antioxidant capacity (TAC) was determined spectrophotometrically using the FRAP method (Ferring Reducing Ability of Plasma) described by Benzie and Strain (1996) and modified as described in Herrera-Dueñas et al. (2017). The parameter was corrected for the uric acid concentration, using the residuals of a linear model between both variables (Costantini, 2011).

Map and weather conditions data

The straight-line distance between the colony and landfill site is strongly correlated with the frequency of landfill use, as demonstrated by Djerdali et al., (2016) and Gilbert et al., (2016). We used free software "QGIS 2.18" and the layer with the position of all the urban household waste landfills in Spain (The European Pollutant Release and Transfer Register) to obtain the minimum distance of each colony to the nearest landfill, reflected in the analysis by the variable "distance".

Daily record of maximum and minimum temperatures and total precipitation from the 7th of May of 2013 to 30th to June of 2013, and from 1st of May of 2014 to 30th to June of 2014 were obtained from the web (https://datosclima.es/), using the nearest automatic climate station to each colony. Data of these periods were chosen because they include the main growth period window of white storks nestling.

Statistical Analysis

We used general linear mixed models fitted with REML (Restricted Maximum Likelihood) to analyse the data. A model was created for each variable, leading to a total of 25 different models. *Distance* (in km) was included as covariate and *Year* (2013 or 2014) as a factor in each model, and the interaction between both was tested and removed from the final model if non-significant. *Nest*, nested in *Locality* (regarding the colony where the nest is located), was included as a random factor in all models to avoid pseudo-replication, since we collected samples from several nestlings at each nest.

To evaluate the differences in weather conditions, one model was created for each variable, leading to a total of 4. *Year* (2013 or 2014) was included as a factor, and *Locality* was included as a random factor.

All models were validated by visual inspection of the residual graphs to verify the assumptions of normality of the residuals and homogeneity of the variances. We used a backward stepwise method to create a final model, but always keeping *Distance* in the final model as the objective of the study was the evaluation of the effect of the use of landfills, being this variable the one representative of this situation. All analyses were performed in R 3.4.1 (R Core Team, 2019) using the R packages "lme4" (1.1-21) and "lmerTest" (3.1-1) (Bates et al., 2015; Kuznetsova et al., 2017).

Results

SMI Regarding nutritional status, the distance to the landfills had a significant effect in the case of the concentration of cholesterol ($F_{7.613}$ = 7.341, p = 0.028), cholesterol HDL ($F_{7.194}$ = 9.877, p = 0.016) triglycerides ($F_{5.851}$ = 7.307, p = 0.036), and urea ($F_{9.572}$ = 7.334, p = 0.023), showing in all cases higher values in individuals near the landfill (Table 2, Figure 2). Both in the case of calcium ($F_{19.95}$ = 4.86, p = 0.039) and phosphorus ($F_{27.94}$ = 7.558, p = 0.01) concentrations, the significant interaction indicates that the effect is not the same on both years of study (Table 2, Figure 2). In the case of calcium, in the year 2014 the effect was close to significance, with lower values in individuals further from landfills ($F_{3.578}$ = 6.509, p = 0.07, Table 2, Figure 2). In the case of phosphorus, the differences were non-significant in any case, but presents a negative trend in 2013 and positive in 2014 (Table 2, Figure 2). There was also a year effect, with lower values in 2014 in the case of the albumin (F_{308} = 58.96, p < 0.001), cholesterol (F_{105} = 9.958, p = 0.002) and magnesium (F_{214} = 14.38, p < 0.001), and an increase for 2014 in the case of glucose (F_{304} = 6.803, p = 0.01) (Table 2).

Figure 2. Significant results of health status variables. Each graph represents the concentration of **A) calcium, B) cholesterol, C) HDL** (high density lipoprotein)**, D) phosphorus** , **E) triglycerides** and **F) urea** in plasma of white stork nestlings in a gradient of use of landfill as a food resource. When the interaction between year and distance is significant, individual regression line for each year were plotted.

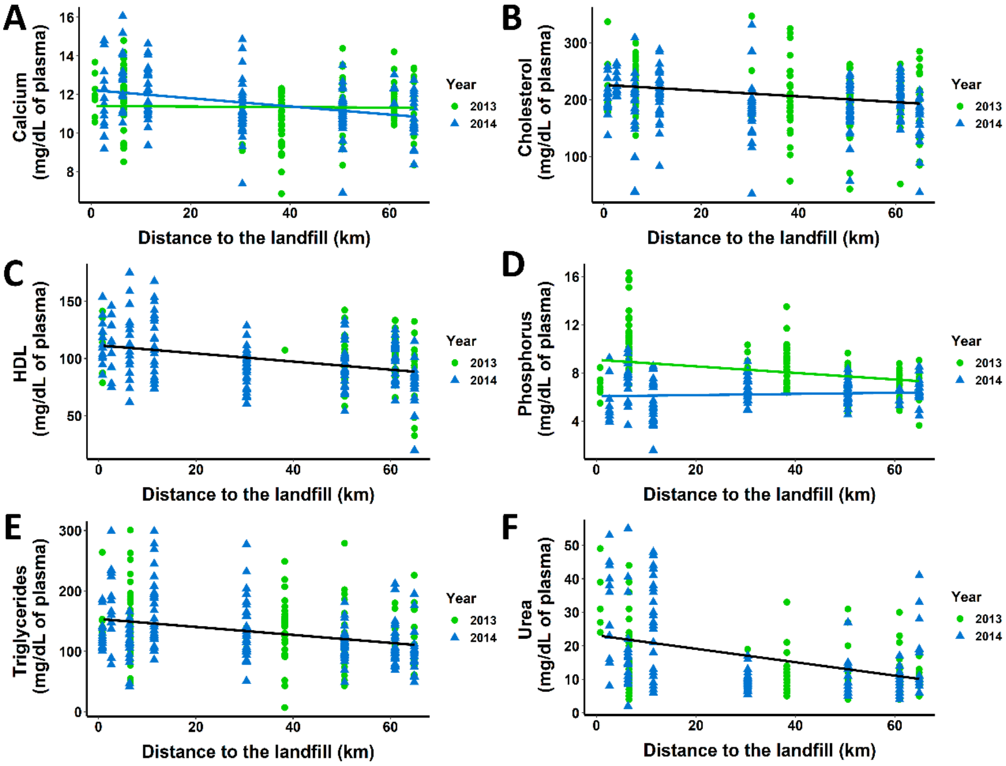


Table 2. Analysis of variables related to nutritional status of nestling white stork in a gradient of use of landfill as a food resource. Significant factors (p ≤ 0.05) have been highlighted in **bold**. Random terms values are in *italic*.

	Nutritional status					
	Terms	**Intercept | Estimate (S.E.)**	**F (d.f)**	**p**	***Random term***	***Variance***
Albumin	Distance	19.4 | -0.018 (0.025)	0.539 (9.888)	0.5	*Nest/Locality*	*1.721*
	Year (2014)	**19.4 | -2.704 (0.352)**	**58.96 (308)**	**<0.001**	*Locality*	*3.384*
	Distance: Year	19.15 | -0.008 (0.017)	0.205 (227)	0.652	*Residual*	*3.9*
Calcium	Distance	11.5 | -0.003 (0.009)	4.02 (7.628)	0.082	*Nest/Locality*	*0.318*
	Year (2014)	11.5 | 0.836 (0.521)	2.574 (12.19)	0.134	*Locality*	*0.216*
	Distance: Year	**11.5 | -0.024 (0.011)**	**4.86 (19.95)**	**0.039**	*Residual*	*1.47*
	2013-Distance	11.4 | -0.001 (0.01)	0.018 (5.483)	0.899		
	2014-Distance	12.23 | -0.021 (0.008)	6.509 (3.578)	0.07		
Cholesterol	**Distance**	**226 | -0.505 (0.186)**	**7.341 (7.613)**	**0.028**	*Nest/Locality*	*616*
	Year (2014)	**226 | -19.76 (6.261)**	**9.958 (105)**	**0.002**	*Locality*	*89.2*
	Distance: Year	226 | 0.003 (0.276)	0.001 (47.54)	0.991	*Residual*	*1672*
Cholesterol HDL	**Distance**	**112 | -0.355 (0.113)**	**9.877 (7.194)**	**0.016**	*Nest/Locality*	*169*
	Year (2014)	115 | -3.473 (3.302)	1.106 (243)	0.294	*Locality*	*38.56*
	Distance: Year	104 | -0.263 (0.164)	2.563 (196)	0.111	*Residual*	*308*
Glucose	Distance	216 | -0.275 (0.454)	0.366 (9.966)	0.559	*Nest/Locality*	*517*
	Year (2014)	**216 | 16.1 (6.172)**	**6.803 (304)**	**0.01**	*Locality*	*1192*
	Distance: Year	203 | -0.436 (0.464)	0.884 (34.17)	0.354	*Residual*	*1091*
Magnesium	Distance	2.876 | -0.006 (0.005)	1.343 (9.667)	0.274	*Nest/Locality*	*0.08*
	Year (2014)	**2.876 | -0.278 (0.073)**	**14.38 (214)**	**<0.001**	*Locality*	*0.159*
	Distance: Year	3.194 | 0.011 (0.006)	3.366 (43.7)	0.073	*Residual*	*0.08*
Phosphorus	Distance	9.192 | -0.032 (0.018)	0.294 (9.489)	0.6	*Nest/Locality*	*1.404*
	Year (2014)	**9.192 | -3.281 (0.922)**	**12.67 (23.25)**	**0.002**	*Locality*	*1.333*
	Distance: Year	**9.192 | 0.047 (0.017)**	**7.558 (27.94)**	**0.01**	*Residual*	*1.002*
	2013-Distance	9.095 | -0.027 (0.021)	1.713 (8.363)	0.225		
	2014-Distance	6.058 | 0.005 (0.011)	0.206 (5.818)	0.667		
Triglycerides	**Distance**	**154 | -0.661 (0.245)**	**7.307 (5.851)**	**0.036**	*Nest/Locality*	*948*

	Year (2014)	158 | -7.155 (6.49)	1.216 (190)	0.272	*Locality*	*226*
	Distance: Year	154 | -0.158 (0.29)	0.295 (63.14)	0.589	*Residual*	*1191*
Urea	**Distance**	**23.12 | -0.2 (0.075)**	**7.334 (9.572)**	**0.023**	*Nest/Locality*	*64.05*
	Year (2014)	22.81 | 0.568 (1.215)	0.218 (226)	0.641	*Locality*	*28.5*
	Distance: Year	20.1 | -0.094 (0.085)	1.229 (20.58)	0.28	*Residual*	*23.27*

HDL: High density lipoprotein.

Regarding health status, the activity of ALT ($F_{8.513}$ = 6.881, p = 0.029) was affected by the distance to the landfills, increasing with the distance in the case of the ALT but decreasing in the case of urea (Table 3, Figure 3). In the year 2014, the values of ALP (F_{290} = 14.74, p < 0.001) and ALT (F_{227} = 7.002, p = 0.009) were higher than in 2013; however, the values of bilirubin (F_{313} = 4.737, p = 0.03), body condition (SMI) (F_{108} = 22.82, p < 0.001), and proteins (F_{273} = 6.484, p = 0.011) were lower than in 2013(Table 3).

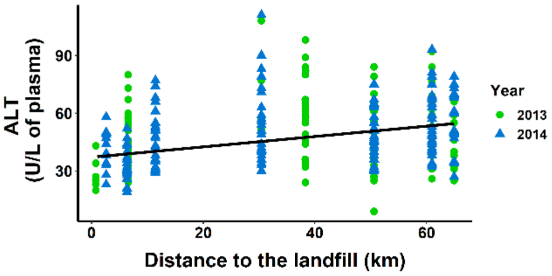


Figure 3. Significant results of ALT (alanine transaminase) regarding the distance to the landfill.

Table 3. Analysis of variables related health status. Significant factors (p ≤ 0.05) have been highlighted in **bold**. Random terms values are in *italic*.

	Health status					
	Terms	**Intercept | Estimate (S.E.)**	**F (d.f)**	**p**	***Random term***	***Variance***
ALP	Distance	672 | -0.834 (1.308)	0.406 (9.191)	0.54	*Nest/Locality*	*10668*
	Year (2014)	**672 | 91.34 (23.79)**	**14.74 (290)**	**<0.001**	*Locality*	*8584*
	Distance: Year	665 | -0.209 (1.232)	0.029 (117)	0.866	*Residual*	*15927*
ALT	**Distance**	**37.16 | 0.269 (0.103)**	**6.881 (8.513)**	**0.029**	*Nest/Locality*	*167.2*
	Year (2014)	**37.16 | 5.814 (2.197)**	**7.002 (227)**	**0.009**	*Locality*	*43.7*
	Distance: Year	41.65 | 0.164 (0.132)	1.542 (17.87)	0.23	*Residual*	*93.99*
AST	Distance	245 | 0.2 (0.312)	0.41 (8.87)	0.538	*Nest/Locality*	*735*
	Year (2014)	246 | -2.72 (6.089)	0.2 (280)	0.656	*Locality*	*471*
	Distance: Year	240 | -0.219 (0.31)	0.502 (102)	0.48	*Residual*	*1037*
Bilirubin	Distance	1.395 | -0.002 (0.005)	0.131 (10.02)	0.725	*Nest/Locality*	*0.113*
	Year (2014)	**1.395 | -0.148 (0.068)**	**4.737 (313)**	**0.03**	*Locality*	*0.118*
	Distance: Year	1.404 | 0.001 (0.003)	0.008 (231)	0.929	*Residual*	*0.105*
Body condition (SMI)	Distance	3303 | -4.354 (4.258)	1.046 (5.382)	0.35	*Nest/Locality*	*34366*
	Year (2014)	**3303 | -315 (65.88)**	**22.82 (108)**	**<0.001**	*Locality*	*71237*
	Distance: Year	3431 | 8.124 (6.66)	1.484 (101)	0.226	*Residual*	*42113*
CK	Distance	663 | -0.979 (1.665)	0.346 (6.676)	0.576	*Nest/Locality*	*14886*
	Year (2014)	707 | -48.74 (41.29)	1.394 (164)	0.240	*Locality*	*12401*
	Distance: Year	642 | -1.489 (2.146)	0.482 (110)	0.489	*Residual*	*20804*
Creatinine	Distance	0.452 | 0.001 (0.001)	0.084 (5.452)	0.783	*Nest/Locality*	*0.003*
	Year (2014)	0.443 | 0.001 (0.001)	0.57 (105)	0.452	*Locality*	*0.003*
	Distance: Year	0.41 | -0.001 (0.001)	1.039 (16.47)	0.323	*Residual*	*0.02*
Proteins	Distance	40.77 | -0.078 (0.035)	4.955 (7.765)	0.058	*Nest/Locality*	*7.912*
	Year (2014)	**40.77 | -1.825 (0.717)**	**6.484 (273)**	**0.011**	*Locality*	*5.931*
	Distance: Year	39.75 | -0.033 (0.034)	0.923 (113)	0.339	*Residual*	*17.34*

ALP: Alkaline phosphatase; ALT: Alanine transaminase; AST: Aspartate transaminase; CK: Creatine Kinase; SMI: Scaled Mass Index.

Regarding oxidative damage, the effect of the distance was only significant in the concentration of MDA in the year 2013 ($F_{185.52}$ = 4.26, p = 0.04) with lower values closer to landfills ($F_{5.087}$ = 6.712, p = 0.048) (Table 4, Figure 4). In the case of the LDH, there was a significant effect of the year (F_{108} = 5.119, p = 0.026), with lower values in 2014 (Table 4).

Focusing on the antioxidant defences, the concentration of tocopherol increased with distance to the landfill ($F_{9.459}$ = 7.271, p = 0.024) (Table 5, Figure 4). The effect of the distance from landfills on the TAC (Total Antioxidant Capacity of plasma) depends on the year (F_{175} = 2.903, p = 0.09), approaching significance in the year 2013, where the values decreased with distance to the landfill ($F_{35.49}$ = 3.396, p = 0.074) (Table 5, Figure 4). Moreover, nestlings sampled in 2014 had higher concentrations of lutein-zeaxanthin (F_{218} = 10.11, p = 0.002), retinol (F_{281} = 20.89, p < 0.001) and tocopherol (F_{251} = 58.55, p < 0.001) (Table 5).

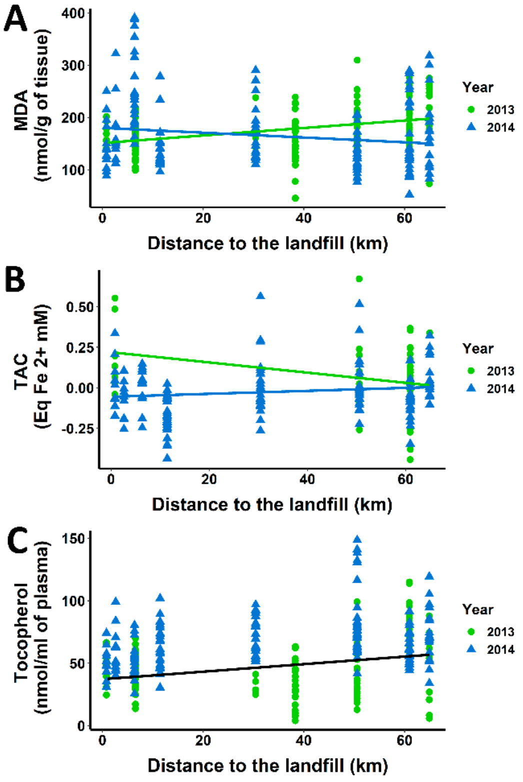


Figure 4. Significant results of the oxidative stress variables. Each graph represents the concentration of **A) MDA, B) TAC** (Total Antioxidant Capacity) and **C) Tocopherol** on blood and plasma of white stork nestlings in a gradient of use of landfill as a food resource. When the interaction between year and distance is significant, individual regression line for each year were plotted.

Table 4. Analysis of variables related to oxidative damage. Significant factors (p ≤ 0.05) have been highlighted in **bold**. Random terms values are in *italic*.

	Oxidative damage					
	Terms	**Intercept | Estimate (S.E.)**	**F (d.f)**	**p**	***Random term***	***Variance***
LDH	Distance	1053 | 0.735 (1.14)	0.416 (6.89)	0.54	Nest/Locality	2065
	Year (2014)	**1053 | -67.72 (29.93)**	**5.119 (108)**	**0.026**	Locality	5017
	Distance: Year	1157 |2.533 (1.405)	3.247 (87.46)	0.08	Residual	16995
MDA (RBCs)	Distance	174 | 0.373 (0.523)	0.001 (8.26)	0.985	Nest/Locality	508
	Year (2014)	174 | -2.128 (19.19)	0.012 (138.27)	0.912	Locality	1196
	Distance: Year	**174 | -0.764 (0.37)**	**4.26 (185.52)**	**0.04**	Residual	2086
	2013-Distance	**151 | 0.717 (0.277)**	**6.712 (5.087)**	**0.048**		
	2014-Distance	180 | -0.461 (0.567)	0.661 (5.973)	0.447		

LDH: Lactate dehydrogenase; MDA: Malondialdehyde; RBCs: Red Blood Cells.

Table 5. Analysis of variables related to antioxidant defence. Significant factors ($p \leq 0.05$) have been highlighted in **bold**. Random terms values are in *italic*.

	Antioxidant defence					
	Terms	**Intercept | Estimate (S.E.)**	**F (d.f)**	**p**	***Random term***	***Variance***
Lutein-Zeaxanthin	Distance	1.945 | 0.028 (0.025)	1.25 (10.96)	0.288	Nest/Locality	10.89
	Year (2014)	**1.945 | 1.025 (0.322)**	**10.11 (218)**	**0.002**	Locality	3.338
	Distance: Year	1.089 | -0.033 (0.021)	2.439 (133)	0.121	Residual	1.678
Retinol	Distance	12 |-0.004 (0.026)	0.022 (8.382)	0.886	Nest/Locality	7.592
	Year (2014)	**12 | 2.265 (0.495)**	**20.89 (281)**	**<0.001**	Locality	2.974
	Rejected: Distance: Year	11.45 | -0.018 (0.024)	0.534 (106)	0.465	Residual	**6.53**
TAC	Distance	0.181 |-0.001 (0.002)	0.105 (7.477)	0.755	Nest/Locality	0.017
	Year (2014)	**0.181 | 0.237 (0.066)**	**12.89 (163)**	**0.001**	Locality	0.004
	Distance: Year	**0.181 | 0.002 (0.001)**	**2.903 (175)**	**0.09**	Residual	0.01
	2013-Distance	0.219 | -0.003 (0.002)	3.396 (35.49)	0.074		
	2014-Distance	-0.055 | 0.001 (0.001)	0.694 (6.703)	0.433		
Tocopherol	**Distance**	**37.24 | 0.301 (0.112)**	**7.271 (9.459)**	**0.024**	Nest/Locality	245
	Year (2014)	**37.24 | 19.09 (2.495)**	**58.55 (251)**	**<0.001**	Locality	45.5
	Rejected: Distance: Year	35.65 | -0.054 (0.119)	0.204 (90.63)	0.652	Residual	153

TAC: Total Antioxidant Capacity (BAP corrected by uric acid concentration)

Regarding weather conditions, there are differences between years in the three variables of study, with higher values of maximum and minimum temperatures on 2014 ($F_{1085} = 44.17$, $p < 0.001$ and $F_{1085} = 50.21$, $p < 0.001$ respectively) and lower values of total precipitations also in 2014 ($X^2 = 61.21$, $p < 0.001$) (Table S1).

Discussion

Our results confirmed the overall positive short term effect of foraging on landfills, with better nutritional status than their counterparts feeding on natural resources and no effect on health status, but we also evidenced how weather differences between reproductive seasons influenced the response of the oxidative stress balance to landfill foraged food in white stork nestlings.

A large amount of food in the landfills, together with its predictability, makes foraging easier for parents and therefore increases the frequency of food intake of nestlings. This higher food intake should be reflected in some biochemical variables, for example, the levels of cholesterol and triglycerides (indicative of fat levels) and the concentration of urea (which informs about protein metabolisms), being all of them related to the nutritional status of animals (Milner et al., 2003; Tauler-Ametller et al., 2019). We observed a negative correlation of all these parameters with the distance to the landfills, indicating that the higher use of landfills implies higher feeding rates of nestlings (Rodríguez, et al., 2011) (Table 2, Figure 2). We found the same result in the HDL, the protective role of which preventing the atherogenic structural modification (Soran et al., 2015) indicates again the good nutritional status of these individuals (Table 2, Figure 2).

Despite the demonstrated transfer of pollutants from landfills to white storks (Muñoz-Arnanz et al., 2011; de la Casa-Resino et al., 2014; de la Casa-Resino et al., 2015) we did not find any increase in oxidative damage indicators in nestlings of colonies nearest to landfills, in accordance with our previous studies (Table 4, Figure 4). However, there was an effect on dietary antioxidants such as tocopherol, which was scarce in the individuals near the landfill (Table 5, Figure 4). The lower presence of this element in waste (Tauller-Ametller et al., 2019) and its sensibility to degradation by heat in the presence of air likely might lead to changes during transport and time remaining in the landfill (Skibsted et al., 2010).

At this point, we can state that we found an effect of the use of landfills as a food resource, with better nutritional status, no effect on health status and no negative disbalance in oxidative stress balance than the ones found in individuals using natural resources. However, the sampling year seems to have a strong influence on some of the analysed traits, and even more important, had an effect of the relation of some of them with the use of landfills. The Mediterranean climate is characterized by dry summers and mild, wet winters. The rains during spring could strongly affect the abundance of the different preys of storks as earthworms, amphibians, and others. Using public data on weather conditions, we found higher temperatures and lower amounts of precipitations in 2014 when compared to 2013 in the study areas (Table S1). During the first weeks after hatching, nestlings must be fed exclusively on natural food, which includes fish, small mammals, and invertebrates (Kosicki et al., 2006; Djeardali et al., 2016). Such dependence of natural food could explain the effect we found in some variables regarding the year of study, as an effect of the weather on potential prey availability (Djerdali et al., 2016).

To counteract the presence of the negative effect of pollutants, organisms could increase their antioxidant defence (Halliwel, 2007). TAC is indicative of the antioxidant capacity of both endogenous and exogenous antioxidants (Benzie and Strain, 1996), with higher values in nestlings near landfills, but only in the year 2013. In addition, in 2013, we found that the same nestlings had lower values of MDA, meaning lower oxidative damage. These results suggest that some of the substances in the waste trigger an effect on oxidative balance, increasing antioxidant defence in a hormetic response to counteract the oxidative damage of these substances (Mattson, 2008; Rattan, 2008). Exogenous antioxidants are the obtained by diet, like retinol and tocopherol and the carotenoids lutein and zeaxanthin. These elements were present in higher concentration in the plasma of nestlings hatched in 2014. This is in accordance with the result obtained for the activity of LDH, where higher values indicate higher oxidative damage, presenting lower values in 2014. The simplest answer to this result is that in 2014 the natural diet was scarcer than in 2013, as demonstrated by the lower values of nutritional status variables such as the plasmatic concentration of albumin, cholesterol and magnesium, and lower values of SMI; but provided more exogenous antioxidants, as reflected by higher values of vitamins and carotenoids in plasma. Thus in both years, there was a protective answer of the organisms elevating endogenous antioxidants to avoid the oxidative damage of the pollutants in waste, but in 2014 it was masked by the higher presence of dietary antioxidants, the reason why we only observed the effect of the distance in the year 2013. However, in general our results do not reveal any direct negative impact of the consumption of landfill foraged food by the white stork nestlings.

This result was in accordance whit the one obtained by Tauler-Ametller et al., (2019) in egyptian vulture (*Neophron percnopterus*), where the ingestion of rubbish produced an increase of the endogenous antioxidant enzymes glutathione peroxidase and superoxide dismutase as a compensatory response for dealing with the ROS, presenting no differences in MDA concentration despite the lower levels of exogenous antioxidants in these individuals.

In conclusion our data shows that nestling's feeding provision from landfills by white storks has a positive effect on nutritional status, independently of the sampling year, and with no apparent effect on the overall health status. On the opposite, the relation of the oxidative stress balance and the use of landfills as a food resource vary between years, due to the dietary antioxidants and the necessity of natural food during early depelopment of nestlings, which indicates the importance of the year and therefore particular weather conditions. Nevertheless, the use of this food resource never produced a disbalance in the oxidative stress balance, which indicates that the potential damage of this use could be counteracted by nestlings.

Supplementary Material

Table S1. Analysis of weather variables. Significant factors (p ≤ 0.05) have been highlighted in **bold**. Random terms values are in *italic*.

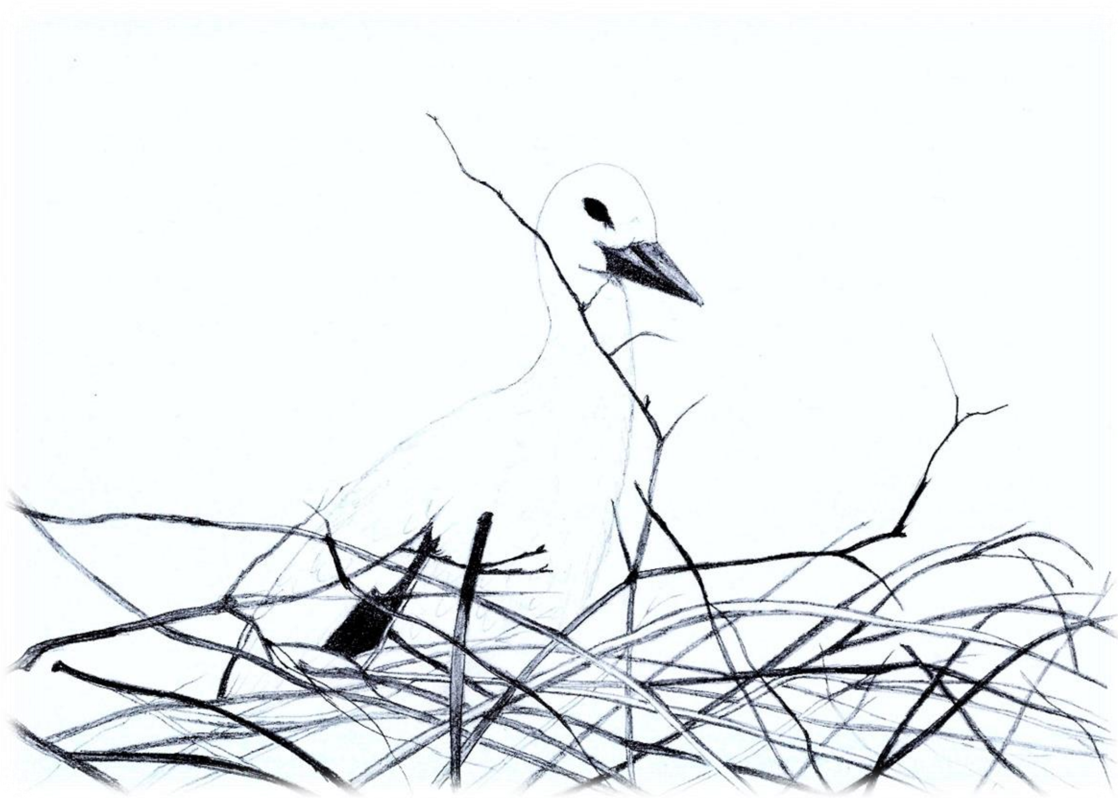
	Terms	**Intercept | Estimate (S.E.)**	**F (d.f)**	**p**	***Random term***	***Variance***
Maximum temperature	Year (2014)	24.08 | 2.023 (0.303)	44.17 (1085)	<0.001	*Colonies*	*9.665*
					Residual	*24.93*
Minimum temperature	Year (2014)	9.778 |1.496 (0.211)	50.21 (1085)	<0.001	*Colonies*	*9.476*
					Residual	*12.149*
	Terms	**Intercept | Estimate (S.E.)**	**χ2 (d.f)**	**p**	***Random term***	***Variance***
Total precipitations	Year (2014)	-0.396 | -0.65 (0.083)	61.21 (1)	<0.001	*Colonies*	*0.184*

Table S2: Description of each variable included in the study.

Variable	**Definition**
Albumin	The most abundant blood plasma protein. It regulates blood volume by maintaining osmotic pressure and serves as a carrier for molecules of low water solubility. Its concentration is influenced by hydration status and it is useful to infer liver function and it is affected by liver, kidney, and gastrointestinal disease.
ALP	Alkaline phosphatase is an enzyme with the role of dephosphorylating different compounds. It is a parameter useful to infer liver function and to infer different bone pathologies (e.g., trauma, osteomyelitis). ALP increases in growth periods.
ALT	Alanine transaminase catalyses the two parts of the alanine cycle. This hepatic enzyme is useful to diagnose hepatic damage and to diagnose muscle damage (e.g. trauma, capture myopathy).
AST	Aspartate transaminase is an important enzyme in amino acid metabolism. This hepatic enzyme is useful to diagnose hepatic damage.
Bilirubin	Compound that occurs in the normal catabolic pathway that breaks down haeme group in vertebrates after the destruction of aged or altered red blood cells. This parameter is useful to infer liver function.
Calcium	Essential element which participates in signal transduction, protein synthesis and bone formation. It is an important parameter to infer nutritional status (e.g., nutritional disorders). It is also affected by different pathologic states.
Cholesterol	Lipid with an essential role as a structural component of animal cell membranes. It is also a precursor of steroid hormones and vitamin D. This parameter is useful to infer nutritional status (e.g. decrease in starvation).
CK	Creatine kinase enhances skeletal, cardiac and muscle contractility. Its measurement is use as a biomarker of cardiac or renal injure.
Creatinine	Breakdown product of creatine phosphate in muscle tissue, a biomarker of renal health, but with a limited diagnosis value in the detection of renal function in birds.
Glucose	The most abundant monosaccharide, being the most important source of energy. It is a metabolic parameter influenced by diet and by stress, being an indicator of fasting periods.

HDL	HDL is known as “good cholesterol”, because high levels are thought to lower the risk of heart disease because carry cholesterol from the cells back to the liver.
LDH	Lactate dehydrogenase catalyses the conversion of lactate to pyruvate and back, and it converts NAD+ to NADH and back. It is a biomarker of common injuries and oxidative damage.
Lutein	A carotenoid that can only be obtained through the diet. An isomer of zeaxanthin. In some species it could be associated with immune system function.
Magnesium	Essential element to all cells and some 300 enzymes.
MDA	Malondialdehyde is an organic compound which is indicative of oxidative damage to lipids. This parameter is useful to infer the oxidative stress balance.
Phosphorus	Essential element due to its structural role in DNA, RNA, ATP and phospholipids.
Proteins	Proteins present in blood plasma which measured is used as changes in plasmatic water or in the synthesis of any of the proteins.
Retinol	Known as vitamin A, essential for eye function and the integrity of skin and mucosal barrier in the respiratory and digestive tract. This parameter is useful to infer the oxidative stress balance.
SMI	Scaled Mass Index
TAC	Total Antioxidant Capacity of plasma. This parameter is useful to infer the oxidative stress balance.
Tocopherol	Known as Vitamin E, fat-soluble antioxidant.
Triglycerides	The main constituents of body fat also present in the blood to enable transference of adipose fat and blood glucose from the liver.
Urea	Is the final result of the metabolism of proteins, formed in the liver from their destruction, being elevated in blood in diets with excess of proteins or renal diseases.
Zeaxanthin	A carotenoidthat can only be obtained by diet. Isomeric with lutein.

Chapter 3. Variation in body condition and redness of leg and beak of white stork nestlings in relation to the use of landfill as feeding resources



This chapter is a transcription of the manuscript: **Javier Pineda-Pampliega**, José I. Aguirre, Ursula Höfle. Variation in body condition and redness of leg and beak of white stork nestlings in relation to the use of landfill as feeding resources. *In preparation*

Abstract

The use of data acquired using non-invasive methods to assess the effect of an external factor has been increased during the last years, due to the growing importance of animal welfare in all kind of research. In our study, we assessed the effect of the use of landfills as a food resource in white stork nestlings on two variables that can be collected using non-invasive methods and could potentially be affected in a similar way, such as body condition based on measures of structural size and weight, and the colouration of beak and legs. Body condition, calculated as Scaled Mass Index, increases as the use of landfills increases, likely due to the higher consumption of food. We found an opposite effect on the redness of legs, while the development of the red colour of the beak did not seem to relate to the use of landfills. These results could suggest that while landfills provide a high amount of easily accessable food, this has a lower concentration of carotenoids than the natural resources. The effect of the presence of carotenoids affects the coloured parts of nestling white storks, but not all of them at the same rate. These results indicate that the measurements of SMI and redness of legs in nestlings of white storks associated with landfills could be used as a proxy of their feeding status without the use of more invasive methodologies.

Introduction

The white stork (*Ciconia ciconia*) suffered a marked decline in the middle of the twentieth century century due to modification of its natural habitat into agricultural areas (Carrascal et al., 1993). In the last four decades, in Spain, the population has recovered, with more than twice the number of individuals than before the decline (BirdLife International, 2015). One of the major drivers of this increase in the population is the use of urban household waste landfills. Such areas are provided by the discards of urban areas (cities and towns). Therefore, a high percentage of organic materials can potentially be used as a food resource by different species (Oro et al., 2013; Plaza and Lambertucci, 2017). Different studies on white storks show that the high concentration of organic resources, along with their constant renewal has increased the food intake of individuals, which is related with better body condition, larger clutch sizes (Djerdali et al., 2008) and higher breeding success (Djerdali et al., 2016).

Previously, we have confirmed good condition along with physiological parameters and variables related to oxidative stress balance (Pineda-Pampliega et al., In preparation) in landfill-fed white stork nestlings. In this study, our objective was to assess if we could obtain equivalent information about the effect of the use of landfills as a food resource in white storks by non-invasive external measurements. For the previous studies, the acquisition of blood samples was essential, which implied invasive sampling, prolonged handling of the individual, and laboratory analysis by qualified personnel, thus increasing economic and ecological costs. All the above make long-term studies challenging, and for this reason, complementary approaches to provide similar information could be of interest. In addition to the use of Scaled Mass Index (SMI) as an indicator of body condition (Peig and Green 2009), which is based on external measurements, we also wanted to evaluate the relation of the use of this food resource and colouration, specifically the redness of beak and legs.

Red coloured ornaments as a proxy for condition has been well stablished in literature (Olson and Owens, 1998; Pérez-Rodríguez 2008). Red colouration is produced by carotenoids (Pérez-Rodríguez, 2008; García-de Blas et al., 2013), which can only be obtained by diet (Pérez-Rodríguez 2008). Their function not only in colouration, but also as antioxidant (Pérez-Rodríguez, 2009; Mougeot et al., 2010; Pérez-Rodríguez et al., 2010) as an immunostimulant (Pérez-Rodríguez et al., 2008; Mougeot et al., 2009, Mougeot et al., 2010) joined with their external origin implies that individuals must trade-off between self-maintenance or ornamental colouration, affecting show casting of individual quality (Pérez-Rodríguez 2008), which could affect mate choice and reproductive decisions (Alonso-Alvarez, et al., 2012). The advantage of the measure of the colour of beak and legs is that living tissues are likely to reflect current physiological condition better than feathers (Pérez-Rodríguez et al., 2010).

Our objective was to evaluate if the measurement of SMI and redness of beak and legs are related to the use of landfills as a food resource by white storks, to provide such measurements as a resource for future evaluations of the individuals in the colonies.

Material and methods

Study area

We sampled four different colonies located in the province of Ciudad Real (Spain), south-central Iberian Peninsula. The colonies show an increasing distance to landfills, which is associated with the intensity of the use of this food resource (Djerdali et al., 2016; Gilbert et al., 2016). We sampled in Alcázar de San Juan (“Closest”; 480827 X, 4362491 Y, at 0.771 km of the landfill), Almodóvar (“ Close”; 396688 X, 4287548 Y, at 50.584 km of the landfill, but less than 1 km to closed landfill); Abenójar (“Far”; 375153 X, 4306733 Y, at 60.950 km of the landfill) and Cabañeros (“Farthest”; 384579 X, 4349217 Y, at 64.914 km of the landfill).

Sampling

A total number of 77 nestlings were surveyed. Each bird was fitted with both a metal ring and a PVC ring for visual recapture. In addition, each bird was weighed (to the nearest 5 gr), and beak and tarsus length (mm) was measured using an electronic calliper (to the nearest 1 mm).

The redness of legs was measured in a surface of 3 x 8 mm located above the tibiotarsal-tarsometatarsal joint on the right exterior side of the left leg, using a portable spectrophotometer CM-2600d (Konica Minolta Sendings, USA), with a measurement of colour in a CIELAB colour space (where L represents lightness, A represents the position between red/magenta and green, and B represents the position between yellow and blue). We selected the value of A, where higher values indicate a red colour.

Due to the surface of white storks beaks and uneven distribution of colour, we decided to evaluate the redness of the beak with high-resolution (6016x4000 pixels) digital photographs of the left side of the head of each bird, using the same camera (Nikon D3200), maintaining settings of shooting speed and diaphragm aperture fixed. To correct for the variability in the environmental level of light received by the nestlings, a standard grey-colour chip was placed close to the head in all pictures.

Processing of samples

Body condition was calculated according to the Scaled Mass Index (“SMI” onwards) proposed by Peig and Green (2009).

Photographs were analysed in Adobe Photoshop CC 2017 (Adobe Systems, San Jose, USA). Briefly, the red area of the beak was selected, obtaining the RGB values, which are transformed into hue (°) values, according to Foley and Van Dam (1984). This value is a measure of colour location that describes the range of wavelengths that most contribute to the reflectance spectrum of an object, measured in degrees of a colour wheel (García-de Blas, et al., 2013). As low values of hue indicate high levels of redness (Foley and Van Dam, 1984; García-de Blas, et al., 2013), the variable evaluated "redness of beak" is the hue value with the sign reversed to simplify the interpretation.

For sex determination, DNA was extracted from the blood of feathers stored in absolute ethanol and used in the PCR described by Fridolfsson and Ellegren (1999).

Statistical analyses

We used general linear mixed models fitted with REML (Restricted Maximum Likelihood) to analyse the data.

To assess how the degree of association of the colony to landfills affected body condition and redness of leg and beak, we constructed three different models in which Redness of the leg, Redness of the beak, and SMI were used as dependent variables (Table 1). In the model of Redness of the beak, the area of the beak evaluated (in pixels) is included as a covariate. In all models Sex (Male or Female) and Age (days) were included as a covariate, and Nest was included as a random factor to avoid pseudo-replication. The three possible permutations of correlations were also evaluated (Table 2).

All models were validated by visual inspection of the residual graphs to verify the assumptions of normality of the residuals and homogeneity of the variances. All analyses were performed in R 3.6.2 (R Core Team, 2019) using the R packages 'lme4' (1.1-21) and ‘lmerTest’ (3.1-1) (Bates et al., 2015; Kuznetsova et al., 2017). Significance was set at $p \leq 0.05$ for all analysis.

Results

While redness of the leg increased significantly with distance from a landfill ($p = 0.031$, $F = 2.276$, Table 1, Figure 1), indicating less redness in individuals more associated with landfills, SMI significantly decreased with distance from the landfill ($p < 0.01$, $F = 6.257$, Table 1, Figure 1), indicating that individuals with a high percentage of food from landfills had higher values of SMI. Both variables are also related between them, showing the expected negative relation ($p = 0.026$, $F = 5.289$, Table 2). Amount of redness in the beak did not relate any of the tested factors.

The SMI was also significantly higher in males ($p<0.01$, $F = 19.52$ Table 2), and increases with nestling age ($p<0.01$, $F = 40.4$, Table 2).

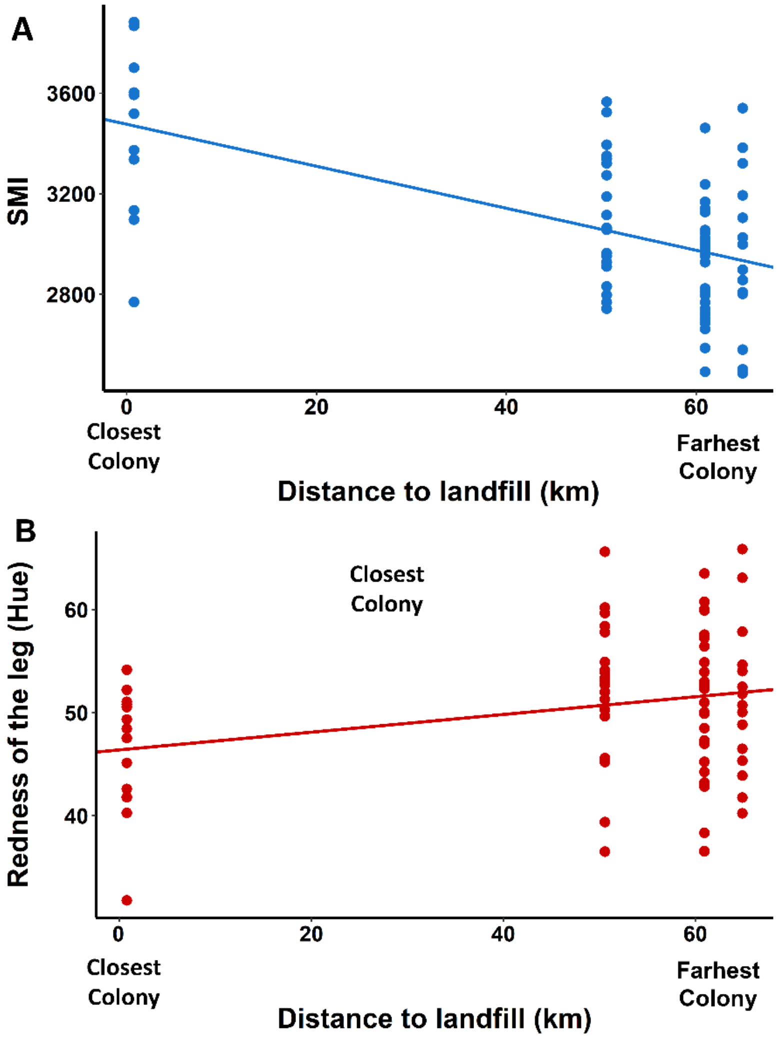


Figure 1. SMI (Scaled Mass Index) and Redness of the leg in white stork nestlings in relation to distance of the colony to the nearest landfill (km).

Table 1. Redness of the leg, redness of the beak, number of fault bars and SMI in relation to Age (days), Distance to closest landfill (km), Sex (Male or Female) and in the case of redness of the beak Coloured area (pixels). Significant factors (p ≤ 0.05) are highlighted in **bold**.

	Terms	Intercept	Estimate (S.E.)	F (d.f)	p	Random terms	Variance
Redness of the leg	**Distance**	**46.39**	**0.086 (0.038)**	**2.276 (28.08)**	**0.031**	Nest	6.068
						Residual	43.228
	Rejected: Sex	48.13	-2.812 (1.619)	3.016 (72.36)	0.087		
	Rejected: Age	45.08	0.051 (0.174)	0.087 (60.02)	0.769		

Redness of the beak	Distance	-29.06	0.0334 (0.027)	1.556 (24.31)	0.224	Nest	8.964
	Area		0.015 (0.012)	1.540 (31.36)	0.224	Residual	6.369
	Rejected: Sex	-28.56	-0.774 (0.948)	0.666 (42.47)	0.419		
	Rejected: Age	-35.39	0.119 (0.107)	1.231 (43.83)	0.273		
SMI	**Distance**	**1064**	**-3.616 (1.446)**	**6.257 (37.25)**	**0.017**	Nest	24962
	Sex		**171.8 (38.89)**	**19.52 (44.34)**	**<0.01**	Residual	14268
	Age		**36.31 (5.713)**	**40.4 (67.49)**	**<0.01**		

SMI: Scaled Mass Index

Table 2. Permutation of all possible correlation between the four variables of study. Significant factors (p ≤ 0.05) are highlighted in **bold**.

Term 1	Term 2	Intercept	Estimate (S.E.)	F (d.f)	p	Random terms	Variance
Redness of the leg	**Redness of the beak**	5	0.021 (0.291)	0.005 (47.98)	0.944	Nest	8.765
						Residual	49.69
Redness of the leg	**SMI**	**68.65**	**-0.006 (0.003)**	**5.289 (51.52)**	**0.026**	Nest	3.699
						Residual	41.92
Redness of the beak	**SMI**	-21.95	-0.002 (0.002)	0.945 (41.39)	0.337	Nest	7.475
						Residual	7.803

SMI: Scaled Mass Index

Discussion

The information obtained by our non-invasive data collection indicates that in white stork nestlings the distance, and therefore the proportion of landfill-foraged food in the diet, had a significant effect on the redness of the legs and the body condition, measured as SMI. However, while SMI increased, leg redness decreased with proximity to landfills.

To evaluate the effect of an external factor on free-living individuals, we need a technique that reliably measures the condition of the animals. Body condition has been widely used in different studies and has been related to survival, reproduction, and behaviour (Labocha and Hayes, 2012). Body condition is an ambiguous term, as it is used for different measurements and parameters (Labocha and Hayes, 2012). For this study, we considered only variables that could be obtained by external measurements of the individuals. In this case, we chose the SMI, defined by Peig and Green in 2009, which has been validated with data of body components such as fat and protein. The distance to, and therefore the use of landfills as a food resource, had increased the SMI of individuals, with higher values in nestlings from colonies near the landfills, indicating a better body condition. This result was consistent with previous studies in white storks, in which the high concentration of organic resources and their continuous renewal allows for easily found food, which is related to better body condition and increased reproductive success (Djerdali et al., 2008, 2016).

The red colouration of beak and legs, an additional non-invasive technique, provides information about the physiological status and quality of the individuals (Pérez-Rodríguez, 2008). In our study we found a significant positive relationship between redness of legs and distance to landfills; however, no relation was found between redness of beak and distance to landfills, when both characters are produced by the presence of carotenoids (Pérez-Rodríguez, 2008). This fact can be caused by the differential carotenoid turnover between living tissues, which makes that each one responds in different ways to changes in status (Pérez-Rodríguez and Viñuela, 2008; Pérez-Rodríguez, 2010). For example, in a food-restriction experiment with red-legged partridges (Alectoris rufa), the eye-ring pigmentation changed markedly, meanwhile, beak redness was more stable and likely needs more time to be altered in response to changes in individual condition (Pérez-Rodríguez and Viñuela, 2008).

Even though red coloured ornaments reflect condition (Olson and Owens, 1998; Pérez-Rodríguez 2008), we found that the SMI and redness of legs respond in an opposite way to the use of food from landfills, and therefore are negatively correlated. Most of the studies found a positive relationship between body condition and coloured areas; however, they are performed on granivores and frugivores species, which have a carotenoid-rich diet. In the study of Sternalski et al., (2010) in nestlings of another carnivorous species such as the Montagu´s harrier (*Circus pygargus*) they also found opposite trends between body condition and colour regarding the kind of diet. Thus, a possible explanation could be that the amount of food in landfills is high, but not its quality, when latter is measured by the concentration of vitamins and micronutrients (Tauler-Ametller, 2019). As far as the redness is due to the presence of carotenoids (Pérez-Rodríguez 2008; García-de Blas et al., 2013), which are only obtained by diet, this result indicates that the concentration in food from landfills is lower than in natural areas. This fact could be due to low concentration of carotenoids in landfill food resources (Tauler-Ametller, 2019), high values in natural areas, possibly due to the use as prey of the invasive Red Swamp Crayfish (*Procambarus clarkii*) which is rich in carotenoids (Negro and Garrido-Fernández, 2000) or both.

The objective of our study was to evaluate if the SMI and redness of beak and legs are related to the use of landfills and can be used as non-invasive measurements to control the effect of feeding behaviour in our study colonies. Higher values of SMI indicates that the individuals that feed on landfills have higher food intake. However, the lower values of redness in legs indicate that the same individuals have lower carotenoids, which could lead to considering the lower quality of such feeding resources regarding carotenoid concentrations. The use of both measurements (SMI and redness of legs) could be used as an early alert concerning food quality and intake in the future without the need for a more invasive evaluation, reducing the time of manipulation of individuals, which implies better animal welfare. Despite we can use both measurements to monitor the situation, we do not have a real understanding the way in which the use of landfills impacts colour and carotenoid dynamics in white storks or the future fitness of the individuals. For that, additional studies on redness (both in beak and legs) and the concentration of carotenoids in peripheral blood and coloured tissues would be necessary

Chapter 4. Antioxidant supplementation slows telomere shortening in free-living white stork chicks

This chapter is a transcription of the manuscript: **Javier Pineda-Pampliega**, Amparo Herrera-Dueñas, Ellis Mulder, José I. Aguirre, Ursula Höfle, Simon Verhulst. 2020. Antioxidant supplementation slows telomere shortening in free-living white stork chicks. Proceedings of The Royal Society B, 286: 20191917. Doi:10.1098/rspb.2019.1917

Abstract

Telomere length and shortening is increasingly shown to predict variation in survival and lifespan, raising the question what causes variation in these traits. Oxidative stress is well known to accelerate telomere attrition in vitro, but its importance in vivo is largely hypothetical. We tested this hypothesis experimentally by supplementing white stork (*Ciconia ciconia*) chicks with antioxidants. Individuals received either a control treatment, or a supply of tocopherol (vitamin E) and selenium, which both have antioxidant properties. The antioxidant treatment increased the concentration of tocopherol for up to 2 weeks after treatment but did not affect growth. Using the TRF technique we evaluated erythrocyte telomere length and its dynamics. Telomeres shortened significantly over the 21 days between the baseline and final sample, independent of sex, mass, size and hatching order. The antioxidant treatment significantly mitigated shortening rate of average telomere length (-31% in shorter telomeres (percentiles 10th, 20th and 30th). Thus, our results support the hypothesis that oxidative stress shortens telomeres in vivo.

Introduction

Telomeres are located at the ends of linear chromosomes to protect chromosome ends from degradation and maintaining stability (Blackburn, 1991). With each cell division, telomere length (TL) decreases; when they reach a critically short length, the cell enters a degenerative process of senescence, eventually followed by apoptosis (Blackburn, 1991; Rodier et al., 2005). Also, on the level of the whole organism there is an association between lifespan/annual survival and TL and its attrition (Boonekamp et al., 2013; Dantzer and Fletcher, 2015; Tricola et al., 2018; Eastwood et al., 2019; Wang et al., 2018; Wilbourn et al., 2018). There is accumulating evidence that telomere attrition during development is of particular importance for later health and survival (Boonekamp et al., 2014; Benetos et al., 2018). Different external factors could affect telomere attrition, but it is generally assumed that most of these exert their effect through oxidative stress (Mizutani et al., 2013; Quirici et al., 2016; Watson et al., 2015).

Oxidative stress results from an imbalance between the production of reactive oxygen species (ROS) and the antioxidant capacity of an organism (Halliweel, 2007) and accelerates telomere attrition in cell cultures (Zglinicki, 2002). The nucleobase guanine is relatively sensitive to oxidation, and its high proportion in the (TTAGGG)n repeats that constitute vertebrate telomeres makes them particularly vulnerable to oxidative attack (Zglinicki, 2002). This high sensitivity, together with the deficiency in repairing single-strand breaks in telomere areas, makes telomeres particularly susceptible to oxidative damage (Houben et al., 2008). This process, together with the oxidative stress enhancing effects of various physiological and psychological stressors, makes telomeres potential biomarkers of lifespan and of the exposure to environmental challenges and individual lifestyle (Monaghan and Haussmann, 2006).

However, the apparently simple relation between oxidative stress and telomere dynamics cited above is largely based on in vitro studies of cell cultures (Zglinicki, 2002; Richter and Zglinicki, 2007) and the extent to which oxidative stress accelerates telomere shortening in vivo has recently been questioned (Boonekamp et al., 2017; Pérez-Rodríguez et al., 2019). To date, few studies attempted to evaluate this correlation using an experimental approach. Thus, the extent to which oxidative stress accelerates telomere attrition in vivo remains an open question (Reichert and Stier, 2017).

In this study, we evaluated the effect of antioxidant supplementation on TL and their attrition in free-living white stork (*Ciconia ciconia*) chicks. First, we analysed whether the antioxidant supplementation affected oxidative stress biomarkers to verify its efficacy. We subsequently described telomere dynamics in relation to age, sex, hatching order, mass, size and treatment. Because we measured TL using the TRF method, i.e. using a smear on a gel, we can characterize TL not only by the average TL but also by distribution. This can be useful, because telomere shortening is faster at the high end of the telomere distribution within individuals (Salomons et al., 2009; Bauch et al., 2014), suggesting that treatment effects may also vary depending on location in the telomere distribution.

Material and methods

Experimental design and sampling

The study was carried out in a breeding colony located in the Northern area of the Community of Madrid, in the centre of Iberian Peninsula (40°44´N, 3°49´ E).

In total, 55 chicks from 20 nests were used in the experiment (165 samples, of which 129 were analysed for the present study). Hatching order was established through frequent visits during the hatching period based on size differences between the nestlings within broods that are due to the high asynchrony in this species. White storks begin incubation with the first or second egg, and laying occurs at intervals of two days. Furthermore, egg mass also tends to decrease with laying order, and this effect, combined with hatching asynchrony, results in a marked size hierarchy among nestmates (Romero and Redonde, 2016). Birds were randomly assigned to two treatment groups, while ensuring that both groups were represented in all nests. The

“Vitamins” group was supplied with a subcutaneous dose of a commercial mixture for veterinarian use (Selevit, Syva laboratories, León, Spain). Each dose contained 5 mg of α-tocopherol acetate (aka vitamin E) and 50 μg of sodium selenite per kg body mass, calculated for each dose depending on the actual mass. Their vitamin E radical-scavenging activity causes both substances to be considered a chain-breaking antioxidant (Cadenas and Pacer, 2002; Traber and Stevens, 2011; Jiang, 2014). Selenium is a trace mineral, essential for the function of different antioxidant enzymes (Cadenas and Pacer, 2002; Sahin, 2002). The “Control” group was administrated with an equivalent dose of a sterile saline solution. Sampling was carried out under a special permit of the regional park Cuenca Alta del Manzanares and of the regional government (Comunidad de Madrid).

The time between the first and last sample was limited to twenty days. Chicks were weighed, measured and bled at the start of the experiment, at which time they were on average 20 days old (S.D. = 8 days; termed ‘day 1’ below), after which the first treatment was administered. This process was repeated 14 and 21 days later, except that no treatment was provided on day 21 (Figure S1). Blood samples (±1 ml) were collected from the brachial vein with a heparinised syringe, immediately transferred to sterile tubes and kept at 4 °C until centrifugation (10 min at 1800g at 4 °C). Blood cells were separated from the plasma and washed three times in ice-cold physiological (0.9%) sodium chloride solution and, both plasma and cells, were aliquoted and stored at -80 °C until analysis.

Oxidative stress biomarkers

Levels of tocopherol in plasma and malondialdehyde (MDA, indicator of lipid peroxidation) were measured using high-performance liquid chromatography (HPLC, Agilent Technologies 1100 Series) (Rodríguez-Estival, 2010; Romero-Haro and Alonso-Alvarez, 2014, respectively). The intra-assay coefficient variation (CV) of tocopherol and MDA were 1.52% and 3.96% respectively.

Total antioxidant capacity (TAC) was determined spectrophotometrically using the FRAP method (Ferring Reducing Ability of Plasma) described by (Benzie and Strain, 1996) and modified as described in (Herrera-Dueñas et al., 2017). The parameter was corrected for the uric acid concentration by using the residuals of the linear model between both variables (Costantini, 2011). Uric acid was measured with a commercial kit (Spinreact; Girona, Spain). The intra-assay and inter-assay CV were respectively 6.71% and 3.08%.

Telomere length assays

TL was measured as described in Salomons et al. (2009). Briefly, 7 µl of erythrocytes were suspended in an agarose solution to form an agarose plug of 0.8% (CHEF Mammalian Genomic DNA Plug kit, Bio-Rad Laboratories, Inc., USA) and digested overnight with proteinase K at 50 °C. Subsequently, DNA was simultaneously digested with Hinf I (30 U), Msp I (60 U) and Hind III (60 U) restriction endonuclease overnight at 37 °C in NEB2 buffer (New England Biolabs, Inc., Beverly MS, USA). The digested DNA from each sample and the 32P-labeled size ladders (DNA Molecular Weight Marker XV, Roche Diagnostics, Basel, Switzerland; 1 kb DNA ladder, New England Biolabs Inc., Ipswich, MA, USA) were separated through a 0.8% agarose gel by pulsed-field gel electrophoresis at 14 °C for 24 h. Gels were dried using a gel dryer (model 538, Bio-Rad) and hybridized overnight using a 32P-end-labelled oligo (5´-CCCTAA-3´) that binds to the single strand overhang of telomeres. Unbound oligonucleotides were removed by washing the gel for 30 min with 0.25 x saline-sodium citrate buffer at 37 °C. The radioactive signal was detected by a phosphor screen (MS, Perkin-Elmer Inc., Waltham, MA, USA) and analysed using a phosphor imager (Cyclone TM Storage Phosphor System, Perkin-Elmer Inc.), resulting in a gel picture with a distribution of grey values in a smear, reflecting the distribution of TL in a sample. We included the three samples of each individual in the same gel next to each other in random order, and the same number of the two treatments randomized in every gel used to avoid confounding effects due to gel identity.

Individual TL size distributions were quantified through densitometry using the open-source software ImageJ v. 1.38x. This technique allows us to classify the telomere distribution of each sample into every 10th percentile from 10th to 90th, with the 10th percentile being the shortest telomeres and the 90th the longest, besides the average TL. Between-gel CV was below 5% but note that samples of the same individual were always together on a gel.

Statistical analyses

We used general linear mixed models (GLMMs) fitted with REML (Restricted Maximum Likelihood) to analyse the data. We created a covariate Hatch order, assigning 1 to the first-hatched chick, 3 to the last-hatched chick, and 2 to the intermediate chick(s) (mean ± S.D., brood size on day 1 was 3.76±1.03).

To assess the effect of the treatment on oxidative stress variables, we constructed three different models, with tocopherol concentration, TAC and MDA as dependent variables respectively, Treatment (Control or Vitamins) as factor and Age of individuals (in days) as covariate. Treatment was coded “Control” (zero) for day 1 for all individuals because antioxidant supplementation started after sampling and coded zero or one on days thereafter for control and treated individuals respectively. Individual identity, nested in brood identity, was included as a random factor in all models to avoid pseudo-replication. Analysis of treatment effects on mass and size (tarsus length in mm) followed the same approach.

When analysing telomere dynamics, we tested effects of Sex, Mass, Hatch order and Treatment in a single model, using a stepwise backward procedure to obtain the final model. Gel identity was included as a random effect in all telomere models, in addition to individual identity nested in brood identity. In each analysis, interaction of each variable with age (included as covariate) was tested and removed from the final model if non-significant. The same analysis were carried out for each percentile (Table S1), evaluating at the same time if the loss of telomeres by percentiles differed significantly (Table S3).

Finally, the relation between telomeres and oxidative stress variables was evaluated using TL as dependent variable, and the values of the previous measurement day of Tocopherol, MDA and TAC (mean centered) were used as covariates.

All models were validated by visual inspection of the residual graphs to verify the assumptions of normality of the residuals and homogeneity of the variances. In all tables, the column “Intercept” was added to show how it changed with the exclusion of the rejected terms. All analyses were performed in R 3.4.1 (R Core Team, 2017) using the R packages “lme4” (1.1-17) and “lmerTest” (3.0-1) (Bates et al., 2015; Kuznetsova et al., 2017).

Results

Treatment effect on oxidative stress biomarkers, mass and size

Treatment elevated tocopherol concentration by approximately 10% (p=0.017; Table 1), indicating a correct absorption and assimilation of the product (Figure 1A). Independent of treatment, tocopherol concentration increased with age (p<0.001; Table 1). There was a trend for treatment to increase TAC (p=0.077; Table 1, Figure 1B), further supporting the assumption that the administration of the antioxidants had a functional effect on oxidative stress. It is worth noting that these effects were measured in samples taken 2 (day 14) and one (day 21) weeks after the administration of the antioxidants. It can therefore be assumed that tocopherol concentration and TAC were elevated considerably more over the whole period between administration and sampling than on the sample days. Lipid peroxidation (MDA concentration) did not differ between treatment groups (p=0.77; Table 1, Figure 1C).

Treatment had no significant effect on the mass or size of the individuals in any comparison (Table S2).

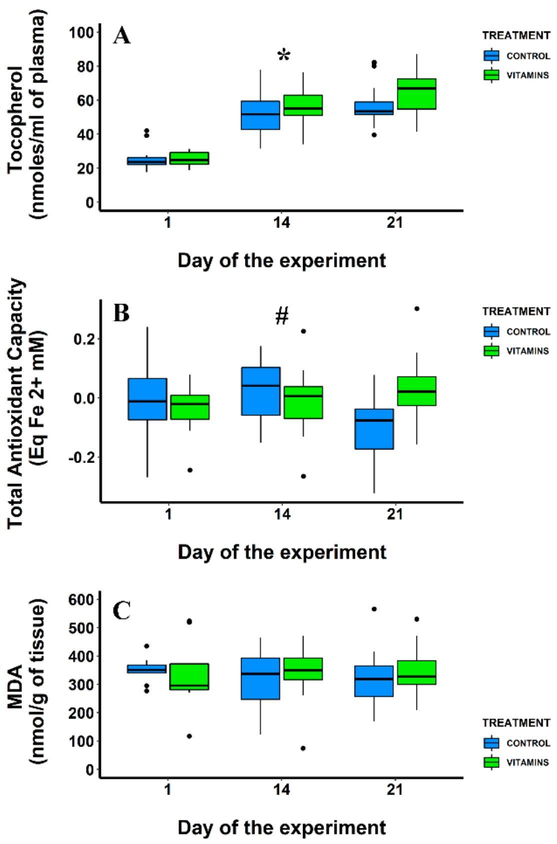


Figure 1. Effects of antioxidant supplementation on oxidative stress markers 1-2 weeks later. **A)** Concentration of *tocopherol* in plasma, **B)** *TAC* (Total Antioxidant Capacity) of plasma, FRAP corrected by uric acid concentration and **C)** *MDA* (malondialdehyde) concentration in erythrocytes. Box plots show the median, upper quartiles, maximum and minimum values, and outliers. Data shown are the values of each variable of the days 1, 14 and 21, but coding day 1 all as Control group. Data shown are the values of each variable depending on the treatment group and the day of measurement (day 1, 14 and 21 respectively). * indicates differences between groups with $p<0.05$. # indicates differences between groups with $p<0.1$.

Table 1. Tocopherol, MDA and TAC in relation to Age (days) and Treatment (Control or Vitamins; note that day 1 measurements are coded as control for all nestlings). Factors in the final model have been highlighted in **bold.** Significant factors (p < 0.05) have an *****. Random terms values are in *italic.*

Oxidative stress and treatment						
			Intercept	**Estimate (S.E.)**	**F (d.f)**	**p**
Tocopherol	**Fixed terms**	**Age**	**2.65**	**1.4 (0.15)**	**91.91 (79.71)**	**<0.001**
		Treatment (Vitamins)	**2.65**	**6.32 (2.58)**	**6.01 (53.47)**	**0.018**
	Rejected terms	Age:Treatment	1.25	-0.4 (0.42)	0.91 (91.5)	0.342
	Random terms (Variance)	Chick/Nest		14.71		
		Nest		64.12		
		Residual		101		
MDA	**Fixed terms**	**Age**	**330**	**-0.03 (0.78)**	**0.01 (74.8)**	**0.968**
		Treatment (Vitamins)	**330**	**4.11 (14.02)**	**0.09 (38.93)**	**0.771**
	Rejected terms	Age:Treatment	342	2.54 (2.26)	1.27 (76.97)	0.263
	Random terms (Variance)	Chick/Nest		209		
		Nest		5622		
		Residual		2775		
TAC	**Fixed terms**	**Age**	**-0.01**	**0.01 (0.01)**	**0.19 (100)**	**0.665**
		Treatment (Vitamins)	**-0.01**	**0.04 (0.02)**	**3.2 (100)**	**0.077**
	Rejected terms	Age:Treatment	0.03	0.01 (0.01)	3.7 (99)	0.057
	Random terms (Variance)	Chick/Nest		0		
		Nest		0		
		Residual		0.01		

MDA: Malondialdehyde.

Telomere dynamics

Average (± S.D.) TL was 8700±688 bp in 32-day-old chicks (i.e. the average age over all measurement), and telomere attrition in all individuals pooled was on average 9.8 bp/day (Table 2, Figure 2). There was no sex difference in telomere length (p=0.59) or attrition (p=0.678; Table 2) while there were large and consistent differences between individuals (Figure 2). Hatching order did not modulate TL (p=0.252) or attrition (p=0.4, Table 2) and neither did weight for either TL (p=0.814) or attrition (p=0.395).

Although we did not find significant differences in telomere shortening rates between percentiles (table S3) we decided to analyze the data for every 10th percentile separately, following (Bauch et al., 2014). This allowed us not only to compare between and within-individual differences in telomere attrition, but also to evaluate if the relation of all our variables of study with telomeres was the same regardless of the length of the telomeres. This decision is based on previous studies in which attrition was shown to differ between percentiles (Salomon et al., 2009; Bauch et al., 2014).

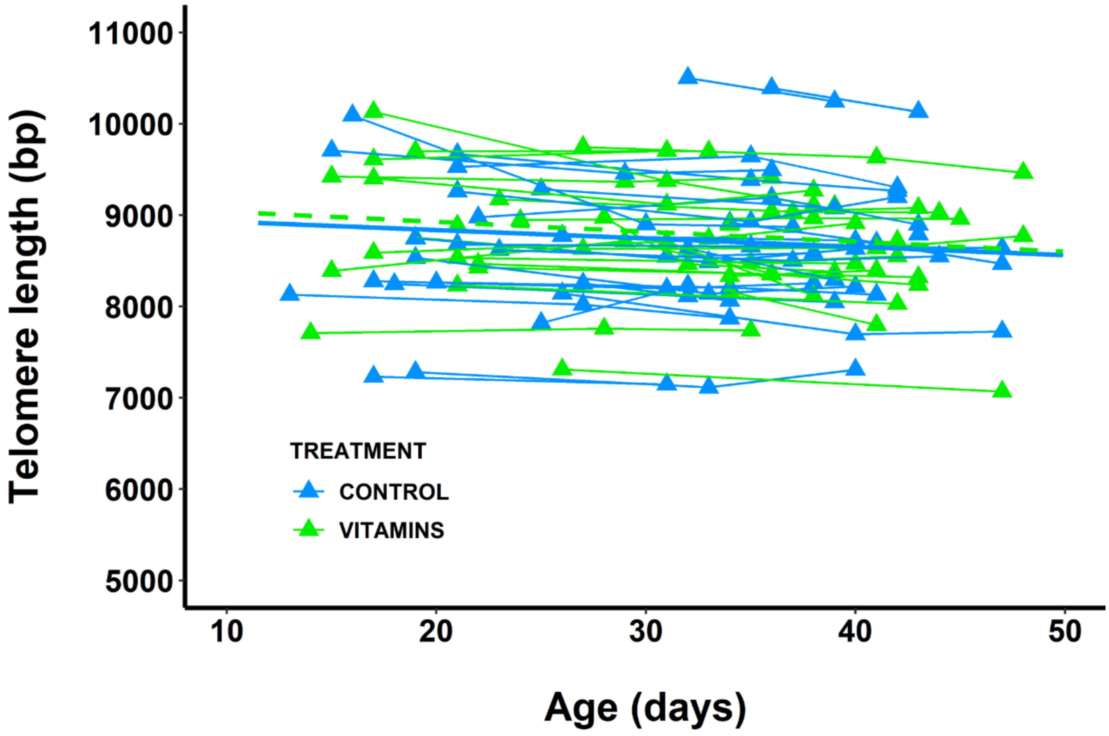


Figure 2. Telomere length in relation to age (days), and the best fitting regression lines for treatment "Vitamins" (green) and control groups (blue).

Table 2. Telomere length (average of each sample) in relation to the covariates Age (days), mass (g) and Hatch Order (1,2,3) and the factors Sex (male and female) and Treatment (Control or Vitamins; note that day 1 measurements are coded as control for all nestlings). Factors in the final model have been highlighted in **bold.** Significant factors (p < 0.05) have an *****. Random terms values are in *italic.*

Fixed terms					**Random terms**	
Terms	**Intercept**	**Estimate (S.E.)**	**F (d.f)**	**P**	**Terms**	**Variance**
Age	**9052**	**-9.816 (1.779)**	**30.43 (78.46)**	**<0.001**	*Chick / Nest*	*438.1*
					Nest	*316.7*
Treatment		**100.2 (46.76)**	**4.592 (87.63)**	**0.035***	*Gel*	*496.1*
					Residual	*126.6*
Rejected: Hatch order	9249	-109.9 (94.34)	1.356 (34.58)	0.252		
Rejected: Sex (Male)	9289	-113 (95.01)	0.295 (43.94)	0.59		
Rejected: Mass	9285	0.013 (0.055)	0.056 (78.4)	0.814		
Rejected: Age:Hatch order	9188	-1.657 (2.329)	0.506 (77.8)	0.479		
Rejected: Age:Mass	9028	-0.002 (0.002)	0.732 (75.65)	0.395		
Rejected: Age:Treatment	9058	-3206 (6122)	0.274 (72.68)	0.602		
Rejected: Age:Sex (Male)	9057	1.378 (3.305)	0.174 (71.79)	0.678		

We found that attrition was faster in the longer telomeres within individuals, increasing from 6.20 bp/day at the 10th percentile, to 10.44 at the 90th percentile (a 68% increase), but this increase did not reach statistical significance (Table S1 and S3, Figure 3A).

Treatment had a positive effect on telomere length (p=0.035), with supplemented birds having 100 bp longer average telomere length than the control group (Table 2, Figure 3B). When testing for treatment effects at the different percentiles separately, we found there to be a significant effect on the 10th, 20th and 30th percentile (p<0.05), and a trend in the 40th percentile (p=0.052, Table S1). Over the 50th to 90th percentile the treatment effect slightly increased to approximately the same extent as at the lower percentiles, but due to the increase in variance the effects were not significant (Table S1).

There were no significant correlations between TL and any of the oxidative stress variables (Table S4) or with mass (Table S5).

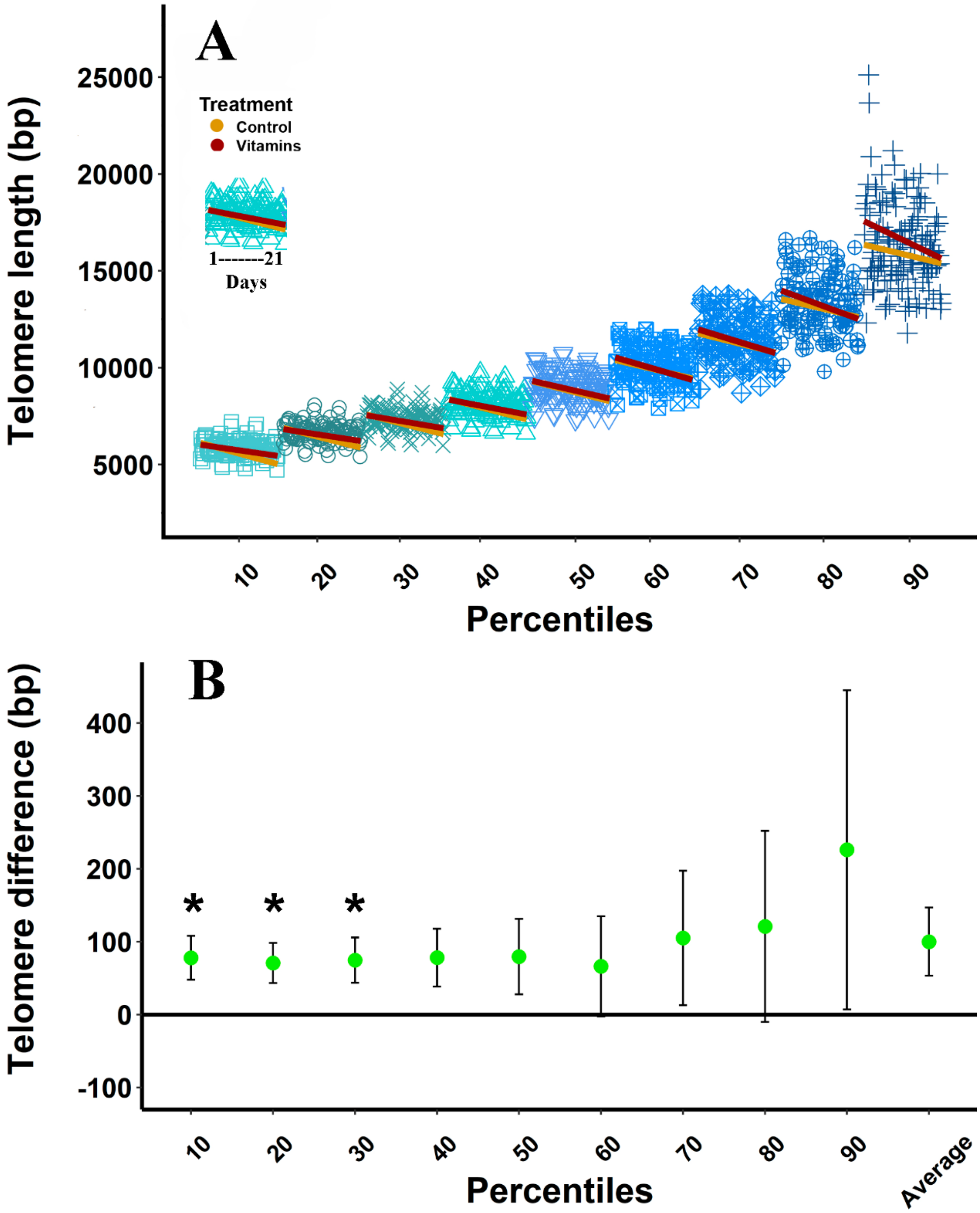


Figure 3. (A) Telomere shortening with age for the 10th to 90th percentile. X axis represents the age of the individuals, repeating the values for each percentile. Regression lines of telomere length by age are yellow for the Control group and red for the Vitamins group. **(B)** Estimate ± S.E. of telomere length in the Vitamins group relative to the Control group. * indicates differences between groups with $p<0.05$.

Discussion

Telomere attrition was 9.8 bp/day (Table 2, Figure 2), close to values obtained in other longitudinal studies of chicks using the same technique (common terns *Sterna hirundo*: 7.5 bp/day (Vedder et al., 2017); jackdaws *Corvus monedul*a: 10.5 bp/day (Boonekamp et al., 2014; Salomons et al., 2009). The rate of telomere loss appears to be higher at the higher percentiles (Table S1 Figure 3A), also in accordance with findings in jackdaws (Salomons et al., 2009) and common terns (Bauch et al., 2013). Longer telomeres are larger targets for damage, and a larger number of base pairs will on average be lost following a double-strand break, which may explain this pattern (Grasman et al., 2011). Previous studies found TL and their rate of loss to be affected by hatching order (Noguera et al., 2016; Stier et al., 2015), sex (Barrett and Richardson, 2011) or mass gain (Monaghan and Ozanne, 2018), but these variables did not noticeably affect TL and/or its attrition in our study (Table 2 and S5).

Our knowledge of the accelerating effect of oxidative stress on telomere attrition is largely based on *in vitro* studies, raising the question whether oxidative stress also modulates telomere attrition *in vivo*. In our study, the treatment we administered was effective in alleviating oxidative stress, in that tocopherol (significantly) and TAC (bordering on significance) were still elevated 1-2 weeks after supplement administration (Table 1, Figure 1A and 1B). Our treatment also had a significant effect on TL, with this group having 100 bp longer telomeres than the control group (Figure 3B). A difference of 100 bp may appear a modest effect when compared to the mean TL of 8700 bp, because it is only 1.15 % of average TL on an absolute scale. However, it is a large effect when one considers that individuals experience on average 150 bp shortening during the experimental period. By percentiles, the treatment had a significant effect on shorter telomeres (from the 10th to 30th percentile, and approaching significance in the 40th (Tables S1, Figure 3A). In the higher percentiles, this difference was not significant, although the “Vitamins” group had a longer TL at all percentiles, and this effect was larger at the longest percentiles, but so was the variance (Table S1, Figure 3A).

We did not find a significant relation between any of the oxidative stress variables and TL (Table S4) although the relation between tocopherol and TL in the 10th percentile approached significance. The lack of significance for the relation between MDA and telomere length could be due to the fact that it is not a marker of oxidative damage specific to DNA. In contrast, 8-oxo-dG is, and its measurement could have yielded a different result. Another potential explanation of the lack of relation between oxidative stress variables and telomere length despite the significant effect of the treatment on the former could be that the reduction in telomere attrition is not directly related to oxidative damage prevention, but rather to changes in redox signalling. It is known that the cellular redox environment (balance between the production of reactive species of oxygen and nitrogen and their removal by antioxidants) has an active role in the control of the cell cycle, which in turn could regulate telomere dynamics (Sarsour et al., 2009).

Observational studies on free-living birds show both significant (Geiger et al., 2012; Taff and Freeman-Gallant, 2017) and non-significant correlations between oxidative stress markers and TL or attrition (Boonekamp et al., 2017; Stier et al., 2015). Experimental studies in free-living animals have yielded mixed effects, with negative (Pérez-Rodríguez et al., 2019) or positive (Badás et al., 2015) results, or affecting only one sex (Noguera et al., 2015), and we contribute a positive result to this small set of studies. There are different potential explanations of this variation: studies differ in methods (including the composition of the antioxidant supplements); in the technique used to measure telomere length, or in the species of study (which present different physiology). Ecology may be very important, because antioxidant supplementation is likely to be of benefit if there is a shortage of antioxidants, either because the diet contains few antioxidants or the need for antioxidants is very high (Reichert and Stier, 2017; Beaulieu and Schaefer, 2013; Simons et al., 2014). For example, in (Badás et al., 2015) the higher shortening caused by reproduction was alleviated by the administration of tocopherol.

A limitation of antioxidant supplementation studies with the aim to alleviate oxidative stress is that the manipulation is indirect, in that the aim is to manipulate oxidative stress through the supplementation with antioxidants rather than manipulating oxidative stress directly. This limitation could in theory be mitigated by manipulating or measuring the molecular cause of oxidative stress directly (e.g. the production of reactive oxygen species), instead of resorting to downstream oxidative stress markers of which the interpretation is not straightforward, but this is difficult in practice, in particular in an ecologically relevant setting. Nevertheless, our results agree with the hypothesis that oxidative stress affects telomere attrition not only in cell cultures, but also *in vivo*, observing a positive effect on TL of the supplementation with antioxidants. This effect could indicate a lack of antioxidants in free-living populations of white stork, which could affect their potential lifespan.

Supplementary Material

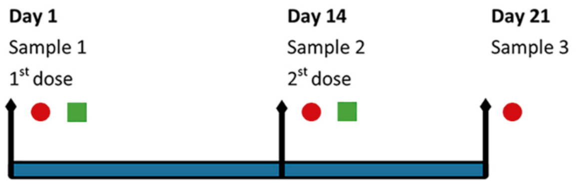


Figure S1: Timeline experimental protocol. The green squares indicate the administration of the treatment, while the red circles indicate that a blood sample was taken. Chicks were on average 20 days old at day 1.

Supplemental Table S1. Telomere length (average of each sample) in relation to the covariates Age (days), mass (g) and Hatch Order (1,2,3) and the factors Sex (Male) (male and female) and Treatment (Control or Vitamins; note that day 1 measurements are coded as control for all nestlings). Factors in the final model have been highlighted in **bold**. Significant factors (p < 0.05) have an *. Random terms are included in all models, and their values are in *italic*. Non-significant interactions were excluded from the models when estimating coefficients of main effects.

Percentile 10						
Fixed terms					**Random terms**	
Terms	**Intercept**	**Estimate (S.E.)**	**F (d.f)**	**p**	**Terms**	**Variance**
Age	**6121**	**-8.026 (1.148)**	**48.91 (78.07)**	**<0.001***	*Chick / Nest*	*76148*
					Nest	*45756*
Treatment (Vitamins)		**78.03 (30.11)**	**6.714 (87.62)**	**0.011***	*Gel*	*66411*
					Residual	*6675*
Rejected: Hatch order	6229	-60.54 (59.94)	1.02 (34.12)	0.32		
Rejected: Sex (Male)	6265	-74.6 (93.68)	0.634 (42.04)	0.43		
Rejected: Mass	6269	-0.014 (0.035)	0.162 (77.43)	0.688		
Rejected: Age:Sex (Male)	6242	-1.901 (1.918)	0.982 (73.65)	0.325		
Rejected: Age:Mass	6212	-0.001 (0.002)	0.127 (73.67)	0.722		
Rejected: Age:Hatch order	6161	0.667 (1.607)	0.172 (76.09)	0.679		
Rejected: Age:Treatment	6147	1391 (3972)	0.123 (70.56)	0.727		
Percentile 20						
Fixed terms					**Random terms**	
Terms	**Intercept**	**Estimate (S.E.)**	**F (d.f)**	**p**	**Terms**	**Variance**
Age	**6908**	**-7.735 (1.04)**	**55.34 (77.9)**	**<0.001***	*Chick / Nest*	*93020*
					Nest	*57931*
Treatment (Vitamins)		**70.88 (27.53)**	**6.629 (84.6)**	**0.012***	*Gel*	*60908*
					Residual	*5415*
Rejected: Hatch order	7017	-60.93 (65.71)	0.86 (35.61)	0.36		
Rejected:Sex (Male) (Male)	7059	-89.03 (102)	0.755 (44.11)	0.390		
Rejected: Mass	7060	-0.005 (0.03)	0.027 (77.23)	0.87		
Rejected: Age:Mass	6994	-0.001 (0.001)	0.738 (74.99)	0.393		

Rejected: Age:Sex (Male) (Males)	6996	-0.855 (1.908)	0.2 (73.53)	0.656		
Rejected Age:Hatch order	6947	0.642 (1.463)	0.192 (76.36)	0.662		
Rejected: Age:Treatment (Vitamins)	6943	0.351 (3.607)	0.01 (71.36)	0.923		
Percentile 30						
Fixed terms					**Random terms**	
Terms	**Intercept**	**Estimate (S.E.)**	**F (d.f)**	**p**	**Terms**	**Variance**
Age	**7622**	**-8.023 (1.172)**	**46.83 (78.37)**	**<0.001***	*Chick / Nest*	*111046*
					Nest	*79409*
Treatment		**74.7 (31.01)**	**5.804 (85.38)**	**0.018***	*Gel*	*76908*
					Residual	*6892*
Rejected: Sex (Male)	7666	-103 (112)	0.849 (44.75)	0.362		
Rejected: Hatch order	7771	-58.32 (71.7)	0.662 (33.17)	0.422		
Rejected: Mass	7769	0.008 (0.036)	0.051 (77.59)	0.821		
Rejected: Age:Mass	7684	-0.001 (0.001)	0.956 (75.27)	0.331		
Rejected: Age:Hatch order	7617	-0.869 (1.639)	0.281 (77.82)	0.597		
Rejected: Age:Treatment	7630	-1.35 (4.043)	0.112 (72.79)	0.739		
Rejected: Age:Sex (Male)	7629	-0.118 (2.184)	0.003 (71.87)	0.957		
Percentile 40						
Fixed terms					**Random terms**	
Terms	**Intercept**	**Estimate (S.E.)**	**F (d.f)**	**p**	**Terms**	**Variance**
Age	**8420**	**-8.473 (1.506)**	**31.64 (79.24)**	**<0.001***	*Chick / Nest*	*141293*
					Nest	*115654*
Treatment		**78.22 (39.62)**	**3.897 (87.93)**	**0.051**	*Gel*	*130402*
					Residual	*11457*
Rejected:Sex (Male)	8474	-129 (129)	1 (43.39)	0.323		
Rejected:Hatch order	8608	-73.28 (81.34)	0.812 (32.29)	0.374		
Rejected:Mass	8602	0.019 (0.047)	0.174 (78.38)	0.677		
Rejected: Age:Treatment	8597	-4.386 (4.567)	0.923 (75.66)	0.34		
Rejected: Age:Hatch order	8526	-1.227 (2.012)	0.372 (77.52)	0.544		
Rejected: Age:Mass	8436	-0.001 (0.002)	0.291 (73.68)	0.591		
Rejected: Age:Sex (Male)	8435	0.739 (2.803)	0.069 (72.22)	0.793		
Percentile 50						
Fixed terms					**Random terms**	
Terms	**Intercept**	**Estimate (S.E.)**	**F (d.f)**	**p**	**Terms**	**Variance**
Age	**9373**	**-8.906 (1.983)**	**20.18 (80.51)**	**<0.001***	*Chick / Nest*	*185604*
					Nest	*169804*
Treatment		**79.62 (51.78)**	**2.364 (91.38)**	**0.128**	*Gel*	*240977*
					Residual	*20044*
Rejected:Hatch order	9546	-96.78 (94.96)	1.039 (33.67)	0.315		
Rejected:Sex (Male)	9623	-159.9 (151.4)	1.115 (41.42)	0.297		
Rejected:Mass	9612	0.034 (0.061)	0.314 (79.71)	0.577		
Rejected:Treatment	9604	-7.946 (5.989)	1.76 (76.33)	0.189		

Rejected:Hatch order	9482	-2.078 (2.626)	0.626 (78.26)	0.431		
Rejected:Sex (Male)	9511	1.431 (3.453)	0.172 (73.51)	0.68		
Rejected:Mass	9430	-0.001 (0.003)	0.149 (73.53)	0.7		
Percentile 60						
Fixed terms					**Random terms**	
Terms	**Intercept**	**Estimate (S.E.)**	**F (d.f)**	**P**	**Terms**	**Variance**
Age	**10515**	**-9.060 (2.646)**	**11.72 (82.14)**	**<0.001***	*Chick / Nest*	*265472*
					Nest	*231185*
Treatment		**66.43 (68.6)**	**0.938 (95.11)**	**0.335**	*Gel*	*424537*
					Residual	*36055*
Rejected:Hatch order	10741	-126.7 (114.1)	1.232 (33.63)	0.275		
Rejected:Sex (Male)	10808	-138.4 (183.5)	0.569 (41.84)	0.455		
Rejected:Mass	10790	0.052 (0.082)	0.404 (81.74)	0.527		
Rejected: Age:Treatment	10780	-0.116 (7.977)	2.125 (77.67)	0.149		
Rejected: Age:Hatch order	10590	-3.323 (3.482)	0.911 (79.53)	0.343		
Rejected: Age:Sex (Male)	10658	3.612 (4.577)	0.623 (74.78)	0.433		
Rejected: Age:Mass	10550	-0.002 (0.004)	0.143 (74.92)	0..707		
Percentile 70						
Fixed terms					**Random terms**	
Terms	**Intercept**	**Estimate (S.E.)**	**F (d.f)**	**P**	**Terms**	**Variance**
Age	**11929**	**-10.04 (3.575)**	**7.888 (83.76)**	**0.007***	*Chick / Nest*	*427914*
					Nest	*304942*
Treatment		**104.8 (92.2)**	**1.292 (97.98)**	**0.259**	*Gel*	*744645*
					Residual	*66301*
Rejected:Hatch order	12202	-152.7 (145.1)	1.107 (34.28)	0.3		
Rejected: Mass	12170	0.078 (0.11)	0.503 (84.59)	0.48		
Rejected: Sex (Male)	12210	-77.04 (233)	0.109 (43.9)	0.743		
Rejected: Age:Treatment	12190	-17.14 (10.73)	2.551 (79.16)	0.114		
Rejected: Age:Sex (Male)	12310	8.038 (6.078)	1.749 (77.17)	0.19		
Rejected: Age:Hatch order	12060	-4.109 (4.713)	0.76 (79.98)	0.386		
Rejected: Age:Mass	11820	-0.003 (0.005)	0.426 (76.58)	0.516		
Percentile 80						
Fixed terms					**Random terms**	
Terms	**Intercept**	**Estimate (S.E.)**	**F (d.f)**	**P**	**Terms**	**Variance**
Age	**13824**	**-10.72 (5.045)**	**4.516 (82.92)**	**0.037***	*Chick / Nest*	*953071*
					Nest	*371546*
Treatment		**120.7 (130.5)**	**0.855 (96.15)**	**0.357**	*Gel*	*1433344*
					Residual	*131713*
Rejected:Hatch order	14245	-234.7 (212.3)	1.222 (35.56)	0.276		
Rejected:Mass	14210	0.094 (0.154)	0.374 (84.51)	0.542		
Rejected:Sex (Male)	14210	-2.335 (332.7)	0 (46.84)	0.994		
Rejected: Age:Treatment	14190	-24.35 (15.09)	2.605 (78.67)	0.111		
Rejected: Age:Sex (Male)	14380	13.69 (8.524)	2.578 (76.69)	0.112		
Rejected: Age:Hatch order	14050	-5.358 (6.622)	0.655 (79.33)	0.421		

Rejected: Age:Mass	13710	-0.005 (0.007)	0.426 (76.33)	0.516		
Percentile 90						
Fixed terms					**Random terms**	
Terms	**Intercept**	**Estimate (S.E.)**	**F (d.f)**	**P**	**Terms**	**Variance**
Age	**17092**	**-15.61 (8.318)**	**3.522 (73.48)**	**0.065**	*Chick / Nest*	*4678974*
					Nest	*451097*
Treatment		**255.9 (218.8)**	**1.065 (82.04)**	**0.305**	*Gel*	*6731578*
					Residual	*350493*
Rejected: Hatch order	18281	-659 (449)	2.153 (33.93)	0.152		
Rejected: Mass	18250	0.0985 (0.254)	0.151 (75.91)	0.699		
Rejected: Sex (Male)	18328	-149.8 (670.2)	0.05 (44.07)	0.824		
Rejected: Age:Sex (Male)	18600	19.35 (13.89)	1.942 (69.99)	0.168		
Rejected: Age:Treatment	18604	-25.38 (25.61)	0.982 (69.48)	0.325		
Rejected: Age:Hatch order	18187	-6.816 (11.11)	0.376 (71)	0.542		
Rejected: Age:Mass	18090	-0.001 (0.012)	0.012 (68.34)	0.913		

Supplemental Table S2. Mass (g) or Tarsus (mm) in relation to the factor Treatment (Control or Vitamins) and Day (1, 14 or 21). Factors in the final model have been highlighted in **bold**. Significant factors ($p < 0.05$) have an *. Random terms are included in all models, and their values are in *italic*. Non-significant interactions were excluded from the models when estimating coefficients of main effects.

	Fixed terms					**Random terms**	
	Terms	**Intercept**	**Estimate (S.E.)**	**F (d.f)**	**p**	**Terms**	**Variance**
Mass model	**Treatment**	**1560**	**-56.57 (70.09)**	**0.65 (119)**	**0.421**	*Chick/Nest*	*78883*
	Day (14)	**1560**	**1378 (58.80)**	**412 (95.64)**	**<0.001***	*Nest*	*74645*
	Day (21)	**1560**	**1697 (60.08)**	**412 (95.64)**	**<0.001***	*Residual*	*49684*
	Rejected: Treatment:Day	1560	2.7 (92.69)	0.001 (85.28)	0.977		
Size model	**Treatment**	**90.87**	**0.09 (2.93)**	**0.01 (114.8)**	**0.976**	*Chick/Nest*	*120*
	Day (14)	**90.87**	**69.13 (2.46)**	**631 (92.99)**	**<0.001***	*Nest*	*230*
	Day (21)	**90.87**	**89.09 (2.54)**	**631 (92.99)**	**<0.001***	*Residual*	*87*
	Rejected: Treatment:Day	90.87	-0.42 (3.96)	0.01 (83.28)	0.916		

Supplemental Table S3. Telomere length at the 10th, 20th, etc. to 90th percentile in relation to Age (days) and its interaction. Factors in the final model have been highlighted in **bold**. Significant factors (p < 0.05) have an *****. Random terms are included in all models, and their values are in *italic*. Non-significant interactions were excluded from the models when estimating coefficients of main effects.

	Fixed terms					**Random terms**	
	Terms	**Intercept**	**Estimate (S.E.)**	**F (d.f)**	**p**	**Terms**	**Variance**
Percentiles model	**Percentiles**	**4092**	**121 (1.21)**	**10035 (1152)**	**<0.001***	*Sample ID / Chick / Nest*	*0*
	Age	**4092**	**-7.15 (3.96)**	**3.27 (1209)**	**0.071**	*Chick / Nest*	*270754*
						Nest	*195406*
	Percentiles:Age	3780	-0.19 (0.13)	2.09 (1151)	0.15	*Gel*	*425398*
						Residual	*1185284*

Supplemental Table S4. Telomere length (average of each sample) in relation to the oxidative stress variables (corresponding to the values of the previous day of the experiment). Factors in the final model have been highlighted in **bold**. Significant factors (p < 0.05) have an *****. Random terms are included in all models, and their values are in *italic*.

	Fixed terms					**Random terms**	
	Terms	**Intercept**	**Estimate (S.E.)**	**F (d.f)**	**p**	**Terms**	**Variance**
Average	**AGE**	**8873**	**-4.495 (3.64)**	**1.527 (46.34)**	**0.223**	*Chick / Nest*	*106730*
	Tocopherol		**-47.2 (32.63)**	**2.093 (47.13)**	**0.155**	*Nest*	*189285*
	MDA		**44.73 (31.71)**	**1.99 (48.03)**	**0.165**	*Gel*	*228414*
	TAC		**-4.73 (16.02)**	**0.087 (40.39)**	**0.769**	*Residual*	*15680*
Percentile 10	**AGE**	**6116**	**-6.913 (2.621)**	**6.95 (47.54)**	**0.011***	*Chick / Nest*	*51368*
	Tocopherol		**0.34 (23.49)**	**0.001 (48.51)**	**0.989**	*Nest*	*68063*
	MDA		**15.48 (22.74)**	**0.464 (50.08)**	**0.499**	*Gel*	*75439*
	TAC		**-11.97 (11.61)**	**1.062 (40.6)**	**0.309**	*Residual*	*8267*
Percentile 20	**AGE**	**6858**	**-5.547 (2.275)**	**5.945 (43.89)**	**0.019***	*Chick / Nest*	*63002*
	Tocopherol		**-16.52 (20.43)**	**0.654 (44.42)**	**0.423**	*Nest*	*88696*
	MDA		**20.45 (19.85)**	**1.062 (45.24)**	**0.308**	*Gel*	*77586*
	TAC		**-8.38 (9.911)**	**0.715 (39.62)**	**0.403**	*Residual*	*5951*
Percentile 30	**AGE**	**7528**	**-4.738 (2.437)**	**3.78 (43.54)**	**0.058**	*Chick / Nest*	*75492*
	Tocopherol		**-30.69 (21.89)**	**1.97 (44.02)**	**0.168**	*Nest*	*120653*
	MDA		**25.29 (21.29)**	**1.41 (44.67)**	**0.241**	*Gel*	*94541*
	TAC		**-5.249 (10.6)**	**0.245 (39.58)**	**0.623**	*Residual*	*6797*
Percentile 40	**AGE**	**8258**	**-3.512 (3.031)**	**1.343 (44.69)**	**0.253**	*Chick / Nest*	*93376*
	Tocopherol		**-50.08 (27.21)**	**3.387 (45.28)**	**0.072**	*Nest*	*175525*
	MDA		**32.07 (26.48)**	**1.467 (45.92)**	**0.232**	*Gel*	*142318*
	TAC		**-3.287 (13.25)**	**0.062 (39.98)**	**0.805**	*Residual*	*10658*
Percentile 50	**AGE**	**9147**	**-2.507 (3.816)**	**0.432 (49.9)**	**0.515**	*Chick / Nest*	*121624*
	Tocopherol		**-66.45 (34.24)**	**3.767 (46.62)**	**0.058**	*Nest*	*255819*
	MDA		**45.28 (33.32)**	**1.846 (47.26)**	**0.181**	*Gel*	*246389*
	TAC		**0.316 (16.76)**	**0.001 (40.39)**	**0.985**	*Residual*	*17136*
Percentile 60	**AGE**	**10247**	**-2.012 (4.893)**	**0.169 (46.98)**	**0.683**	*Chick / Nest*	*177420*
	Tocopherol		**-77.91 (43.87)**	**3.153 (47.81)**	**0.082**	*Nest*	*358043*
	MDA		**64.16 (42.67)**	**2.26 (48.62)**	**0.139**	*Gel*	*443578*
	TAC		**6.529 (21.59)**	**0.092 (40.77)**	**0.764**	*Residual*	*28524*
Percentile 70	**AGE**	**11565**	**-0.477 (6.357)**	**0.006 (47.48)**	**0.94**	*Chick / Nest*	*297541*
	Tocopherol		**-102 (56.99)**	**3.194 (48.36)**	**0.08**	*Nest*	*510348*
	MDA		**84.96 (55.34)**	**2.357 (49.44)**	**0.131**	*Gel*	*737970*
	TAC		**18.34 (28.1)**	**0.426 (40.96)**	**0.518**	*Residual*	*48359*
Percentile 80	**AGE**	**13226**	**4.009 (8.546)**	**0.22 (47.04)**	**0.641**	*Chick / Nest*	*626268*

	Tocopherol		**-149 (76.62)**	**3.77 (47.89)**	**0.058**	*Nest*	*752358*
	MDA		**112 (74.2)**	**2.293 (49.38)**	**0.136**	*Gel*	*1069014*
	TAC		**28.56 (37.69)**	**0.574 (40.83)**	**0.453**	*Residual*	*86786*
Percentile 90	**AGE**	**15934**	**9.242 (13.17)**	**0.492 (46.62)**	**0.486**	*Chick / Nest*	*1864367*
	Tocopherol		**-217 (118)**	**3.382 (47.49)**	**0.072**	*Nest*	*1041406*
	MDA		**184 (114)**	**2.623 (49.99)**	**0.111**	*Gel*	*1370390*
	TAC		**35.72 (58.05)**	**0.379 (40.5)**	**0.542**	*Residual*	*205278*

Supplemental Table S5. Telomere length (average of each sample) in relation to the covariate Age (days) and Mass (g). Factors in the final model have been highlighted in **bold**. Significant factors (p < 0.05) have an *****. Random terms are included in all models, and their values are in *italic*. Non-significant interactions were excluded from the models when estimating coefficients of main effects.

	Fixed terms					**Random terms**	
	Terms	**Intercept**	**Estimate (S.E.)**	**F (d.f)**	**p**	**Terms**	**Variance**
Average	**Mass**	**9005**	**0.03 (0.06)**	**0.318 (79.56)**	**0.574**	*Chick / Nest*	*193368*
	Age	**9005**	**-9.91 (4.55)**	**4.746 (78.17)**	**0.032***	*Nest*	*98761*
	Rejected Mass:Age	8930	-0.01 (0.02)	0.288 (76.68)	0.593	*Gel*	*237984*
						Residual	*16916*
Percentile 10	**Mass**	**6088**	**0.01 (0.04)**	**0.0001 (78.73)**	**0.99**	*Chick / Nest*	*75324*
	Age	**6088**	**-6.22 (2.98)**	**4.357 (77.239**	**0.04***	*Nest*	*46637*
	Rejected Mass:Age	6023	-0.01 (0.01)	0.5 (76.65)	0.48	*Gel*	*66258*
						Residual	*7266*
Percentile 20	**Mass**	**6877**	**0.01 (0.03)**	**0.05 (78.65)**	**0.825**	*Chick / Nest*	*93408*
	Age	**6877**	**-6.65 (2.69)**	**6.09 (77.5)**	**0.016***	*Nest*	*58238*
	Rejected Mass:Age	6808	-0.01 (0.01)	0.69 (76.35)	0.41	*Gel*	*59639*
						Residual	*5866*
Percentile 30	**Mass**	**7587**	**0.02 (0.04)**	**0.31 (79.19)**	**0.579**	*Chick / Nest*	*111605*
	Age	**7587**	**-7.87 (3.02)**	**6.8 (78.04)**	**0.011***	*Nest*	*79433*
	Rejected Mass:Age	7499	-0.01 (0.01)	0.91 (76.87)	0.344	*Gel*	*74104*
						Residual	*7379*
Percentile 40	**Mass**	**8382**	**0.03 (0.05)**	**0.46 (80.11)**	**0.5**	*Chick / Nest*	*141595*
	Age	**8382**	**-9.11 (3.84)**	**5.63 (78.83)**	**0.02***	*Nest*	*114565*
	Rejected Mass:Age	8296	-0.01 (0.01)	0.536 (77.41)	0.466	*Gel*	*125615*
						Residual	*11995*
Percentile 50	**Mass**	**9333**	**0.05 (0.06)**	**0.59 (81.34)**	**0.446**	*Chick / Nest*	*185368*
	Age	**9333**	**-10.68 (5.01)**	**4.55 (79.96)**	**0.036***	*Nest*	*167437*
	Rejected Mass:Age	9242	-0.01 (0.01)	0.35 (78.18)	0.55	*Gel*	*234155*
						Residual	*20592*
Percentile 60	**Mass**	**10480**	**0.06 (0.08)**	**0.63 (83.08)**	**0.43**	*Chick / Nest*	*265358*
	Age	**10480**	**-12.49 (6.63)**	**3.55 (81.47)**	**0.063**	*Nest*	*228172*
	Rejected Mass:Age	10380	0.01 (0.01)	0.24 (79.36)	0.625	*Gel*	*418090*
						Residual	*36314*
Percentile 70	**Mass**	**11872**	**0.1 (0.1)**	**0.868 (85.1)**	**0.354**	*Chick / Nest*	*431596*
	Age	**11872**	**-15.49 (8.94)**	**3 (83.28)**	**0.087**	*Nest*	*297863*
	Rejected Mass:Age	11700	0.01 (0.01)	0.39 (80.91)	0.533	*Gel*	*732195*
						Residual	*66695*
Percentile 80	**Mass**	**13758**	**0.12 (0.15)**	**0.65 (84.98)**	**0.423**	*Chick / Nest*	*962022*
	Age	**13758**	**-17.49 (12.57)**	**1.94 (83.02)**	**0.168**	*Nest*	*362559*
	Rejected Mass:Age	13550	0.01 (0.01)	0.29 (80.72)	0.591	*Gel*	*1415561*
						Residual	*131917*
Percentile 90	**Mass**	**16977**	**0.15 (0.25)**	**0.35 (76.23)**	**0.554**	*Chick / Nest*	*4735283*
	Age	**16977**	**-22.08 (20.74)**	**1.13 (74.51)**	**0.291**	*Nest*	*408396*
	Rejected Mass:Age	17140	0.01 (0.01)	0.07 (72.94)	0.798	*Gel*	*6635976*
						Residual	*353073*

General discussion

Landfills are easily detectable areas that offer large amounts of organic resources that can be used as a food supply by white storks among other species. This impressive amount of feeding resources is also constantly renewed. Such characteristics make landfills rich feeding patches, easily accessible, not only for adult individuals but also for their offspring, which enables higher feeding rates (Djerdali et al., 2016). With this in mind, this thesis aimed to disentangle the current and potential individual effects of the use of this food resource on nestlings using different approaches. Since we used an integrative approach it most valuable to discuss the results from general point of view.

If the use of landfills enables higher feeding rates, it should be reflected by different nutritional variables. The results of chapter one and two support this premise. In chapter one, nestlings feed from landfills showed higher albumin, cholesterol, glucose, and triglycerides concentrations in plasma, which are indicative of higher food intake (Milner et al., 2003; Rodriguez et al., 2011; Tauler-Ametller et al., 2019). The same pattern was found in chapter two, by a negative correlation between the distance to the landfill (considered an indicator of the use of it as a feeding resource (Djerdali et al., 2016; Gilbert et al., 2016)) and the concentration of cholesterol, cholesterol HDL, triglycerides and urea in plasma, which again indicates higher feeding rates in nestlings located near landfills. None of the relations found in chapter two differs significantly between years, which indicates that the effect of feeding on landfills on these nutritional variables is consistent over time. A possible explanation could be that, even in years with a high presence of natural prey, the amount and availability of food is always higher in landfills compared to natural areas. These results are in accordance with previous studies in other bird species such as silver (*Larus novaehollandiae*) and yellow-legged (*Larus michahellis*) gulls, where the use of landfills increased body condition (Auman et al., 2018; Steigerwald et al., 2015). The same results were found in american black vulture (*Coragyps atratus*), that not only were in a good body condition, but also had a good nutritional status (Plaza and Lambertucci, 2018) similarly to the results in the white storks of the present study. To our knowledge, this is the first times that the relation between physiological variables related to nutritional status was evaluated in relation to the use of landfills in white storks. Nevertheless, previous studies reported that egg volume, clutch size and breeding and hatching success are higher in individuals near rubbish dumps (Djerdali et al., 2008 and 2016; Tortosa et al., 2002 and 2003).

In the food found on landfills, different pollutants can be found (Plaza and Lambertucci, 2017) which can eventually be transferred to white storks (Muñoz-Arnanz et al., 2011; de la Casa-Resino et al., 2014; de la Casa-Resino et al., 2015). If such toxic substances (mainly heavy metals) enter the organism they could affect different tissues and systems. To provide an overall idea of the general impact on the organism, we chose to evaluate the effect on renal and hepatic systems due to their role in the detoxification process of the organism. Higher values of AST, ALT and AMP are indicative of liver function, while the increase in the activity of CK and creatinine concentration relate to renal function. Our results for the former parameters were consistent with the ones reported in individuals of the same species (Puerta et al., 1989), also in nestlings more closely associated with landfills. These results indicate an absence of measurable negative effects of this food resource on liver and kidney functionality, in the variables used. The evaluation of some of these variables in egyptian vultures did not show any differences between individuals which fed in landfills or those who did not (Tauler-

Ametller et al., 2019); and in the case of american black vultures, similarly to our situation: if any variables show differences, the higher values were in individuals unrelated to landfills (Plaza and Lambertucci, 2018).

Any indication of renal or hepatic functional damage reflects significant damage to the organism and potentially a harmful situation. However, long before any serious functional damage is evident in an organism, other systems could be affected as they are more sensitive. The search for these early indicators is important in medicine and veterinary fields, being some of the most interesting variables associated with the oxidative stress balance. We have mentioned earlier that the presence of pollutants in landfills is well established, especially heavy metals (Plaza and Lambertucci, 2017) the uptake of which by white storks has also been shown (de la Casa-Resino et al., 2014 and 2015). The direct relation of heavy metals and oxidative stress is well-known (Ercal et al., 2011), and has been largely demonstrated in white storks (Kaminski et al., 2007 and 2009; Tkachenko and Kurhaluk, 2012 and 2013).

Our findings suggested that the oxidative stress balance of white stork nestlings which use landfills as food resource showed a hormetic response; a dose-response phenomenon in which exposure to a low dose of a chemical agent, that is damaging at higher doses, induces an adaptive positive effect. This is typical in the exposure to many toxic substances (Mattson, 2008; Rattan, 2008).

The interpretation of oxidative stress balance requires the integration of both, indicators of oxidative damage and the measurement of antioxidant defences. The only parameter indicative of oxidative damage which showed differences is the concentration of metHb in the nestlings examined in chapter one, with higher values in the colony associated with a landfill. MetHb is formed by the oxidation of the iron atom in haemoglobin from ferrous to ferric (Patton et al., 2016), which renders the transport of oxygen impossible, producing tissue hypoxia and possibly cell death which isthe reason why high values could produce seizures, coma or death (Patton et al., 2016). Its percentage in the blood is used as a biomarker of exposure to nitrates, nitrites, and N-nitroso compounds (Martínez-Haro and Mateo, 2008). Nitrate is a precursor to nitrite, which in cured meat contributes to the flavour, inhibits the growth of microorganisms, controls rancidity and fixes colour; being the reasons why the meat and poultry industry use it frequently in their products (Sindelar, 2012). Its presence in meat product remains of which white storks possibly feed, could explain the higher presence of metHb in these individuals.

Regarding antioxidants, in chapter one, chicks related to landfills had higher values of both GSH and retinol, being an endogenous and an exogenous antioxidant, respectively. In chapter two this pattern was not the same, with lower values of the exogenous antioxidant tocopherol in individuals from landfills, but almost higher “total antioxidant capacity” in one of the years. Our results suggested that white storks are developing a hormetic response, as individuals who feed on landfills have higher levels of endogenous antioxidants but lower values in the indicators of oxidative damage. It looks like the pollutants are producing oxidative damage, which is fully counteracted by antioxidants. This explains why we did not detect an increase in indicators of oxidative damage, but instead we detected an increase in the antioxidant defence. This scenario coincides with the first part of a hormetic situation (the chemical increases the defences, but it does not reach a point where the defences are overwhelmed until they fail). This situation could suggest an adaptation to these pollutants and could also explain the lack of effect on previously commented parameters related to hepatic and renal function.

Due to the importance of dietary antioxidants on oxidative stress balance, it is logical to expect yearly differences in their concentration in peripheral blood, in addition to the potential lack of antioxidants in food from landfills (Tauler-Ametller et al., 2019). The Mediterranean climate is characterized by mild wet winters and dry summers, and the quantity of rain in spring could affect the amounts of different types of prey of white storks, such as earthworms and amphibians. This is important as, despite the proximity to landfills, during the first days after hatching all white stork nestlings must be fed exclusively on natural prey (Djerdali et al., 2016; Kosicki et al., 2006). An important part of the antioxidant system which is completely related to food is the exogenous components, such as retinol, tocopherol, and carotenoids, which presented higher concentrations in all individuals in 2014, independently of the distance to the landfill. While no differences were evident in stork nestlings from 2014, in 2013 we found a positive relation between the distance to the landfill and the total antioxidant capacity of plasma, and a negative relation with the concentration of MDA, respectively. A possible explanation could be that the higher concentration of dietary antioxidants in 2014 makes the elevation of the endogenous protection against the pollutants in landfills unnecessary, or, if it occurs, is masked by them. This result is in accordance with previous data in egyptian vultures (Tauler-Ametller et al., 2019), where landfill feeding increased endogenous antioxidants as a compensatory response to pollutants, with no differences in MDA despite the lower levels of exogenous antioxidants in these individuals.

An additional problem of the use of landfills could be the presence of pathogenic microorganisms and/or the presence of multiresistant strains, which could be transmitted to nestlings, as indicated by the higher presence of antibiotic resistant *E.coli* in the individuals associated to landfills. The exposure to different pathogens in wildlife associated with landfills has been previously documented, including the presence and prevalence of bacteria that carry mechanisms of antibiotic resistance (ABR) (Barbara et al., 2017; Plaza and Lambertucci, 2017; Migura-García et al., 2019). Antibiotics were widely used in the treatment of bacterial infections and for growth promotion of livestock although the former has been banned in the EU since 2007 (Wang et al., 2019). Also, antibiotic overuse in private households, their uncontrolled disposal on landfills and the disposal of sludge from wastewater treatment facilities lead to the presence of residues of antibiotics in the landfills systems (Ahlstrom et al., 2019; Wang et al., 2019). The presence of antibiotics on the landfills leads to an increase in the prevalence of ABR (Borquaye et al., 2019), causing it to be a priority global research need (Chung et al., 2018) due to the potential reduction in the efficacy of antimicrobial treatments (with its subsequent increase in morbidity and mortality from minor bacterial infections), as well as a general increase in health care expenditure (Borquaye et al., 2019). The relation these ARB and avian species have is particularly dangerous, as birds could play a role in their dissemination; in particular in the case of the white stork, because of their close contact to humanized and natural habitats which could enhance the possibility of transmission and dispersal (Gómez et al., 2016). In chapter one we observed a higher prevalence of *E. coli* with multiresistant phenotypes in nestlings associated with landfills than in others associated with more natural areas. Its presence in nestlings could be due to direct acquisition through contaminated food items, or by transmission due to the high level of interaction between individuals (inter or intraspecies), but also due to selection of the more resistant phenotypes in the intestinal microbiota by ingestion of residues of antibiotics (Borquaye, 2019). Finally, the pressure of exposure to heavy metals in landfill foraged food could also lead to co-selection of antibiotic resistance in intestinal bacteria (Li et al., 2019). Our results are consistent with previous data on white storks that shows that individuals from landfill-associated colonies are carriers of *S. aureus* resistant to methicillin, an agent that causes subclinical to severe infections (Gómez et al., 2016). There is also a positive relationship between the use of landfills and the prevalence of colistin-resistant *E. coli*, which is the last-line antimicrobial for the treatment of multidrug-resistant *Enterobacterales* in human medicine (Migura-García, et al., 2019) an extended spectrum lactamase producing *E.coli* (Höfle et al., 2020). All these results indicate the importance of ABR in bacteria, not only for the health of the storks but also from a public health point of view, due to the possibility that white storks act as carriers of these microorganisms to wildlife and livestock. The dissemination of antimicrobial resistance genes and plasma would be a problem not only for free-living animals but also for humans, due to higher pressure in the big problem of the discovery of new antibiotics, due to the quick development of new resistances in pathogenic microorganisms. The great move-range of white storks, and their contact with both anthropized and natural habitats, make them dangerous vectors, and new studies about their microbiota its resistance are needed. A good approximation would be the evaluation of the entire gut microbiome using NGS techniques, which ensures the determination of the complete microbiome and the presence of genes associated with resistance.

Previous studies about the effect of the use of landfills on the health status of wildlife were based on external measurements, mainly body condition (Plaza and Lambertucci, 2017), and also the colour of living tissues, which are likely to reflect current physiological condition better than feathers (Pérez-Rodríguez, 2009). Differences in these variables, as we found in chapter three, could be used to monitor the effect of the use of landfills as a food resource by white storks from colonies with previous baseline data, as in our case, with less expenditure of money and time. Colour reflects an animals' physiological condition because yellow-red pigmentation is produced by carotenoids (García-de Blas et al., 2013). Carotenoids can only be acquired through the diet and they are considered indicators of condition and foraging ability (Pérez-Rodríguez et al, 2010). Carotenoids also have a role as antioxidants and immune stimulants (Mougeot et al, 2009; Pérez-Rodríguez et al., 2010), the reason why individuals must allocate them to either self-maintenance or ornamental colouration. This need to allocate causes carotenoid dependent pigments to reveal individual quality, affecting mate choice and reproductive decisions, which could affect future reproductive events (Negro and Garrido, 2000).

We found a negative association between redness of the legs and the use of landfills, which should indicate differences in the concentration of carotenoids. Unfortunately, we did not have data of carotenoids in the peripheric blood of the individuals from which we obtained the colour measurements; however, our results from previous years in the same colonies suggest we would not have found any significant differences. The logical explanation could be that the concentration of carotenoids in food from landfills is lower than in natural areas (Tauler-Ametller, 2019), in addition to the increase in natural areas of the invasive Red Swamp Crayfish (*Procambarus clarkii*), a prey of white storks which is rich in carotenoids (Negro and Garrido, 2000). These results indicate that we can use external measurements as a tool in a monitoring program of white storks related to landfills (previously studied); however, to have a better understanding of the relation of colour and carotenoids regarding landfills, the next logical step should be to quantify it;

to evaluate the colour in both legs and bill, accompanied by an evaluation of the concentration of carotenoids in peripheral blood and coloured tissues.

With these results we can see the current situation like a “fixed picture” with no information about the carry-over effects of the use of landfills on the life of the stork nestlings. In this context, our results in chapter four, where we observed the relation between telomeres and exogenous antioxidants, could indicate that the use of this resource could have a potential long-term effect.

It is well known that the early-development conditions can influence different life-history traits later in life; the most important being the lifespan of individuals (English and Uller, 2016). Ideally, we should monitor individuals from hatch to death. However, this is difficult to realise, monitoring an especially long-lived species such as the white stork. And even more so during the short duration of a Doctoral Thesis. As a proxy to this effect, we chose to measure the telomeres, which have been suggested to be responsible for the link between early-life conditions and potential lifespan (Monaghan and Haussmann, 2006; Eastwood, et al., 2019). Telomeres are noncoding sequence repeats (TTAGGG) located at the end of chromosomes to maintain chromosomal integrity and prevent damage to coding DNA in each cell division by shortening themselves instead of the coding DNA (Blackburn 1991; von Zglinicki et al., 2001). Finally, when they reach a critically short length, the cell enters a degenerative process of senescence, followed by apoptosis (Blackburn 1991). In the past, it was established that the attrition followed a constant rate; however, it is known that different external factors may alter the rate of attrition during the nestling period, mainly through oxidative stress (Mizutani et al., 2013). However, this relation is largely based on *in vitro* studies (von Zglinicki, 2002; Houben et al, 2008). Our results showed that by administring antioxidants in free-living white storks, theirtelomeres remain longer. This is among the first few studies to support that this relation also occurs *in vivo*. Furthermore, this effect of the antioxidant administration could suggest that possibly free-living population of white storks show a lack of dietary antioxidants.

However, it is very difficult to make any assumption of the future of this species in our country, due to changes in legislation surrounding landfills. We mentioned that the use of landfills was one of the reasons of the population increase after the decline which was brought on by the destruction of their natural habitats. European legislation (Council Directive 1999/31/EC; Council Directive 2008/98/EC; Council Directive 2018/851) and therefore Spanish legislation (Ministerio de Medio Ambiente, y Medio Rural y Marino, 2011), indicates that the countries must reduce levels of residual waste to close to zero, with the replacement of open-air landfills by covered processing facilities that are inaccessible by birds including white storks (Gilbert et al., 2016). This will supposedly cause a sharp reduction in the availability of food, which could affect demography, as well as behaviour, forcing adult individuals to migrate again or forage for original natural food resources once again. Nevertheless, in many cases the return to their natural habitats is not possible, due to their disappearance or drastic modifications; or if possible, the return to these areas would imply problems to their natural prey. For these reasons, there is a need to look for options that allow the sustainable management of our waste without affecting the species benefiting from them, including the white stork as one of the most remarkable examples for which the Iberian Peninsula is of particular importance due to its emblematic role for migratory routes of most of the Western part of the European population.

Conclusions

- As expected, the use of landfills as resource by white storks has some effects, not only at population level as has been observed in previous studies, but also at the individual level. Using landfills has some short-term advantages but may have long-term disadvantages. However, the higher reproductive success caused by the use of landfills appears to offset this disadvantage at a population level, as the increase in the number of individuals observed in our country might indicate.
- Use of landfill foraged food implies better nutritional status in nestlings, possibly due to higher food intake, which is important for self-maintenance and future reproduction.
- The use of this resource affected oxidative stress balance, which in turn produced an increase in the antioxidant defences but not in the indicators of damage, which suggests an adaptation of this system to the use of this landfill forage food.
- In no case did the administration of food from landfills have a detrimental effect on blood chemistry. Thus, no apparent short-term damage due to the use of landfills can be detected.
- Exposure to this kind of resources produced an increase in the presence of *E.coli* with a phenotype of multiresistance to antibiotics, which could pose a problem for the health of the stork nestlings. It can also pose a problem from a public health point of view because it could play a role in the dissemination of these microorganisms to other wildlife and livestock.

- Oxidative stress balance and the use of landfills are related, and this relation depends on the year of the study. This result supports the idea of the importance of the role of the dietary antioxidants in natural food.
- The detection of telomere attrition in white stork nestlings indicated the potential use of this technique to evaluate long-term effects produced by external factors in this long-lived species.
- Our finding of a relation between the administration of antioxidants and telomere dynamics indicated that free-living populations of white storks have a lack of dietary antioxidants, which could have long-term effects such as potential effects on lifespan.
- The use of landfills as food resource produces external differences between nestlings, as happened in the SMI and the redness of the legs. Both measurements could be used in non-invasive monitoring of previously studied colonies regarding the effect of the use of landfills.

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