Universidade do Minho
Escola de Ciências

Rute Alexandra Pais Costa

Forest Avifauna as a Bioindicator of Heavy Metal Pollution

Outubro de 2012

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Forest Avifauna as a Bioindicator of Heavy Metal Pollution

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Professor Doutor Tapio Eeva

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ABSTRACT

Air pollution has become a widespread problem in the last century, becoming necessary the monitorization of several habitats. Air pollution was found to have direct and indirect effects on forest passerines (Eeva et al. 1997), but there is very little information on the effects of emissions from the paper and pulp industry.

The present work includes a series of studies which main goals were to use non-invasive procedures in the evaluation of forest passerines as bioindicators of heavy metal pollution and to assess the possible influence of pollution in birds' breeding biology and health status in industrial and rural sites in maritime pine forests on the west coast of Portugal.

We found higher arsenic levels in the rural area and higher mercury levels in the industrial area but we also found several differences with significantly lower levels of contamination in 2010 and 2011. We found that Great tits bred earlier, laid more eggs and produced more fledglings in the industrial area, where we also found higher caterpillar biomass, which are an important food source for tits. Health indices presented similar results in both areas and comparing to other studies in Europe the values are consistent with good health conditions.

Our results suggest that there are no direct toxic effects of emissions from the paper industry on the study species. However, invertebrate food availability seems to be related to pollution levels, which indirectly affect the breeding performance of the Great tit.

RESUMO

A poluição atmosférica tornou-se um problema generalizado no século passado, tornando-se necessária a monitorização de diversos habitats. Foi já demonstrado que a poluição atmosférica pode ter efeitos diretos e indiretos sobre passeriformes florestais (Eeva et al., 1997), mas há muito pouca informação sobre os efeitos das emissões de indústria de pasta e papel.

O presente trabalho inclui uma série de estudos cujo objetivo principal foi usar procedimentos não-invasivos na avaliação de passeriformes florestais como bioindicadores de poluição por metais pesados e avaliar a possível influência da poluição na biologia de reprodução das aves e no seu estado de saúde, em zonas de pinhal com influência de indústria e de agricultura, na costa oeste de Portugal.

Encontrámos níveis mais elevados de arsénio na área rural e níveis mais elevados de mercúrio na área industrial, mas também encontrámos várias diferenças com níveis significativamente mais baixos de contaminação em 2010 e 2011. Descobrimos que o Chapim-real, coloca mais ovos e tem mais crias na área de influência industrial, onde também se pode encontrar um maior número de larvas de insectos, que são uma fonte importante de alimento para os chapins. Os índices de saúde apresentaram resultados semelhantes em ambas as áreas e a sua comparação com outros estudos na Europa demonstra que os valores são consistentes com boas condições de saúde.

Os resultados sugerem que não há nenhum efeito tóxico direto de emissões da indústria de papel sobre a espécie de estudo. No entanto, a disponibilidade de alimentos parece estar relacionada com os níveis de poluição, que indiretamente afetam o desempenho reprodutivo de Chapim-real.

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II. Costa, R.A., Eeva, T., Eira, C., Vaqueiro, J. and Vingada, J.V. Trace Elements in Faeces of Great Tit Nestlings in Relation to Breeding Performance in Coastal Areas in Central Portugal. Archives of Environmental Contamination and Toxicology 63 (4): 594-600 (2012).

III. Costa, R.A., Eeva, T., Eira, C., Vaqueiro, J. and Vingada, J.V. Assessing heavy metal pollution using great tits (*Parus major*): feathers and excrements from nestlings and adults. Environmental Monitoring and Assessment DOI 10.1007/s10661-012-2949-6 (2012).

IV. Costa, R.A., Eeva, T., Eira, C., Vaqueiro, J. and Vingada, J.V. Passerines breeding in nest-boxes: Long-term study in coastal pine stands, central Portugal. Under review for publication in Ornis Fennica.

V. Costa, R.A., Eeva, T., Eira, C., Vaqueiro, J., Medina, P. and Vingada, J.V. Great tit as a bioindicator of mercury contamination from the paper and pulp industry. Submitted to Chemistry and Ecology.

CHAPTER 1

INTRODUCTION

1. Introduction

Air pollution has increasingly become an expanding problem in the last century, significantly affecting terrestrial ecosystems, changing the physical structure and / or chemical habitats, making them less suitable for wildlife species (Pitelka 1994; Eeva and Lehikoinen 1996; Newton 1998). One of the main concerns are heavy metals, which are frequent waste products of industrial processes, often resulting in the contamination of the surrounding environment and potentially affecting the environment for decades after the actual pollution has occurred, since they can accumulate in organisms, particularly those living near industrial areas (Fowler 1990). Also, when above certain levels, heavy metals can cause decreased reproductive success and survival of wild species (Esselink et al. 1995; Eeva and Lehikoinen 1996). Because of their persistence and bioaccumulation potential, monitoring possible effects on wildlife is important. Although monitoring substances in air, soil and/or water gives valuable information on possible threats to the environment, the use of living organisms as biomarkers of metal contamination is often more advantageous. In fact, contamination levels are higher in biological tissues in relation to samples of water or sediment, while allowing to record ranges of concentrations in species consumed by Humans, and contributing to the assessment of potentially dangerous levels concerning public health (Burger 1993; Burger et al. 1994; Eens et al. 1999).

1.1 Metals

About 80% of the elements are metals. Metals are ubiquitous in the global environment, as a result of human activities (e.g. pollution released by anthropogenic emissions, or fossil fuel combustion) and natural processes (e.g. volcanic activities and processes of metal volatilization from the surface).

Some metals, with special focus on mercury and arsenic, are bioaccumulative, persistent in the environment and may also become toxic at high doses.

Arsenic

Arsenic compounds are naturally present in the environment at low levels. Arsenic is used in the manufacture of wood preservatives, glass and non-ferrous alloys, and is also used in bronzing and pyrotechnics. The use in agricultural products (including pesticides) is banned in almost all western countries, although some compounds like sulphide Paris green, calcium arsenate and lead arsenate have been used as agricultural insecticides. Excessive exposure to arsenic can lead to health effects on the digestive and central nervous system, heart and kidneys and some of its compounds may cause cancer and genetic damage (EPER 2009).

Mercury

Mercury has been used in the manufacture of thermometers, barometers, diffusion pumps and other instruments. The main sources of mercury release to the atmosphere are waste incineration, non-ferrous metal production, coal combustion, crematoria and chlorine manufacturing plants using mercury cells. Mercury is also released to waste water by industrial processes using the metal and its compounds and from dental surgeries, hospitals and clinics. Excessive exposure to mercury and its compounds may cause effects on the brain, digestive system, eye, heart, kidney, lung, reproductive system, skin, and higher miscarriage rates. Organic compounds of mercury are very toxic to wildlife, while the metallic form and the inorganic compounds are less toxic. Organic mercury compounds tend to bioaccumulate and can have adverse effects on aquatic species. Low levels of mercury contamination in lakes can lead to high concentrations in insects, fish and birds. Mercury is also toxic to plants and micro-organisms, hence its former use as a fungicide and bactericide. However, hazards depend upon the form and bioavailability of mercury. Because of the persistence and bioaccumulation potential of mercury and its compounds in the environment, mercury is regarded as a global pollutant. Mercury and its compounds are listed as priority hazardous substances in of the Water Framework Directive (EPER 2009).

1.2 Birds as indicators of metal pollution

Birds have been suggested to be an important indicator species of environmental contaminants and have been used extensively to monitor metal polluted areas in a variety of habitats (Furness and Greenwood 1993; Dauwe et al. 2000; Jassens et al. 2002; Eeva et al. 2009; Berglund et al. 2011). Also, it is possible to use non-invasive procedures like collecting feathers or excrements since birds excrete elements into growing feathers (Burger 1993) and can also eliminate metals through excrements or by depositing them in the uropygial gland and salt gland (Burger and Gochfeld 1985).

In feathers heavy metals are sequestered in the sulfhydryl groups of keratin as the feather grows. Once the feather growth is completed, the blood supply atrophies, and the metal content in the feather remains extremely resistant to further change (Jassens et al. 2002).

In birds, exposure to heavy metals may cause effects on different levels of organization from biochemical responses to changes in population levels (reviewed in Scheuhammer 1987). Reproductive dysfunctions such as smaller clutches, reduced fertility, hatching failure and nestling mortality can be a direct result of pollution and may have profound effects on the stability of avian populations (Dauwe et al. 2005). However, depending on the profile and level of exposure the concentrations found may only cause indirect effects rather than direct toxic effects (Scheuhammer 1987).

According to Beeby (2001) there are three types of species that can be used for assessing different forms of pollution. These are monitoring species, which measure the impact by reducing its performance, indicator species, which absence or abundance indicates the pollution impact, and sentinel species, which are insensitive to pollutants (at least in the ranges of environmental concentration), in other words its performance and abundance are not affected, but show a simple correspondence between tissues and environment, allowing for the quantification of pollution levels in a certain area and over a range of time.

1.3 Advantages and disadvantages of using birds as indicator species

There are both positive and negative aspects to the use of birds as indicator species. Listed below are the most important traits considered positive and negative features of birds as indicator species (Chambers 2008):

- Birds are easy to detect and observe, since many species are diurnal, brightly-coloured and advertise their presence by call (Hutto 1998; Carignan and Villard 2002; Mac Nally et al. 2004);
- The classification of birds is well determined and species are generally easy to identify in the field (Furness et al. 1993; Gregory et al. 2005);
- Birds are widely distributed and occupy a broad range of habitat types and ecological niches (Chambers 2008);
- The distribution, biology, ecology and life history of birds are well known compared with other taxa (Furness et al. 1993; Gregory et al. 2005);
- Several bird species are ecologically well-studied, making well-founded ecotoxicological interpretations possible (Berglund 2010);
- Birds are considered to be high in the food chain. This makes birds sensitive to changes at lower levels of the food chain and to environmental contaminants that accumulate at each level of the food chain (Furness et al. 1993; Mac Nally et al. 2004; Gregory et al. 2005);
- Birds in general are popular amongst the public and any reported hazards may receive particular attention (Mac Nally et al. 2004; Gregory et al. 2005; Berglund 2010);
- Bird survey techniques are simple compared to other taxa and it is also possible to collect information on several species at the same time (Hutto 1998);
- Birds are usually less expensive to monitor than other taxa such as invertebrates, reptiles and mammals (Landsberg et al. 1999; Mac Nally et al. 2004);
- Birds are highly mobile allowing monitoring to be conducted over broad spatial scales; on the other hand, their high mobility makes it difficult to link responses of birds to specific conditions or stressors on the ground (Furness et al. 1993; Mac Nally et al. 2004; Gregory et al. 2005);

- Birds often respond to secondary or tertiary effects of stressors (Morrison 1986; Koskimies 1989; Temple and Wiens 1989). For example, meteorological events such as warm nutrient-poor water currents may lower breeding success and cause crashes in some populations of fish-eating birds due to a reduction in food availability (Barber and Chavez 1983; Schreiber and Schreiber 1984; Duffy 1990). Under these circumstances, time lags exist between the onset of the stressor and the response of birds. Therefore, the impact of the stressor may be more difficult to mitigate and more difficult to trace back to the correct cause of an indicator species' response (Temple and Wiens 1989);
- Birds possess behavioural and physiological traits that may make them less sensitive to ecosystem changes than some other taxa. Traits such as regulation of fat stores and metal concentrations in their tissues can help birds to buffer the impacts of ecosystem changes, thus limiting the ability of birds to indicate ecosystem changes and their effects on other taxa (Furness et al. 1993).

1.4 The Great tit as the study species

Species of the family *Paridae* are among the best-known songbirds (e.g. Perrins 1979; Cramp and Perrins 1993). According to Navarro (2010), from 1997 to 2010 more than 1200 studies have been published directly or indirectly implying Great tits *Parus major* (ISI Web of Knowledge) covering several research topics, from genes to habitat selection.

The Great tit is a resident species, predominantly using forests and woodlands, with great expansion throughout Europe, being a common representative of forest birds. It feeds mainly on insects and therefore, the Great tit occupies a high place in the food chain.

This passerine preferably nests in cavities. Because of the cavity shortage in pine forests, it easily occupies nest boxes and so breeding populations can be easily monitored (Cramp and Perrins 1993; Eens et al. 1999; Dauwe et al. 2000). It is a ubiquitous and abundant species, and sometimes the only species of forest passerines available at appropriate densities in polluted areas. Great tit nestlings are also easily monitored because they are relatively insensitive to the nest disturbances (Janssens et al. 2002). Another advantage in using great tit nestlings is that metal levels reflect local pollution because they are restricted to their nest, receiving their food from the immediate vicinity of the nest box. Therefore the metal contamination refers to a clearly defined time period and a restricted area around the nest (Furness and Greenwood 1993).

Great tits have been used in numerous ecological and behavioural studies throughout Europe (e.g. Perrins 1991; Hõrak 1993; Naef-Daenzer et al. 2000; Mand et al. 2005; Lambrechts et al. 2008) and they have also been successively used in many studies performed in polluted habitats (e.g. Eeva and Lehikoinen 1996; Eens et al. 1999; Dauwe et al. 2000, 2004, 2006; Eeva et al. 2009; van den Steen et al. 2009). However, there are very few studies performed in European southern coastal maritime forests (Fidalgo 1990; Belda et al. 1998; Pimentel and Nilsson 2007; Costa et al. 2011). Such forests represent relatively barren coniferous habitats and may therefore be particularly sensitive to pollution.

1.5 Indirect effects of pollution

Environmental pollution can indirectly affect birds through habitat changes (Morrison 1986), increased amount of parasites (Eeva et al. 1994) or reduced amount of suitable food (Graveland 1990; Hörnfeld and Nyholm 1996; Eeva et al. 1997). Food availability is one of the main environmental factors limiting avian reproduction (Newton 1998), and higher food availability during brood-rearing has been shown to result in better growth and survival of Great tit nestlings (Keller and van Noordwijk 1994; Naef-Daenzer 2000; Mägi et al. 2009).

So, because metal concentrations alone do not give any information about the biological stress caused by pollution, it is very important to assess the health status and breeding biology of organisms in relation to environmental pollution (Peakall 1992). Body mass, tarsus and wing length reflect the condition of birds and are also indicative of survival and reproductive success (Tinbergen and Boerlijst 1990). Assessing haematological variables also provides important information on health and physiological status. Several factors such as nutrition, stress, seasonal cycles and toxic chemicals can alter haematological values. A weakened haematological status can have serious implications on the fitness and breeding capacity of a bird. Low haematocrit values have been shown to reflect low body condition (Svensson and Merilä 1996), infections with blood parasites (Booth and Elliott 2002) and low aerobic and flight performance (Saino et al. 1997).

1.6 Objectives

The main objective of this thesis was to assess the effects of metal exposure on health and breeding performance of the Great tit *Parus major* in two areas (rural *vs* industrial) while comparing the performance of different monitoring procedures (feathers, faeces and blood sampling) to assess metal contamination. Therefore, the following particular objectives were defined:

1- To monitor pollution by several monitoring procedures, such as collecting feathers, faeces and blood of forest passerines, and compare levels between industrial and non-industrial / rural area.

2- To evaluate pollutants accumulation and its effect on the breeding biology of the insectivorous birds in the study areas.

3- To analyse the role of food availability on the breeding biology of the insectivorous birds in polluted and unpolluted environments.

4- To evaluate Tits' health indices against environmental stress caused by pollution.

5- To relate the possible changes in Tits' health indices with changes detected in the breeding parameters.

1.7 Thesis Outline

The thesis consists of six chapters, starting with an introduction to the theme and corresponding objectives and perspectives, followed by a second chapter describing the methodology used for this work. The third chapter includes the discussion of the obtained results and some conclusions drawn from the documents included in annexes I to V. The fourth chapter compiles some concluding remarks and future perspectives. The fifth chapter lists the references used in this thesis and chapter 6 includes annexes I to V, each representing manuscripts presented as scientific papers (3 published papers, 2 under review in international scientific journals).

CHAPTER 2

METHODOLOGY

2. Methodology

2.1 Study areas

In Portugal, the forested area with maritime pine *Pinus pinaster* covers about 1 million ha, half of which are even-aged monocultures, as a result of a deliberate forestry policy dating from the end of the 19th century (DGF 1999). The study was carried out in two maritime pine forests located in Figueira da Foz, in the central coast of Portugal – MQ *vs* MU.

MQ – National Pine Forest of Quiaios (40º14'N 8º47'W):

Situated in the northern limit of the city of Figueira da Foz, MQ is a 6,000 ha forested /rural area included in a Natura 2000 network site "Dunas de Mira" (Natura 2000 PTCON055) (Fig.1). It is essentially a forest of 70 – 80 year-old pine plantations dominated by *Pinus pinaster* interspersed by some *Pinus pinea* patches. Pine density varied between 600 to 1250 individuals per ha (average 875 individuals per ha) and pine tree DBH between 22 and 41 cm (average 32 cm). The soil is sandy and the shrub layer is dominated by *Myrica faya*, *Halimium halimifolium*, *Cytisus scoparius*, *Ulex* spp, *Cistus* spp and *Acacia* spp. The area does not present direct influences of industrial pollution, despite the existence of agriculture fields in the forest border, which may be a factor of concern.

Fig.1 – General landscape view of the National Pine Forest of Quiaios (MQ).

MU – National Pine Forest of Urso (40º02'N 8º52'W):

Situated in the southern limit of the city of Figueira da Foz, MU is a 9,000 ha forested area sited near a pulp mill industrial complex (<1km) (Fig.2). MU is approximately 20 km to the south of MQ and both areas share the same altitude (±50 m asl) and average temperature. MU is very similar to MQ and also presents a sandy soil and 70 – 80 year-old pine plantations dominated by *Pinus pinaster* interspersed by some *Pinus pinea* patches. Pine density varied between 675 to 1300 individuals per ha (average 1,000 individuals per ha) and pine tree DBH between 15 and 26 cm (average 19 cm). The shrub layer is also dominated by *Myrica faya*, *Halimium halimifolium*, *Cytisus scoparius*, *Ulex* spp, *Cistus* spp and *Acacia* spp.

The pulp mill industrial complex present in MU has been active since 1967 and for many years the effluents were released to the sea through an open-air conduit (Vala do Estremal) (Celulose Billerud, undated). In the nineties, an underground pipe was built that released the effluent discharges several miles off the coast.

The manufacturing process is digestion with continuous prehydrolysis kraft and the raw materials used are sodium sulphate, caustic soda, chloride, sodium chloride, sulfuric acid, sulfur, limestone, fuel oil, sodium hypochlorite and chlorine dioxide (Cellulose Billerud, undated).

Fig.2 - General landscape view of the of National Pine Forest of Urso (MU).

2.2 Study species

The main focus of this thesis was the Great tit. However, because Coal tits *Periparus ater*, Crested tits *Lophophanes cristatus*, Blue tits *Cyanistes caeruleus* and Short-toed treecreepers *Certhia brachydactyla* also bred in the nest boxes, the information on these species was also used.

These five species breed naturally in tree holes. However, when nest boxes are available, these are strongly preferred over natural cavities, especially in coastal pine areas where the absence of natural holes is one of the limiting factors in Paridae reproduction (Fidalgo 1990). They readily nest in manmade nest boxes and so breeding populations can easily be monitored. Also, nestlings are very easy to manipulate and very insensitive to human disturbances. All study species are sedentary, performing short movements, mainly during the winter (Cramp 1998).

The **Great tit** is the largest among tit species. It is a small insectivorous bird that breeds in most forest habitats from northern Africa, across Europe, to western Siberia and southwestern Asia. It commonly feeds on a wide variety of insects, especially Lepidoptera and Coleoptera and also spiders during the breeding season. They also ingest a significant amount of seeds and fruits in wintertime. Both sexes are similar in plumage, but males present a wider black stripe down the underparts (Cramp 1998). Great tits have been successfully used in biomonitoring studies (Eens et al. 1999; Dauwe et al. 2006; Eeva et al. 2009; Berglund et al. 2011) being potentially good biomonitors for heavy metal pollution because they are ubiquitous and abundant (Dauwe et al. 2000; Janssens et al. 2002), and sometimes the only forest passerine species available in reasonable densities in polluted areas.

The **Blue tit** is easily recognizable by its blue and yellow plumage. It is also an insectivorous bird, smaller but generally similar to the Great tit. It breeds throughout temperate and subarctic Europe and western Asia in deciduous or mixed woodlands. It mainly eats insects and spiders, also fruits and seeds outside the breeding season, nectar and pollen, especially in spring, and sap of trees. Both sexes are similar in plumage, but males present a brighter blue crown and brighter wing-coverts discernible in close comparison (Cramp 1998).

The **Crested tit** is a small, rather compact tit, with a large head and backward-pointing crest unique in small arboreal passerines of the western Palearctic. It breeds from upper to lower middle latitudes of the western Palearctic, mainly in dry cool or warm continental temperate climates, preferably in coniferous forests. It mostly feeds on insects, spiders and plant material especially in periods other than the breeding season (mainly conifer seeds). Both sexes are similar in plumage (Cramp 1998).

The **Coal tit** is slightly smaller and shorter-tailed tit. It breeds in the western Palearctic from boreal through temperate to Mediterranean zones, in continental and oceanic upper and lower middle latitudes, preferably in coniferous forests. It feeds on adult and larval insects and spiders, plus seeds in autumn and winter. Both sexes are similar in plumage, although females can present a narrower black throat than males (Cramp 1998).

The **Short-toed treecreeper** is a small brownish tree-dwelling bird with a long slim down-curved bill. It breeds in the middle and lower middle latitudes of the southwestern Palearctic in continental and oceanic temperate and Mediterranean zones. It feeds mainly on insect larvae and pupae and also on spiders, throughout the year. Both sexes are similar in plumage with no seasonal variation (Cramp 1998).

2.3 Breeding data collection

The study areas (MQ, MU) have been monitored since 2003 and both areas were established with the aim of performing long-term studies, especially those related to pollution levels.

Three homogeneous even aged plots were selected in each study area. Plots were within 2 km of each other. Similar nest-boxes were placed at an average density of 9/ha, at equal distances from each other, resulting in 20 to 50 nest boxes per plot. The wooden nest boxes, with a 2.8 cm entrance and cavity measurements of 20 x 15 x 15 cm (height, width, length), were checked from the beginning of February till the end of July. In the beginning of the breeding season each site was visited once per week. After the first signs of nest occupation, the visits started to be planned in function of the working schedule that was needed for each nest box, according to nestling's development. In each visit, hatching date, clutch size, brood size and number of fledglings were registered for each box. Finally, it was possible to calculate the hatching success (% of eggs that hatched successfully excluding predated clutches), survival rate (number of fledglings/numbers of hatched eggs) and breeding success (number of fledglings/numbers of eggs laid). Nests where egg laying was initiated, whether successfully or not, were considered as occupied nests. Laying dates were calculated assuming that Great tits lay one egg per day and have an average incubation period of 12 days (Costa et al. 2005). On day 15 (±1) post-hatching, all nestlings were measured for their body mass (g) with a spring scale, and marked with an individually numbered aluminium ring.

2.4 Measurements of food availability

Food availability was studied in the two areas by monitoring caterpillar frass-fall and the abundance of ground-living arthropods through time. The amount of herbivorous caterpillars and sawfly larvae in tree foliage was measured by collecting their droppings during the breeding seasons of 2009 and 2010, using round plastic funnels attached with wire to trunks of pine trees (the dominant trees) at a 2-meter height. Under the funnel there was a container where the frass accumulated during the collection period. Contents were dried and stored in paper bags until the frass was separated from the litter and weighed. The abundance of ground-living arthropods was measured during the breeding seasons of 2009 and 2011, using pitfall traps, where plastic jars, containing ethylene glycol, were randomly buried in the ground.

2.5 Metal analyses

Metal concentrations in feathers, faeces and blood of Great tit nestlings were analysed, in order to make comparisons between areas and sampling method. In addition, metals in the feathers of Great tit adult females and soil samples were also analysed. Eight elements (As, Cd, Cu, Hg, Ni, Pb, Se and Zn) were determined with

ICP-MS (Elan 6100 DRC, PerkinElmer-Sciex, Boston, USA) and the detection limits for all metals were around 1 ng/l and below.

CHAPTER 3

MAIN RESULTS and DISCUSSION

3. Main Results and Discussion

The main focus of this thesis was to study the effects of metal exposure on the health and breeding of Great tits in both study areas (rural *vs* industrial) while comparing the performance of the non-invasive procedures to assess metal contamination. Therefore the discussion in this section is mainly focused on similarities and differences between the results from the two study areas.

3.1 Metal analyses: feathers, faeces, blood, soil (annexes II, III and V)

One of the objectives was to evaluate the performance of different sample types. However, as explained in papers II, III and V, feathers, faeces and blood represent different times of accumulation and/or absorption. Feathers reflect the amount of metals present in the blood at the time of feather growth, either from current dietary sources or from mobilization of metals from internal organs (Burger 1993), faeces represent the unabsorbed remnants of multiple food items (Spahn and Sherry 1999; Morrissey et al. 2005), and metal levels in blood reflect the input of metals through immediate (< 1 week) dietary intake (Ek et al. 2004).

Considering the various sample types, we found different results even though there was some consistency in Great tit nestlings feathers and faeces, with higher concentration of mercury in the industrial area MU and higher concentration of arsenic in the rural area MQ (Papers II and III). Considering blood samples, these metal elements were similar in the two study areas (Table 1) and mercury was not detected in soil samples (Table 2). One must also consider that faeces and feathers were sampled in 2009, while soil was collected in 2010 and blood in 2010 and 2011. Therefore, the results obtained from the different sample types along the sampling years can be related to a progressive decrease of pollution levels.

Table 1 - Comparison of metal elements concentrations (mean ± standard deviation, ppm) in blood samples of Great tit nestlings collected in 2010 and 2011 between sites near a pulp mill (MU) (n=14 samples from 11 nests) and at the reference site (MQ) (n=44 samples from 33 nests) using generalized linear models (nest was used as a random factor in the analyses).

	MQ	MU	Df	F	p
As	0.02±0.02	0.02±0.01	1,37	0.01	0.91
Hg	0.15±0.09	0.12±0.05	1,53	0.79	0.38
Pb	0.74±1.29	0.43±0.18	1,55	2.02	0.16
Ni	1.37±2.79	0.75±0.34	1,55	0.77	0.39
Cu	0.82±1.49	0.31±0.31	1,55	14.05	0.0004
Cd	0.02±0.01	0.02±0.01	1,23	0.17	0.68
Zn	2.82±1.65	2.68±0.82	1,54	0.07	0.79
Se	1.36±3.21	0.63±0.49	1,55	0.09	0.77

Table 2 - Comparison between metal elements concentrations (mean ± standard deviation, ppm) in soil samples collected in 2010 at sites near a pulp mill (MU) (n=15 samples) and at the reference site (MQ) (n=15 samples) using generalized linear models.

	MQ	MU	Df	F	p
As	0.61±0.28	0.58±0.14	1,28	0.00	0.95
Hg	0.02±0.001	n.d.	-	-	-
Pb	2.62±2.23	3.22±1.94	1,28	1.96	0.17
Ni	0.62±0.47	0.90±0.55	1,28	4.20	0.05
Cu	0.80±0.98	0.39±0.28	1,28	2.82	0.10
Cd	0.02±0.03	0.01±0.007	1,28	0.06	0.81
Zn	3.61±4.29	3.87±2.09	1,28	1.75	0.20
Se	0.09±0.12	0.03±0.03	1,25	5.70	0.02

Either way, no mercury emissions were indicated on the report given by the paper and pulp facilities in 2004 (EPER 2009). The contamination by mercury in MU could be a result of a past long-term emission, when environmental concerns were not considered a priority and there was a lack of regulations for this kind of emissions. Alternatively, current emissions from factories are also a possibility, since paper and board mills can release low concentrations of heavy metals originating mainly from energy generation (steam and electricity). Also the incineration of different types of RCFs (recovered cellulose fibres) paper mill residues can release mercury in the process (European Commission 2001). The presence of arsenic in the rural area MQ can be accounted by the presence of agriculture fields in the surroundings of the study area, where pesticides and herbicides are most likely still used.

When we compared arsenic and mercury levels between study years we found several differences with significantly lower levels of contamination in 2010 and 2011 (Table 3, Fig.3). Explanations to the decrease of contamination levels in MQ are sparse mostly due to the unknown use of agrochemicals in the agriculture fields bordering the study area. With respect to MU, the paper mill has undergone several improvements in terms of “cleaner” production technologies in 2008 and 2009, and so the significantly lower levels of contamination in 2010 and 2011 can be the result of this transformation. At the same time weather factors like rainfall can alter contamination levels, since the aerial dispersal of pollutants is favored under warm, dry conditions with steady side winds (Walker et al. 2006). The study area, as most of Portugal, has been subject to high differences in annual rainfall levels, with normal levels of rain in 2003 and severe droughts in 2005 and 2010 (Instituto de Meteorologia 2011).

The non-detection of mercury in the soil in 2010 is also a major contributor to the idea that mercury contamination has indeed decreased. However, one must take into consideration that the sandy soil is highly permeable, difficulting a correct evaluation.

Table 3 - Two-way ANOVA showing the effects of area (industrial vs. rural) and year (2003 – 2011) on levels of Hg and As in feathers, faeces and blood samples of Great tit nestlings.

	Hg				As		
	num d.f.	den d.f.	F	P	den d.f.	F	P
Feathers							
Area	1	127	17.61	<0.0001	78	1.84	0.18
Year	2	127	21.57	<0.0001	78	25.06	<0.0001
Area * year	2	127	9.84	0.0001	0	-	-
Faeces							
Area	1	74	3.72	0.06	78	0.24	0.63
Year	2	74	2.97	0.06	78	0.45	0.64
Area * year	2	74	0.11	0.89	78	0.78	0.46
Blood							
Area	1	51	1.39	0.24	35	0.11	0.75
Year	1	51	6.62	0.01	35	0.81	0.37
Area * year	1	51	1.63	0.21	35	0.48	0.49

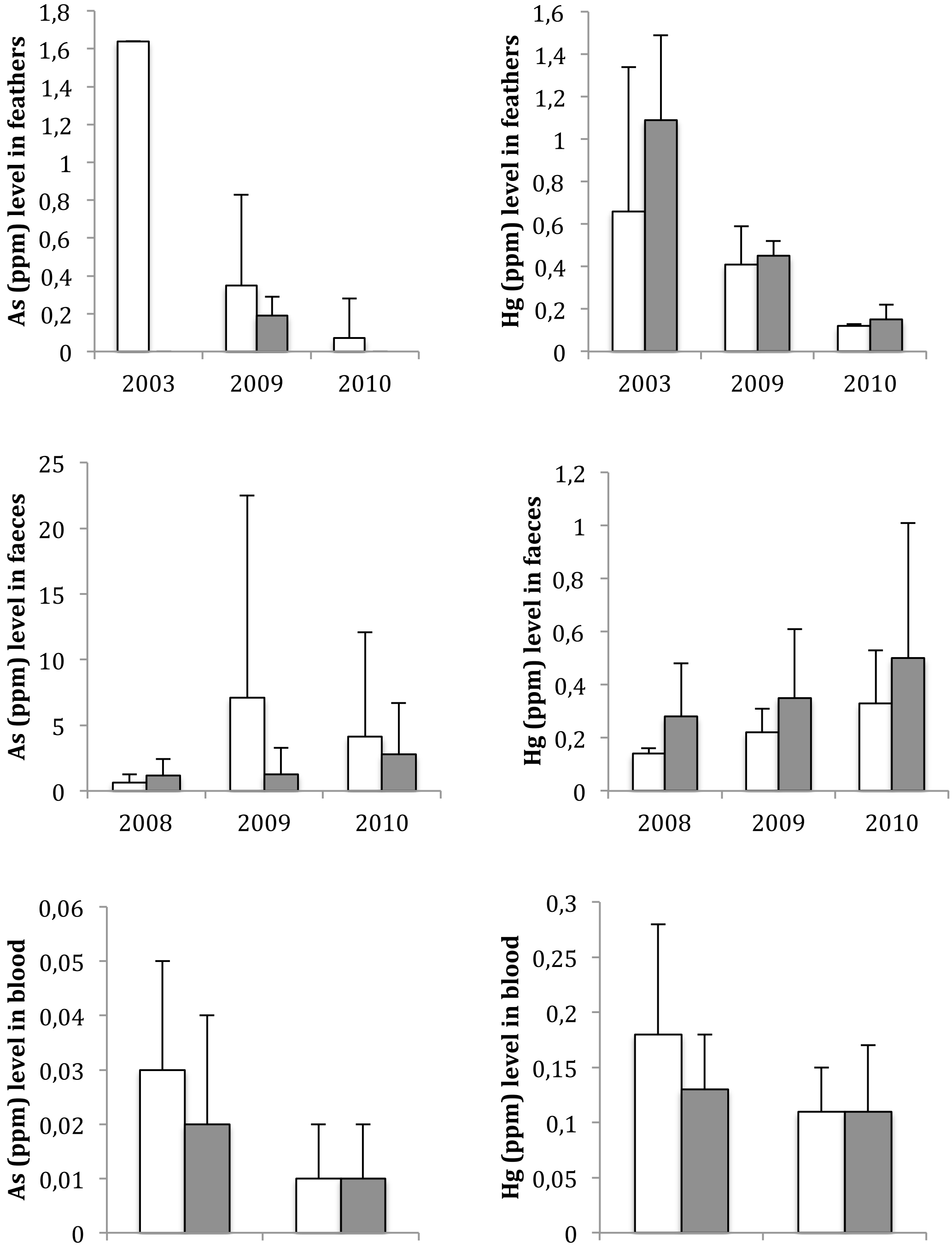

Fig.3 – Average level of As and Hg (±SD) in feathers, faeces and blood samples of Great tit nestlings during the study years, at areas MU (grey bars) and MQ (white bars).

In conclusion, since mercury and arsenic levels showed a consistent pattern in feathers and faeces of nestling Great tits, these substrates seem to present a good method for the evaluation of these elements in the study areas. Arsenic is a toxic nonessential element that readily bioaccumulates. Several reported values for arsenic in the feathers and faeces of Great tits are higher than the levels found in the present study (Janssens et al. 2003; Dauwe et al. 2004; Eeva et al. 2006, 2009) and no toxic effect was found to be directly related to arsenic values. Mercury is considered to be very toxic for wild animals and at higher levels of contamination it can adversely affect birds by reducing their fecundity, growth and body length (Eisler 1987). However, although the mercury values found in the present study were within other previously reported ranges in feathers and faeces of Great tits inhabiting metal contaminated sites (Janssens et al. 2002, 2003), we found no direct adverse effects in our populations (Papers I and II). Nevertheless, considering the well-known hazardous effect of these elements on the environment, the regular monitoring of the study areas is essential.

In 2009 we also collected feathers from Great tit adult females, breeding in the study areas. Female adult feathers presented no significant differences in arsenic or mercury between study areas, with only significantly higher levels of nickel in MU (Table 4). However, the patterns of higher arsenic in MQ and higher levels of mercury in the industrial area (MU) were the same as those found in nestlings. The different values can also be due to external contamination and also due to differences in metabolism, since some elements like nickel can be regulated by homeostatic control in nestlings (Nyholm 1995; Dauwe et al. 2004; Berglund et al. 2011).

Table 4 - Comparison of heavy metal concentrations (ppm) in feathers of female adult Great tits at a site near a pulp mill (MU) (n=6) and at a reference site (MQ) (n=7) using a t test (mean ± standard deviation).

	MQ	**MU**	**t**	**p**
As	0.98±1.16	0.48±0.22	0.90	0.40
Hg	0.39±0.12	0.65±0.66	-0.77	0.47
Pb	2.49±1.31	8.86±16.0	-0.99	0.36
Ni	1.66±0.15	2.20±0.39	-3.23	0.02
Cu	5.72±0.82	7.11±2.55	-1.30	0.24
Cd	0.11±0.04	0.10±0.03	0.37	0.72
Zn	101.6±11.8	104.2±12.7	-0.37	0.72
Se	0.90±0.18	0.97±0.26	-0.55	0.39

When we compared contaminant levels of adult and nestling feathers (Table 5), we expected levels of metals to be higher in adults than nestlings, especially because adults have had longer time to acquire and bioaccumulate contaminants (Burger et al. 2009). However, we found significantly higher levels of zinc and copper in nestlings, and only arsenic, lead and cadmium were significantly higher in adults. Interestingly the metals are here divided by their redox activity (Koivula et al. 2010). Copper is a redox active metal while arsenic, lead and cadmium are redox inactive. Adult and nestling feathers present a slightly different composition because at the time of sample collection adult feathers are completely formed while in 15-day old nestlings feathers are still growing and have an active blood circulation supporting the growth. Once the feather has reached its full size, the blood supply is no longer needed and the vessels shrivel up. The redox inactive metals have strong affinity to the sulfhydryl groups of keratin (a key protein in feather) and this is probably why we found higher levels of these metals in adult feathers. Nestling feathers on the other hand include blood vessels and blood that might contain more copper and zinc than pure keratin.

Table 5 - Comparison of metal elements concentrations (ppm) in feathers of female adult (n=13) and nestling (n=70) Great tits at the study area (MQ+MU) using generalized linear models (mean ± standard deviation).

	Adult feathers	**Nestling feathers**	**df**	**F**	**p**
As	0.75±0.87	0.26±0.34	1,80	22.56	<0.0001
Hg	0.51±0.46	0.43±0.13	1,80	0.06	0.81
Pb	5.43±10.9	1.28±0.42	1,80	47.52	<0.0001
Ni	1.91±0.39	2.30±0.70	1,80	3.24	0.08
Cu	6.36±1.89	8.72±2.94	1,80	16.29	0.0001
Cd	0.10±0.03	0.03±0.01	1,80	118.9	<0.0001
Zn	102.8±11.7	111.9±5.05	1,80	27.84	<0.0001
Se	0.93±0.46	1.00±0.22	1,80	1.13	0.29

In conclusion, nestlings seem a more optimal choice for the evaluation of local pollution, since we can sample data from a defined area and time period. Furthermore, because the nestlings stay within the nest boxes there is a very limited possibility of external airborne deposition from industrial sources, which can happen to adults. In addition, we found that nestlings' feathers show a different metal profile, which is partly due to the fact that their feathers are still growing and show a different tissue composition in comparison to fully grown feathers. Therefore, the developmental stage of feathers is important to consider when such results are interpreted.

3.2 Health status of Great tit nestlings (annex V)

Measuring metal concentrations in bird tissues is very useful, however, it may not be sufficient because stress caused by exposure to metals can be both direct and indirect, and extrapolating metal concentrations in tissues to possible effects on the health and condition of free-living birds can be very difficult (Eeva et al. 2000). For this reason in 2010 and 2011, when we collected blood samples for metal concentration analyses, we also assessed white blood cell (WBC) counts, heterophil-to-lymphocyte (H/L) ratio and haematocrit (HTC) levels (Paper V).

HCT is the percentage of the packed volume of erythrocytes in the total volume of the blood and is indicative of the oxygen transport capacity of the blood (Ots et al. 1998). WBC count indicates the overall state of the immune system (Campbell 1995) and H/L ratio is used as a stress indicator in birds (Gross and Siegel 1983).

Low haematocrit levels may indicate anaemia and can arise as a consequence of blood loss, the activity of blood and gastrointestinal parasites, and deficiencies of certain minerals (Campbell 1995; Dubiec and Cichoñ 2001).

Some studies have found a reduction in haematocrit levels due to heavy metal pollution (Hoffman et al. 1985; Nyholm 1998; Henny et al. 2000), other studies did not detect any effects of heavy metals on haematocrit values (Pain 1989; Blus et al. 1995; Fair and Ricklefs 2002) and other studies have revealed significant effects of heavy metal pollution on reproductive success and immunocompetence (Janssens et al. 2003; Snoeijs et al. 2004).

In our study the estimates of the total white blood cell count (MQ: 2.54±1.59; MU: 2.54±2.51; $F_{1,32}$=0.00; p=0.98), H/L ratio (MQ: 0.24±0.28; MU: 0.18±0.11; $F_{1,25}$=0.01; p=0.98) and haematocrit level (MQ: 48.35±6.30; MU: 50.01±5.34; $F_{1,57}$=1.61; p=0.21) presented similar results for both study areas. Although haematocrit levels in nestlings are difficult to compare, since blood variables change with nestling growth (Gayathri et al. 2004; Nadolski et al. 2006), the values found in our study were similar to those detected in other studies made on 15±1 days Great tit nestlings, in which no differences were found between polluted and control areas (Hõrak et al. 1999; Janssens et al. 2003; Kalinski et al. 2011), which suggests that in our study haematocrit values were not affected by metal pollution.

3.3 Food availability (annexes I and II)

It has been shown that food availability is one of the main constrains for a good breeding performance (e.g. Perrins 1991; Seki and Takano 1998). For this reason we included in papers I and II results from two methods, frassfall and pitfall, evaluating not only caterpillar biomass, one of the main preys of Great tits, but also ground-dwelling arthropods like spiders and beetles. In fact, among arthropods, spiders are considered very significant in tits' diet (Betts 1955; Royama 1970; Török 1985; Lambrechts et al. 2008). Millipedes are also considered important sources of calcium for insectivorous birds (Bureš and Weidinger 2003), calcium being a crucial factor for successful breeding, possibly limiting the reproductive output of birds (Graveland and Van Gijzen 1994; Tilgar et al. 2002; Bureš and Weidinger 2003; Eeva and Lehikoinen 2004).

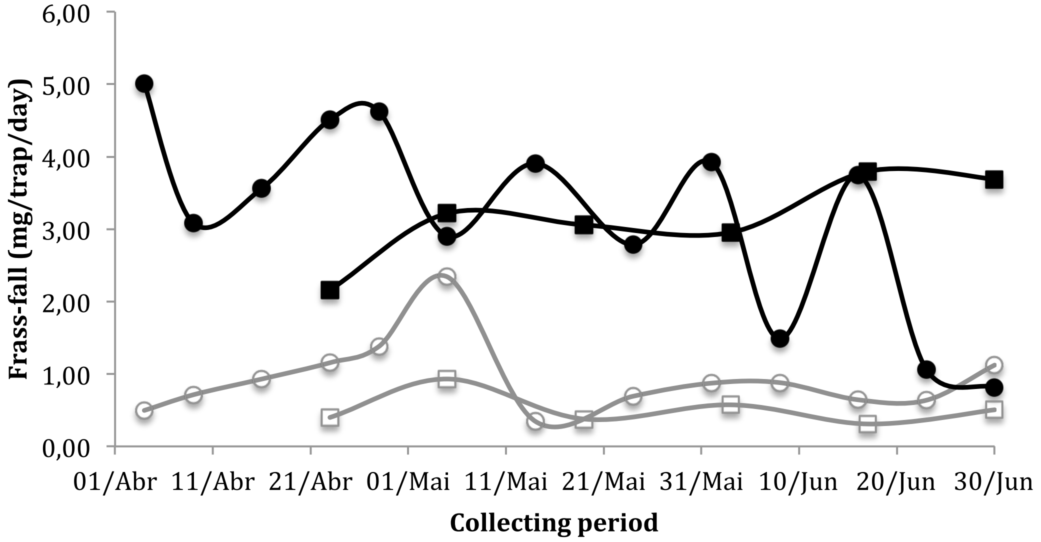

Fig.4 – Frass-fall abundance (mg/trap/day) in industrial (MU – black line) and non-industrial areas (MQ – grey line), in 2009 (circles) and 2010 (squares).

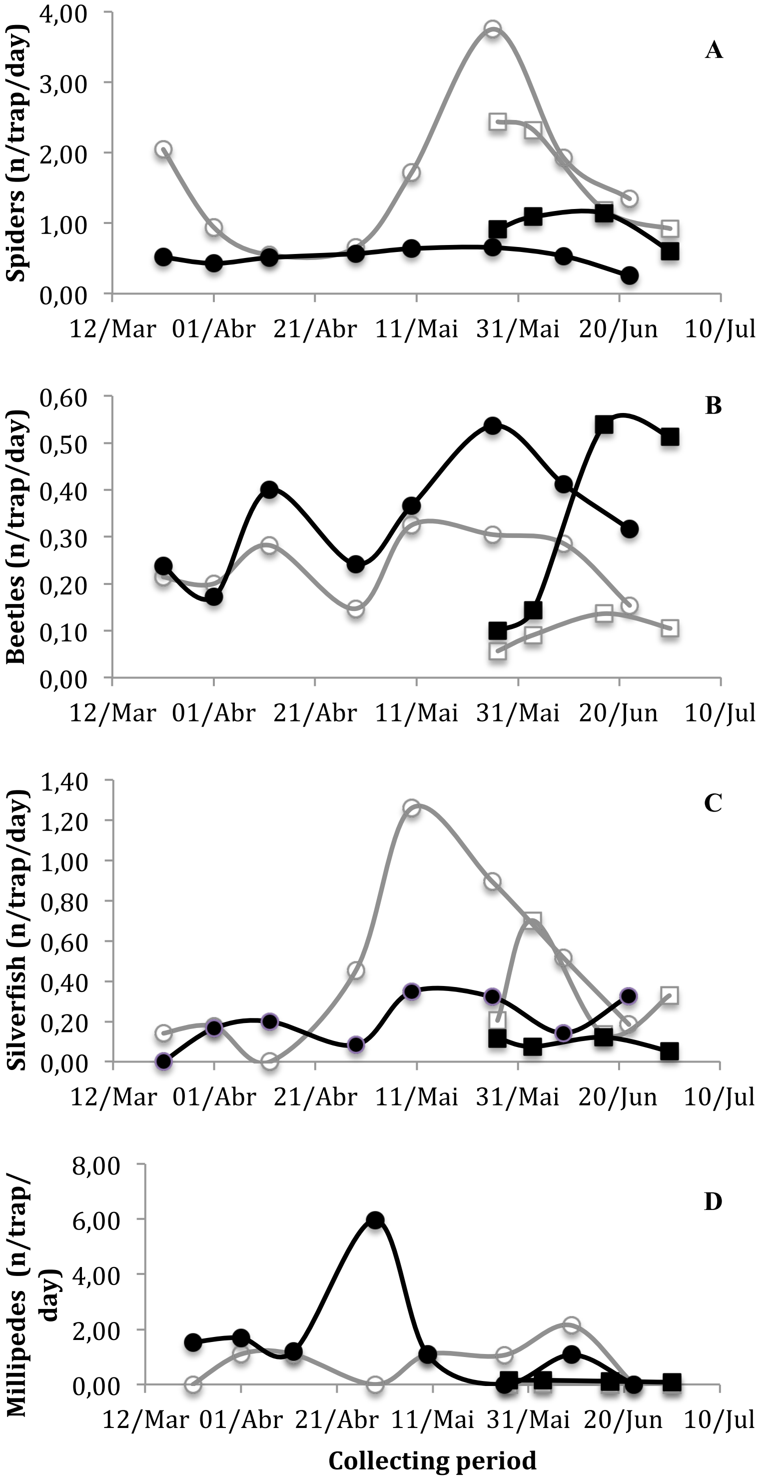

Fig.5 – Arthropod abundance (n/trap/day) (A- spiders, B- beetles, C- silverfish, D- millipedes) in industrial (MU – black line) and non-industrial area (MQ – grey line), in 2009 (circles) and 2011 (squares).

Although both study areas presented a very similar habitat, MU appeared to be a more productive environment, with values of frass-fall almost doubling the amount found in MQ (Fig.4). Also, we found significant differences in arthropod abundance with MQ having more spiders and silverfish and MU more beetles and millipedes (Fig.5). As explained in papers I and II the paper and pulp mill industry in the vicinity of MU since 1967 has most likely affected the relationships between trees and herbivorous insects, making the area more profitable to some invertebrate species. Several species of herbivorous insects, which are an important food resource for many insectivorous birds, may be affected by severe pollution although their abundance is known to increase in moderately polluted environments, probably due to decreased tree vigour (Heliövaara and Väisänen 1990; Eeva et al. 1997). However, due to time and budget constrains, it was not possible to perform tests directly on plants in order to prove this theory.

3.4 Breeding biology MQ vs MU (annexes I and II)

We found that Great tits bred earlier, laid more eggs and produced more fledglings in the industrialized area (MU), while breeding success and fledglings body mass were similar in both study areas (Table 6, Fig.6).

Nest-box occupation rate by Great tits was similar in both areas, but we found a significant difference in the number of other species that occupied the nests (lower in MU). Coal tits, Crested tits, Blue tits and Short-toed treecreepers also bred in the nest boxes, and these species largely overlapped the Great tit in terms of resource use (nest boxes, food types, breeding period), which could imply interspecific competition particularly when resources are scarce. The difference in occupation rates by other species can also be related to pollution, since some species can be more sensitive and hence appear in lower number in polluted areas. On the other hand, Great tits are less specialised than other tits in terms of feeding, having a larger range of prey, which is probably one of the reasons why Great tits settle more often in less advantageous habitats (Török 1985).

Table 6 - Two-way ANOVA showing the effects of area (industrial vs. rural) and year (2003 – 2011) on laying date, clutch size, number of fledglings, breeding success and fledglings body mass of Great tit, using generalized linear models.

Laying date	num d.f.	den d.f.	F	p
Area	1	197	46.7	<0.0001
Year	8	197	3.63	0.0006
Area * year	8	197	2.38	0.02
Clutch size				
Area	1	197	40.8	<0.0001
Year	8	197	0.63	0.75
Area * year	8	197	0.82	0.58
Number of fledglings				
Area	1	197	28.7	<0.0001
Year	8	197	2.16	0.03
Area * year	8	197	0.67	0.72
Breeding success				
Area	1	197	1.75	0.19
Year	8	197	1.25	0.27
Area * year	8	197	0.31	0.96
Fledglings body mass				
Area	1	174	1.12	0.29
Year	8	174	1.68	0.11
Area * year	8	174	0.82	0.59

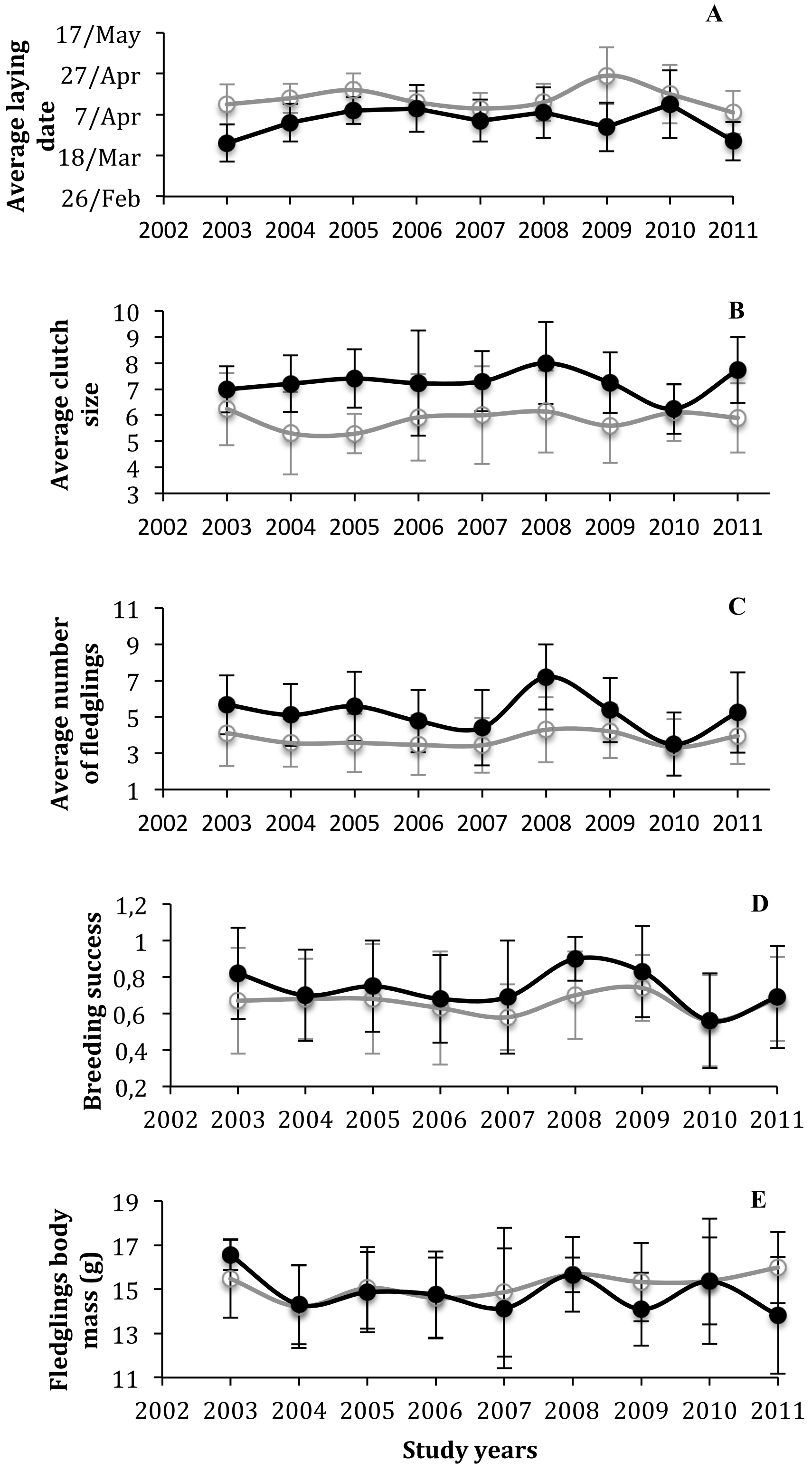

Fig.6- Annual average (± standard deviation) laying date of the first egg (A), clutch size (B), number of fledglings (C), breeding success (D) and fledglings body mass (E) of Great tit during the study years in industrial - MU (black circles) and rural - MQ (empty circles) area.

This low number of other species that occupied the nests in MU inhibits us from performing comparisons between areas for all breeding species. This was possible in MQ only (Paper IV), and we found that clutch size, number of fledglings, breeding success and fledglings' body mass were similar between study years for all breeding species, likely indicating that food availability is quite constantly low in the area. The lack of extreme temperatures at this coastal zone (Almeida 1997; Ferreira 2000) and the low temperature variation recorded during springtime in the study area may account for the constant food availability and consequently for the similarity of breeding parameters throughout the years.

Considering Great tits, in both areas, laying dates differed between study years (Table 6). However, yearly variation in laying dates seems not to influence the other breeding parameters, since breeding values were similar throughout the study years. It seems that Great tit breeding parameters are more closely dependent upon caterpillar phenology (which shows temporal variation) than the other studied species breeding parameters.

The breeding parameter values of Great tits are remarkably lower in our study than in many other studies made in European coniferous forests (Lemel 1989; Tilgar et al. 2002; Mänd et al. 2005; Mägi et al. 2009). However, most of these studies were performed at higher latitudes, in Central and Northern Europe, where average daily temperatures are considerably lower and more variable than in the Iberian Atlantic coast. Breeding conditions may be much more challenging in southern habitats because, apart from daytime variations, other constraints such as poor food supply and climatic factors (high temperature and low water supply) may be strongly limiting the reproductive output of Great tits (Sanz et al. 2000).

Our results suggest that Great tit females adopt a different strategy in each area due to different food availability, producing smaller clutches in the rural pine forests and thus enhancing nestling survival in an environment scarce of caterpillars.

We found no evidence of direct toxic effects of emissions from the paper industry on Great tits. However, since both study areas were otherwise homogeneous, the variation in food availability seems to be related to the pollution levels, indirectly affecting the breeding performance of the Great tit. It is known that at moderate pollution levels only indirect effects on birds may occur (Morrison 1986; Furness and

Greenwood 1993; Eeva et al. 1997), but these effects can change important parameters of bird reproduction, such as timing of laying or clutch size.

Therefore, our study emphasizes the importance of further research in order to clarify ecological effects of moderate pollution levels on wildlife.

CHAPTER 4

CONCLUDING REMARKS and FUTURE PERSPECTIVES

4. Concluding remarks and Future perspectives

The present work focused on the evaluation of metal concentrations, breeding biology and health status of Great tits in the surroundings of a pulp and paper industry and in a rural area. The evaluation of metal levels and their effect on the health and breeding of Great tits was accompanied by a comparison of several non-invasive procedures to assess metal contamination. The main findings are summarized below, followed by some perspectives for the future.

Considering the analysed elements (As, Cd, Cu, Hg, Ni, Pb, Se, Zn) the main differences between study areas were found in arsenic and mercury, with higher arsenic levels in the rural area and higher mercury levels in the industrial area.

During the study years we found several differences with significantly lower levels of contamination in 2010 and 2011 and although some reasonable explanations are presented, the role of droughts is still questionable. Nevertheless, considering the well-known hazardous effect of mercury and arsenic on the environment, it is important to monitor the study areas regularly.

Feathers and faeces of nestling Great tits presented a consistent pattern of results indicating that these substrates constitute a good method for evaluating trace elements in the study areas. Also, nestlings seem a more optimal choice for the evaluation of local pollution, because we can sample data from a defined area and time period. However, since nestlings' feathers show a different metal profile than adults, the developmental stage of feathers is important to consider when such results are interpreted.

We found no evidence of direct toxic effects of emissions from the paper industry on Great tit breeding biology or health status, with similar breeding success and health indices in both areas. However, Great tits bred earlier, laid more eggs and produced more fledglings in the industrialized area. The higher food availability of the industrial area corroborates the hypothesis that Great tit females adopt a different strategy in each area, producing smaller clutches in rural pine forests, thus enhancing nestling survival in an environment scarce of caterpillars.

Since both study areas were otherwise homogeneous, the variation in food availability seems to be related to the pollution levels, indirectly affecting the breeding performance of the Great tit.

In future studies, it would be interesting to analyse metal levels in trees and shrubs, thus investigating the relation between plant quality and caterpillar availability. This would allow for accurate conclusions regarding the food availability differences between the studied areas.

CHAPTER 5

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CHAPTER 6

ANNEXES

6.1 Paper I

Effects of air pollution from pulp and paper industry on breeding success of Great tit in maritime pine forests.

Rute A. Costa, Tapio Eeva, Catarina Eira, Jorge Vaqueiro and José V. Vingada (2011) Effects of air pollution from pulp and paper industry on breeding success of Great tit in maritime pine forests. *Écoscience* 18(2): 115-123 doi: http://dx.doi.org/10.2980/18-2-3382

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Effects of air pollution from pulp and paper industry on breeding success of Great tit in maritime pine forests[1]

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Abstract: Air pollution has been found to have direct and indirect effects on forest passerines, but there is very little information on the effects of emissions from the pulp and paper industry. This long-term (7 y) study compares breeding parameters of Great tits in industrial and rural sites in maritime pine forests on the west coast of Portugal. We found that Great tits bred earlier, laid more eggs, and produced more fledglings in the industrial area, where we also found a higher biomass of caterpillars, an important food source for tits. There were also differences in ground arthropod numbers, the industrial area having more beetles and millipedes and the rural area more spiders and silverfish. Our results suggest that there are no direct toxic effects of emissions from the paper industry on the study species. However, invertebrate food availability is clearly related to pollution levels, which indirectly affect the breeding performance of the Great tit.

Keywords: air pollution, breeding biology, food limitation, invertebrate abundance, *Parus major*.

Résumé : Il est connu que la pollution atmosphérique a des effets directs et indirects sur les passereaux forestiers, mais il existe très peu d'information sur les effets des émissions polluantes de l'industrie des pâtes et papiers. Cette étude à long terme (7 ans) a comparé les paramètres de reproduction de mésanges charbonnières dans 2 pinèdes maritimes, l'une située dans un secteur industriel et l'autre dans un secteur rural, sur la côte ouest du Portugal. Nous avons observé que les mésanges charbonnières s'accouplaient plus tôt, pondaient plus d'oeufs et produisaient plus de jeunes dans les sites industriels, où nous avons également observé une plus grande biomasse de chenilles, une importante source de nourriture pour les mésanges. Il y avait aussi des différences dans les quantités d'arthropodes terrestres, les sites industriels ayant plus de coléoptères et de millipèdes et les sites ruraux plus d'araignées et de lépismes argentés. Nos résultats suggèrent qu'il n'y a pas d'effet toxique direct des émissions de l'industrie des pâtes et papiers sur l'espèce à l'étude. Cependant, la disponibilité des invertébrés est reliée de façon évidente au niveau de pollution, ce qui a un effet indirect sur la performance de reproduction de la mésange charbonnière.

Mots-clés : abondance des invertébrés, biologie de la reproduction, *Parus major*, pollution atmosphérique, restriction de nourriture.

Nomenclature: Cramp & Perrins, 1993.

Introduction

The pulp and paper industry is known for the emission of malodorous sulphurous air pollutants such as hydrogen sulfide (H_2S), methyl mercaptan (CH_3SH), and methyl sulfides, but the available information concerning the effects of these pollutants on wildlife is still sparse (Haahtela *et al*., 1992). Air pollution may have both direct and indirect effects on avian reproduction, and at moderately polluted sites only indirect effects may occur (Morrison, 1986; Furness & Greenwood, 1993; Eeva, Lehikoinen & Pohjalainen, 1997). Breeding parameters of passerine birds (timing of breeding, clutch size, nestling growth and survival) can be affected by several factors, such as food resources and competition (Perrins, 1991a; van Noordwijk, McCleery & Perrins, 1995; Forsman, 1998; Seki & Takano, 1998). Many Parid species have adopted the clutch adjustment

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strategy, *i.e.*, opportunistic parents lay large clutches and raise more offspring in good food conditions and fewer in poor food conditions (O'Connor, 1978; Pettifor, Perrins & McCleery, 1988; Tinbergen & Daan, 1990; Rytkönen & Orell, 2001). For example, female Blue tits (*Cyanistes caeruleus*) laid smaller clutches in poor quality sites than in good quality sites (Dhondt, Kempenaers & Adriaensen, 1992). Similarly, Great tits (*Parus major*) can adjust their offspring number to the food resources available on their territory. The correct timing of laying is very important for successful breeding in the Great tit, since even a relatively small temporal mismatch between timing of breeding and an abundant occurrence of food invertebrates may lead to measurable differences in nestling weight and local recruitment rates of the resulting offspring (van Noordwijk, McCleery & Perrins, 1995). Furthermore, as a facultative double-brooded passerine, Great tits adjust their reproductive investment between successive breeding attempts, presenting variations of clutch size and number of fledglings between breeding attempts (Pimentel and Nilsson, 2007; Lambrechts *et al*., 2008). Air pollution might affect these important life history traits, *e.g*., via changes in resource levels.

Great tits have been the subject of numerous ecological, behavioural, and environmental studies throughout Europe (Perrins, 1991a,b; Hõrak, 1993; Eeva & Lehikoinen, 1996; Dauwe *et al*., 2000; van den Steen *et al*., 2009).

However, there are very few studies from southern coastal maritime forests (Fidalgo, 1990; Belda *et al*., 1998; Pimentel & Nilsson, 2007; Norte *et al*., 2010), which are relatively barren coniferous habitats and may therefore be sensitive to pollution. Also, the effects of emissions from the pulp and paper industry on the breeding of a common forest passerine like the Great tit are still little known. A preliminary study of Great tit breeding parameters (based on data from one season) in central Portugal suggested that breeding performance of *P. major* was actually better in an industrial than in a rural area (Costa, Petronilho & Vingada, 2005). The present long-term study aims to determine 1) whether emissions from the pulp industry affect the breeding parameters or nest box occupation rates of Great tits, and 2) whether the possible effects on tits are related to invertebrate food abundance during breeding.

Methods

STUDY AREA

In Portugal, the forested area with maritime pine (*Pinus pinaster*) covers about 1 million ha, half of which consists of even-aged monocultures as a result of a deliberate forestry policy dating from the end of the 19th century (DGF, 1999). The study was carried out in 2 maritime pine forests located in Figueira da Foz, on the central coast of Portugal, from 2003 until 2009: the National Pine Forest of Quiaios (MQ) and the National Pine Forest of Urso (MU). MU is approximately 20 km to the south of MQ. Both areas share the same altitude (±50 m asl) and average temperature, and both have sandy soil and feature 70–80-y-old pine plantations dominated by *Pinus pinaster* interspersed by some *Pinus pinea* patches, with a tree density varying from 666 to 1066 individuals per ha. The shrub layer is dominated by *Myrica faya*, *Halimium halimifolium*, *Cytisus scoparius*, *Ulex* spp., *Cistus* spp., and *Acacia* spp.

MQ (40° 14' N, 8° 47' W) is a 6000-ha forested area without the direct influence of industrial pollution that forms part of the "Dunas de Mira" Natura 2000 site (PTCON055). MU (40° 02' N, 8° 52' W) is a 9000-ha forested area sited near a pulp mill industrial complex (< 1 km to the south). In 2004 the paper mill reported emissions of carbon monoxide (999 t), nitrous oxide (13 t), nitrogen oxides (405 t), and PM10 particulates (205 t) (EPER, 2004). Also, previous studies focusing on the same study area (MQ and MU) detected higher mercury concentrations in feathers of both nestling (Costa, 2005) and adult Great tit (Norte *et al*., 2010) in MU. Because of the north-northwest prevailing winds in this area, MQ is not exposed to any emissions from the pulp mill complexes.

Three homogeneous, even-aged plots (trees aged 70–80 y) were selected in each study area. Plots were within 2 km of each other. Nest boxes were placed at an average density of $9 \cdot ha^{-1}$, at equal distances from each other (40 m), resulting in 20 to 50 nest boxes per plot (2003-2004: 216 boxes; 2005-2006: 208 boxes; 2007-2008: 120 boxes; 2009: 300 boxes).

BREEDING PARAMETERS

The breeding of *P. major* was monitored in both areas between February and July of each year. The wooden nest boxes, each with a 2.8-cm entrance and cavity measurements of 20 × 15 × 15 cm (height, width, length), were checked at least once a week in order to gather information about laying date, clutch size, number of fledglings, and occupation rate. Laying dates were calculated assuming that Great tits lay 1 egg per day and have an average incubation period of 12 d (Costa, Petronilho & Vingada, 2005). Nests where egg laying was initiated were considered occupied nests. On day 15 (± 1) post-hatching, all nestlings were measured for their body mass (g) with a spring scale and marked with an individually numbered aluminum ring. Adult birds were captured while feeding nestlings, enabling their sex and age to be recorded. Since not all parents were captured at the nests, it was assumed that a female captured at the nest at the time of second clutches had completed a first clutch of eggs in the same or in a nearby box. This was a reasonable assumption because the Great tit is a territorial bird with high breeding area fidelity. On average, females move less than 100 m between nesting attempts during the same breeding season (Harvey, Greenwood & Perrins, 1979). Replacement, predated, and deserted clutches were excluded from the data. Coal tits (*Periparus ater*), crested tits (*Lophophanes cristatus*), blue tits (*Cyanistes caeruleus*), and short-toed treecreepers (*Certhia brachydactyla*) also bred in the nest boxes.

MEASUREMENT OF FOOD AVAILABILITY

In 2009 the abundance of herbivorous caterpillars and sawfly larvae in tree foliage was measured by the frass-fall method (Southwood, 1978), in which the amount of falling frass is used as a reliable relative measure of caterpillar biomass in the tree canopy (Fischbacher, Naef-Daenzer

& Naef-Daenzer, 1998). Round plastic funnels (34 cm in diameter) were attached with wire to the trunks of pine trees (the dominant trees) at a 2-m height. The number of collectors per area was 18 (*i.e.*, 6 collectors·site^{-1}). Sampling took place between the 13th of April and the 30th of July, with an average duration of 14 d. Funnels were placed within 20 m of nest boxes occupied by Great tits. A container was placed under each funnel to accumulate the frass during the collection period. Contents were dried and stored in paper bags until the frass was separated from litter and weighed.

In 2009, the abundance of ground-living arthropods was also measured using pitfall traps (12 traps per area; 4 traps·site^{-1}). These were plastic jars (15 cm in diameter) containing ethylene glycol. Sampling took place between the 18th of May and the 1st of July, with an average duration of 14 d, and samples were stored in ethanol. Arthropods were counted and divided into 7 groups: spiders (Aranea), ants (Formicidae), beetles (Coleoptera), woodlice (Isopoda), silverfish (Thysanura), millipedes (Diplopoda), and crickets (Gryllidae).

STATISTICS

Statistical analyses were performed with SAS statistical software 9.1 (SAS Institute, 2003). The effects of area and year on laying date, clutch size, number of fledglings per pair, and nestling body mass were examined using a two-way ANOVA (followed by a Tukey–Kramer *post hoc* test), after checking for normality of distributions. Average nestling body mass per brood was used in the analyses to avoid pseudoreplication. First and second breeding attempts were analyzed separately. In both study areas, the relationships between clutch size and laying date, and between number of fledglings and laying date, were examined by linear regressions. The differences between areas in the frequency of second breeding attempts were assessed using a chi-square test. Wilcoxon's matched pair test (with n = 7 y) was used to compare nest box occupation rates between the 2 areas. Since other species compete with the Great tit for the same nest boxes, we also analyzed the occupation rates of the other breeding species in the study areas. Differences in caterpillar biomass and arthropod numbers were compared between study areas using ANOVA (GLM procedure in SAS). Values were $\log_{10}$-transformed to normalize distributions. In arthropod analyses each prey group was compared separately. The relationship between caterpillar biomass and onset of hatching (number of nests where tits started hatching eggs during the collection period) was examined by linear regression. For all analysis, the significance level was set at $P < 0.05$.

Results

FIRST BREEDING ATTEMPT

Great tits started egg laying on average 10 d earlier in MU (industrial area) than in MQ (Figure 1a). In addition to the significant difference between areas, there were also a significant variation in timing among years and an interaction effect between area and year on the laying date (Table I; Figure 1a). The interaction was due to the fact that in 2006, 2007, and 2008 the date of breeding onset was similar in both areas, while in the remaining years breeding onset occurred earlier in MU than in MQ (Tukey–Kramer *post hoc* comparison, $P < 0.05$). In 2009, the significant interaction was also a result of an earlier laying date for the first egg in MU when compared to 2008 and a considerably later laying date in MQ when compared to 2008, presenting an inversion of the pattern observed in the previous years.

Average clutch size (± SD) was significantly larger in MU than in MQ (MQ: 5.80 ± 1.51; MU: 7.30 ± 1.42), and no significant differences were detected in average clutch sizes among years (Table I; Figure 1b). Average number of fledgling number (± SD) was also larger in MU (MQ: 3.80 ± 1.58; MU: 5.20 ± 1.84), and significant differences were detected among years (Table I; Figure 1c). There were no significant differences in fledgling body mass (± SD) between areas (MQ: 14.97 ± 1.94; MU: 14.76 ± 1.85), but body mass varied between sampling years (Table I; Figure 1d).

Nest box occupation rates of Great tits were not significantly different in the 2 areas (MQ: 13.88%, MU: 15.10%; Wilcoxon's test, $n = 7$, $Z = 0.45$, $P = 0.65$). Other species that occupied the nest boxes included the coal tit, the crested tit, the blue tit, and the short-toed treecreeper. The combined nest-box occupation rate for these species was significantly higher in the unpolluted area (MQ: 7.30%, MU: 3.48%; Wilcoxon's test, $n = 7$, $Z = 2.24$, $P = 0.025$).

There was a significant decrease in clutch size and number of fledglings with the delay of egg laying in MU (Figures 2a and 2c). In MQ there was a significant decrease in clutch size with the delay of egg laying, but no significant changes in the number of fledglings were detected (Figures 2a and 2c).

SECOND BREEDING ATTEMPT

Thirty-six percent of Great tits bred a second time in MQ and 41% in MU ($\chi^2 = 0.25$; $P = 0.61$). Egg laying of the second breeding attempt started on average 7 d earlier in MU (Table I). There was also an interaction between sampling area and year with respect to the start of egg laying; this was due to the fact that the date of breeding onset was earlier in MU than MQ in 2003 and 2004, while in the remaining years the date of onset was similar in the 2 areas (Tukey–Kramer *post hoc* comparison, $P < 0.05$).

Overall average clutch size (± SD) was larger in MU than in MQ (MQ: 5.34 ± 1.05; MU: 6.28 ± 1.05) even though there were no significant differences in clutch sizes among sampling years (Table I). No significant differences were detected between sampling areas (MQ: 4.17 ± 1.31; MU: 4.95 ± 1.49) or years (MQ: 17.62 ± 1.37; MU: 17.89 ± 1.06) with respect to number of fledglings and fledgling body mass (± SD) (Table I).

Nest-box occupation rates of Great tits were again very similar in both areas (MQ: 7.25%, MU: 6.91%; Wilcoxon's test, $n = 7$, $Z = 0.128$, $P = 0.90$). The occupation rate of nest boxes for the other breeding species was higher in the unpolluted site, but there were no significant differences between areas (MQ: 4.16%, MU: 2.60%; Wilcoxon's test, $n = 7$, $Z = 1.087$, $P = 0.28$).

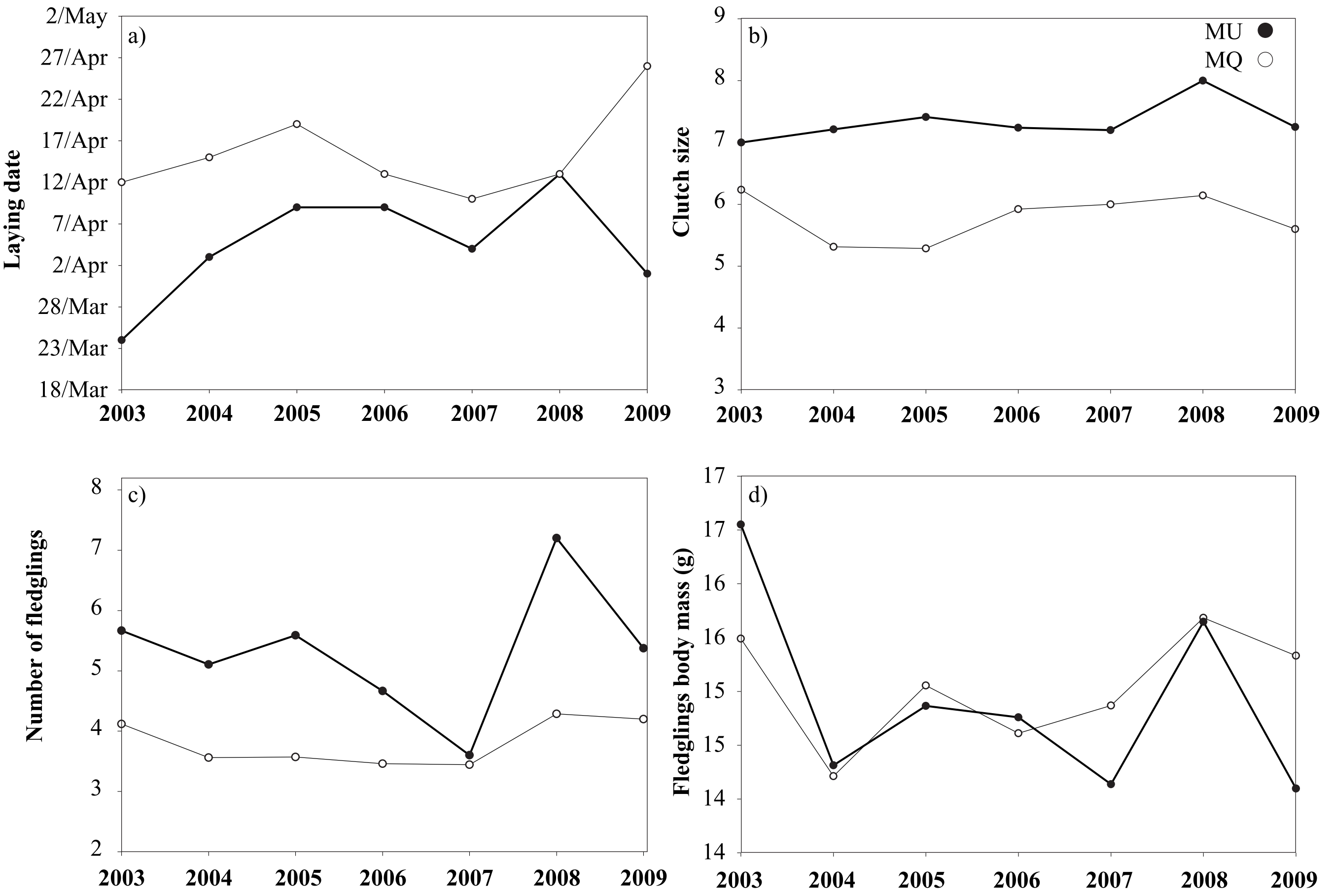

Figure 1. a) Annual average laying date of the first egg; b) clutch size; c) number of fledglings; and d) fledgling body mass for Great tits' first breeding attempt in polluted (MU, filled circles) and unpolluted (MQ, empty circles) areas.

Table I. Two-way ANOVA showing the effects of area (polluted *versus* unpolluted) and year (2003–2009) on laying date, clutch size, number of fledglings, and fledgling body mass of Great tits' first ($n = 160$ nests) and second ($n = 68$ nests) breeding attempts. Significant relationships indicated in bold.

		Laying date			Clutch size			Number of fledglings			Fledgling body mass		
	Num df	Den df	*F*	*P*	Den df	*F*	*P*	Den df	*F*	*P*	Den df	*F*	*P*
First breeding													
Area	1	146	48.2	**< 0.0001**	146	34.1	**< 0.0001**	146	25.5	**< 0.0001**	133	0.13	0.71
Year	6	146	3.13	**0.007**	146	0.52	0.79	146	2.45	**0.03**	133	2.14	**0.05**
Area * year	6	146	3.23	**0.005**	146	0.49	0.81	146	0.83	0.55	133	0.60	0.73
Second breeding													
Area	1	54	7.63	**0.008**	54	9.86	**0.003**	54	1.15	0.29	44	0.37	0.55
Year	6	54	1.52	0.19	54	1.89	0.10	54	0.70	0.65	44	0.70	0.65
Area * year	6	54	2.52	**0.03**	54	2.04	0.08	54	0.96	0.46	44	0.90	0.49

Second clutches were 14% smaller than first clutches in MU (1^{st}: 7.30, 2^{nd}: 6.28), while there was no statistically significant difference in MQ (1^{st}: 5.80, 2^{nd}: 5.34) (Table II). There was also a significant decrease of second clutch sizes with the delay of egg laying in MU, and a corresponding but not statistically significant decrease in the number of fledglings (Figures 2b and 2d). MQ showed no significant temporal change in either clutch size or number of fledglings (Figures 2b and 2d). Although fledgling body mass did not vary between areas, the nestlings of second clutches were heavier than those of the first clutches in both areas (Table II; Figure 3).

Food availability

The overall abundance of caterpillar biomass was significantly higher in MU (ANOVA: $F = 10.75$, $P < 0.0001$; Figure 4). The samples primarily contained the frass of sawflies (Symphyta) and moths (Lepidoptera). There was no relation between caterpillar biomass and the number of nests where hatching started during the collection period in

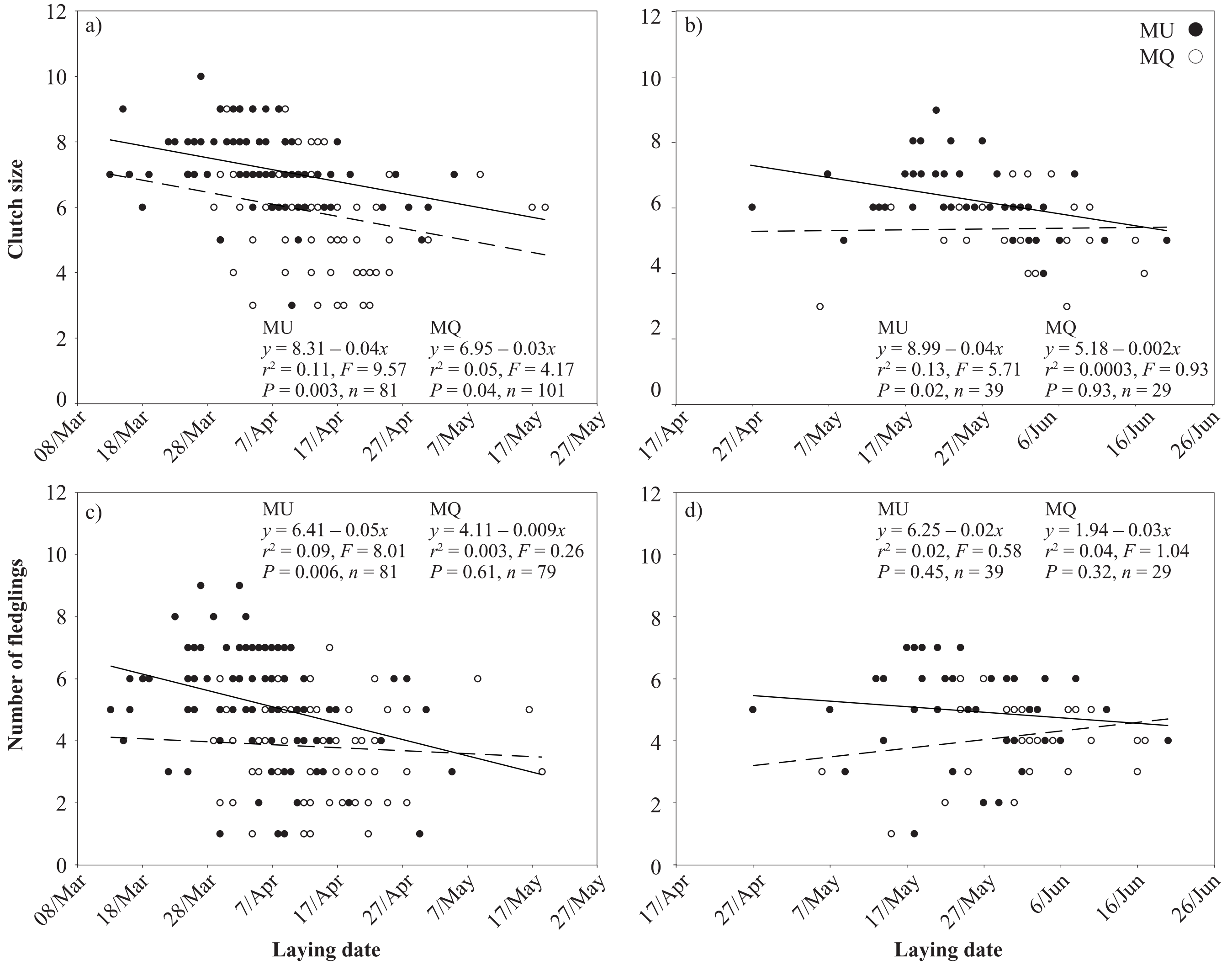

FIGURE 2. The relationship between clutch size and laying date, and between number of fledglings and laying date, for Great tits' first (a, c) and second (b, d) breeding attempt in polluted (MU, filled circles, black line) and unpolluted (MQ, empty circles, dotted line) areas, tested with linear regressions.

TABLE II. Two-way ANOVA showing the effects of breeding attempt (first or second) and year (2003–2009) on nestling body mass at the age of 15 d and clutch size of Great tit in the unpolluted (MQ) and polluted (MU) areas. Significant relationships indicated in bold.

		MQ			MU		
	Num df	Den df	*F*	*P*	Den df	*F*	*P*
NESTLING BODY MASS							
Attempt	1	85	34.56	**< 0.0001**	92	49.77	**< 0.0001**
Year	6	85	1.01	0.43	92	0.90	0.50
Attempt * year	6	85	0.41	0.84	92	0.52	0.79
CLUTCH SIZE							
Attempt	1	94	1.04	0.31	106	10.33	**0.002**
Year	6	94	0.40	0.88	106	1.92	0.08
Attempt * year	6	94	0.54	0.78	106	1.04	0.40

either of the areas (Hatching date: MQ: $F = 0.66$, $r^2 = 0.099$, $P = 0.45$, $n = 8$; MU: $F = 2.46$, $r^2 = 0.29$, $P = 0.17$, $n = 8$).

Ground arthropod abundance varied between the 2 areas, but this variation depended on invertebrate group. Spiders and silverfish were significantly more abundant in MQ (ANOVA: $F = 5.95$, $P < 0.0001$ for spiders; $F = 3.06$, $P = 0.01$ for silverfish; $n = 92$; Figures 5a and 5c), while beetles and millipedes were significantly more abundant in MU (ANOVA: $F = 5.60$, $P < 0.0001$ for beetles; $F = 2.75$, $P = 0.048$ for millipedes; $n = 92$; Figures 5b and 5d). The other groups (ants, woodlice, and crickets) did not show significant differences between the areas.

Discussion

We found that Great tits bred earlier, laid more eggs, and produced more fledglings in the industrialized area (MU). Better availability of invertebrate food in MU is a likely explanation of why these parameters registered higher values in this area. Food abundance and timing of breeding are 2 important and interrelated factors influencing reproduction in birds (Martin, 1987; Naef-Daenzer, Widmer & Nuber, 2001). The breeding success of the Great tit has been observed to decrease considerably after even a short delay in the onset of breeding, due to a mismatch between nestling period and caterpillar peak (Perrins & McCleery, 1989;

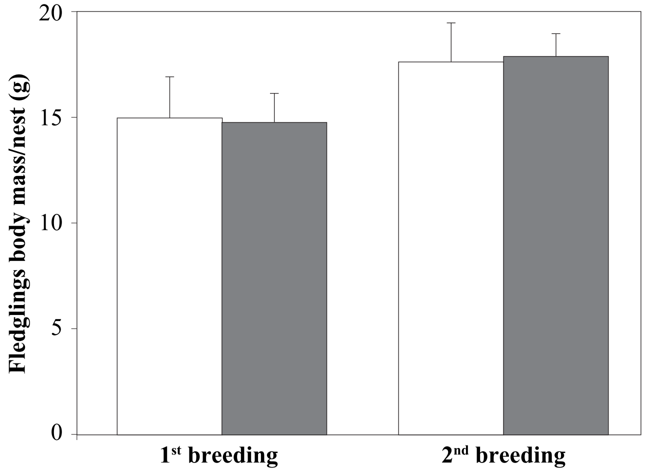

FIGURE 3. Average fledgling body mass (+ SD) for Great tits' first (MU: 14.76 ± 1.85, n = 75; MQ: 14.97 ± 1.94, n = 72) and second (MU: 17.89 ± 1.06, n = 31; MQ: 17.62 ± 1.37, n = 26) breeding attempt in polluted (MU, grey bars) and unpolluted (MQ, white bars) areas.

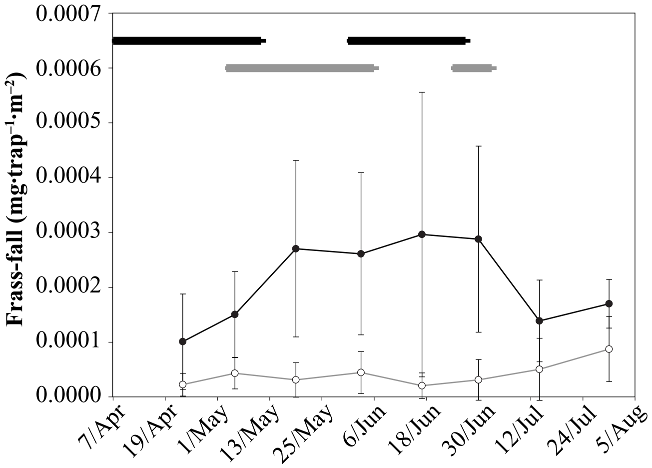

FIGURE 4. Temporal variation in frass production (mg·trap^{-1}·m^{-2} ± SD) of insect larvae in pine (*Pinus* sp.) canopy in polluted (MU, filled circles, n = 110 frass samples) and unpolluted (MQ, empty circles, n = 104 frass samples) areas. The horizontal bars denote the range of hatching dates for the 1st and 2nd breeding of great tits.

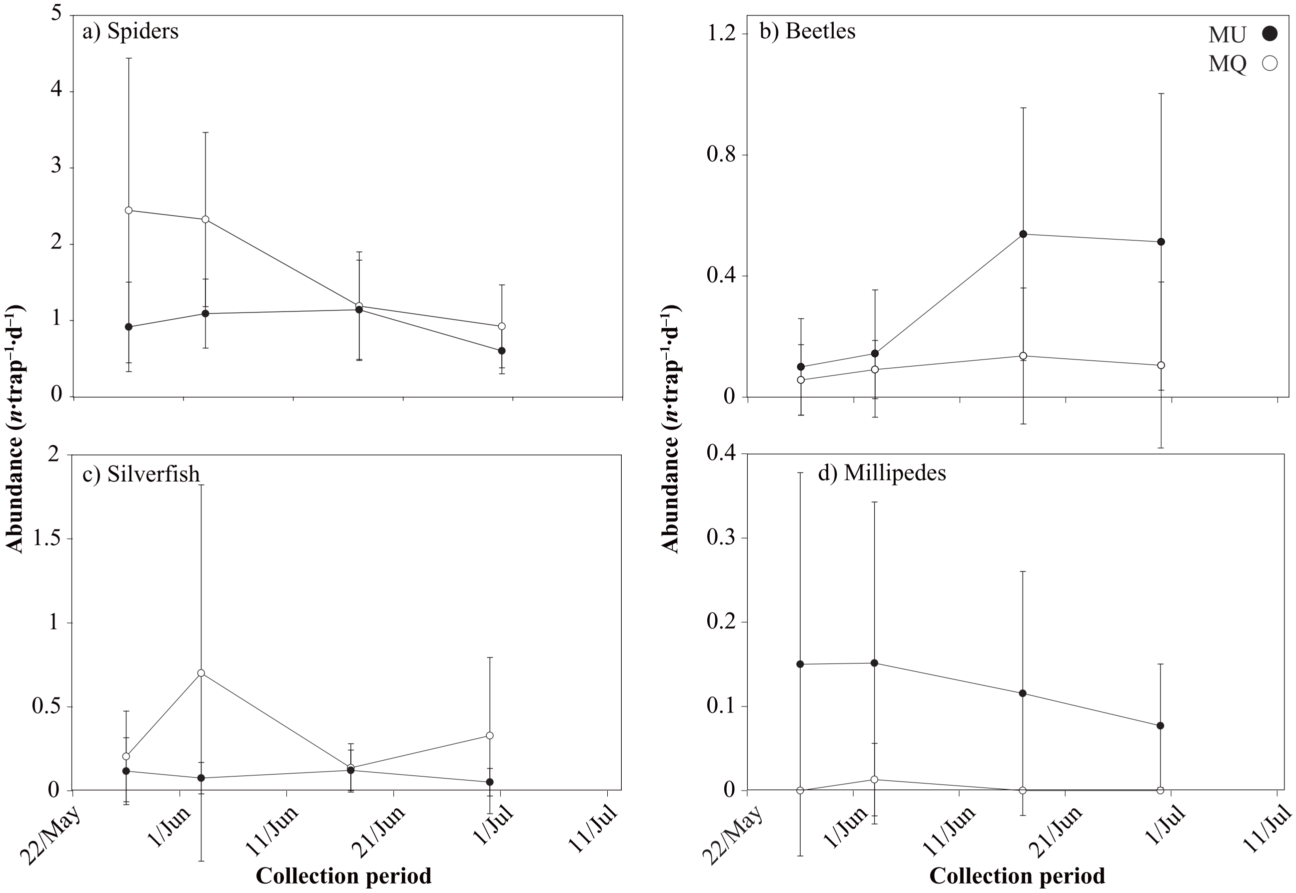

FIGURE 5. Arthropod abundance (n·trap^{-1}·d^{-1} ± SD) in polluted (MU, filled circles) and unpolluted (MQ, empty circles) areas.

Barba, Gil-Delgado & Monrós, 1995). Despite the similarity and proximity of the study areas, we found significant differences in the breeding parameters of Great tits and also in food availability. Caterpillar availability was markedly higher in the industrialized area, and the differences in other invertebrate groups also suggest pollution-related changes in the invertebrate community.

It is interesting to consider why MU, which is under the direct influence of industrial pollution (Costa, 2005; Norte *et al.*, 2010), shows higher food availability than MQ, a relatively unpolluted area. Insect populations and their host plants can be stressed by air pollutants, and significant changes in the ecology of both insects and plants may occur (Kidd, 1990; Pimentel, 1994). Several species of herbivorous insects, which are an important food resource for many insectivorous birds, may suffer from severe pollution, but their abundance is known to increase in moderately polluted environments, probably due to decreased tree vigour (Heliövaara & Väisänen, 1990; Eeva, Lehikoinen & Pohjalainen, 1997). The exposure of plants to airborne pollutants such as photochemical oxidants in smog, SO_2, and fluoride increases the content of free amino acids in the damaged tissues and predisposes plants to successful attack by insects (see White, 1984). Although food abundance was assessed during one season only, it is reasonable to assume similar results for the remaining years, since the breeding success of the Great tit was consistently lower in the unpolluted area during all study years. The relationship between food abundance and breeding parameters is well documented (Perrins, 1991b; Seki & Takano, 1998). In our study, caterpillar biomass was markedly higher in MU, but the abundance of ground arthropods differed between prey groups and areas. The existence of a paper mill in the vicinity of MU since 1967 probably has affected the relationships between trees and herbivorous insects, making the area more profitable to some invertebrate species. Of the arthropod groups collected with pitfall traps, spiders are the most important for tits' diet (Betts, 1955; Royama, 1970; Török, 1985; Lambrechts *et al.*, 2008). Woodlice and millipedes are also considered important sources of calcium for insectivorous birds (Bureš & Weidinger, 2003), calcium being a crucial factor for successful breeding, possibly limiting the reproductive output of birds (Graveland & Van Gijzen, 1994; Tilgar, Mänd & Mägi, 2002; Bureš & Weidinger, 2003; Eeva & Lehikoinen, 2004).

Nest-box occupation rate by Great tits was very similar between the areas, but we found a significant difference in the number of other species that occupied the nests. These species largely overlap the Great tit in terms of resource use (nest boxes, food types, breeding time), which could imply interspecific competition, particularly if resources are scarce. The difference in occupation rates by other species could also be related to pollution, since some species could be more sensitive and hence appear in lower numbers in polluted areas. On the other hand, Great tits are less specialized than other tits in terms of feeding, having a larger range of prey, which is probably one of the reasons why Great tits settle more often in less advantageous habitats (Török, 1985).

The seasonal variation in clutch size detected in the present study indicates that the Great tit adopts different breeding strategies in MQ and MU: while a higher investment is made in the first clutch in MU, investment is similar in both clutches in MQ. We argue that this is explained by lower caterpillar abundance in MQ, especially during the first breeding attempt (see Figure 4). Our results suggest that Great tit females adopt a different strategy in each area due to different food availability, producing smaller clutches in the rural pine forests and thus enhancing nestling survival in an environment where caterpillars are scarce. Mägi *et al.* (2009) verified that variation in food supply during the brood-rearing period could explain not only habitat, but also seasonal differences in breeding performance in Great tits.

We found no evidence of direct toxic effects on the study species from paper industry emissions. However, since both study areas were otherwise homogeneous, the variation in food availability seems to have been related to the pollution levels, indirectly affecting the breeding performance of the Great tit. It is known that at moderate pollution levels the effects on birds may only be indirect (Morrison, 1986; Furness & Greenwood, 1993; Eeva, Lehikoinen & Pohjalainen, 1997), but these effects can change important parameters of bird reproduction, such as clutch size or timing of laying. Therefore, the results of our study emphasize the importance of further research to clarify the ecological effects of moderate pollution levels on wildlife.

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6.2 Paper II

Trace Elements in Faeces of Great Tit Nestlings in Relation to Breeding Performance in Coastal Areas in Central Portugal.

Rute A. Costa, Tapio Eeva, Catarina Eira, Jorge Vaqueiro and José V. Vingada (2012) Trace Elements in Faeces of Great Tit Nestlings in Relation to Breeding Performance in Coastal Areas in Central Portugal. *Archives of Environmental Contamination and Toxicology* 63(4): 594-600. doi: 10.1007/s00244-012-9798-8.

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Trace Elements in Faeces of Great Tit Nestlings in Relation to Breeding Performance in Coastal Areas in Central Portugal

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Abstract This long-term study (2003–2010) compared the breeding parameters of great tits living in a paper-and-pulp–industry area to those of great tits living in a rural area on the west coast of Portugal. We also measured the abundance of caterpillar biomass, an important food source and determinant of breeding success for tits. In 2009, we further analysed trace metal [arsenic (As), calcium (Ca), cadmium, copper, mercury (Hg), nickel, lead, selenium, and zinc] as well as Ca concentrations in excrement of 15-day-old great tit nestlings. Generally, for most trace metals, fecal concentrations were similar at both sites. Nonetheless, greater Hg levels and lower As levels were detected in the industrial area. Great tits laid more eggs and produced more fledglings in the industrial area than in the rural area. Caterpillar biomass was also greater in the industrial area, which likely explains the better breeding success. Our results suggest that there are no direct effects of emissions on the studied species.

Trace metals are frequent waste products of industrial processes, often resulting in the contamination of the surrounding environment. Because of their persistence and bioaccumulation potential, monitoring their possible effects on wildlife is important. In birds, exposure to trace metals may cause effects on different levels of organization from biochemical responses to changes in population levels (reviewed in Scheuhammer 1987). Reproductive dysfunctions—such as smaller clutches, decreased fertility, hatching failure, and nestling mortality—can be a direct result of pollution and may have profound effects on the stability of avian populations (Dauwe et al. 2005). However, depending on the profile and level of exposure, the concentrations found also may cause indirect rather than direct toxic effects (Scheuhammer 1987). Environmental pollution can indirectly affect birds through habitat changes (Morrison 1986), increased amount of parasites (Eeva et al. 1994), or decreased amount of suitable food (Graveland 1990; Hörnfeldt and Nyholm 1996; Eeva et al. 1997).

Insectivorous great tits (*Parus major*) are potentially good biomonitors of pollution by trace metals because they are ubiquitous and abundant and sometimes the only forest passerine species available in reasonable densities in polluted areas. They readily nest in manmade nest boxes; thus, breeding populations can easily be monitored. Although used to a much lesser extent, nestlings are also potentially good monitors for terrestrial local and point-source pollution (Burger and Gochfeld 1993; Burger 1996; Dauwe et al. 2004). Metal levels in nestlings always reflect local pollution because they are restricted to their nest and receive their diet from the immediate vicinity of the nest box. Therefore, the metal contamination found refers to a clearly defined time period as well as a restricted area around the nest (Furness and Greenwood 1993). In addition, great tit nestlings are easily monitored because they are relatively insensitive to nest disturbance (Janssens et al. 2002).

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A previous long-term study on the breeding success of *P. major* in industrial and rural environments in Portugal (Costa et al. 2011a) suggested that pollution levels may lead to an increment on invertebrate availability, thus affecting breeding performance of the great tit. Apart from food availability, dietary calcium (Ca) levels are a crucial factor for successful breeding and may limit the reproductive output of birds (Graveland and Van Gijzen 1994; Tilgar et al. 2002; Bureš and Weidinger 2003; Eeva and Lehikoinen 2004).

Noninvasive procedures, such as collecting feathers and excrements, have been successfully used in biomonitoring studies focusing on metal pollution (Dauwe et al. 2004; Eeva et al. 2009; Berglund et al. 2011). Once a metal has entered the body, it can be stored or accumulated, or it can be excreted (Burger 1993). Birds can rid their body of metals through excrements or by depositing them in the uropygial gland, salt gland (Burger and Gochfeld 1985), and feathers (Burger 1993; Dauwe et al. 2002).

This study was a follow-up of a long-term ecological research, including an ecotoxicology evaluation, of trace elements on forest passerines (Costa et al. 2011a, b) that focused on their breeding performance and determining noninvasive methods for pollution evaluation. The objective of this study was to increase the available data on great tit reproduction parameters and on food availability as well as determine the concentrations of trace metals in great tit nestlings using another noninvasive method that does not affect their breeding success. Therefore, we analysed the excreta of 15-day-old nestlings and related element levels, including Ca, with the number and body mass of *P. major* nestlings from industrial and rural sites located in a pine forest coastal area in Portugal.

Materials and Methods

Study Area

The present work is part of a long-term monitoring study on the effects of air pollution on wild birds that has been ongoing since 2003 (Costa et al. 2011a). The study has been performed at multiple study plots in two maritime pine (*Pinus pinaster*) forest areas located in Figueira da Foz (Portugal). One area is located in the National Pine Forest of Quiaios (MQ), a 6000-ha forested area bordered by agriculture fields without a direct influence of industrial pollution, that is included in the Natura 2000 site “Dunas de Mira” (40°14′N 8°47′W). The second area is located in the National Pine Forest of Urso (MU), a 9,000-ha forested area sited <1 km to the south of a paper-and-pulp–mill industrial complex (40°02′N 8°52′W). MU is situated approximately 20 km to the south of MQ, and both areas share the same altitude (±50 m asl), average temperature, and sandy soils. As a result of a deliberate forest policy dating from the end of the 19th century (DGF 1999), pine trees were planted in both study areas between 1920 and 1930 to stop sand incursions onto agriculture fields. Therefore, in both areas, forests are dominated by even-aged (80- to 90-year-old pine plantations) *P. pinaster* interspersed by some *P. pinea* patches. Pine density in MU varied between 675 and 1,300 individuals/ha (average 1,000 individuals/ha) and pine tree diameter at breast height (DBH) between 15 and 26 cm (average 19). Pine density in MQ varied between 600 and 1,250 individuals/ha (average 875 individuals/ha) and pine tree DBH between 22 and 41 cm (average 32). The shrub layer is dominated by *Myrica faya*, *Halimium halimifolium*, *Cytisus scoparius*, *Ulex* spp, *Cistus* spp, and *Acacia* spp.

The paper-and-pulp mill at MU produces bleached kraft pulp using an elemental chlorine-free method, and reported air emissions include carbon monoxide, nitrous oxide, nitrogen oxides, and PM10 particulates (EPER 2009) without any reference to metals. However, previous studies comparing the same study areas detected greater mercury (Hg) concentrations in feathers of both nestling (Costa et al. 2011b) and adult great tits (Norte et al. 2010) at the industrial area.

Three homogeneous even-aged plots (trees age 70–80 years old) were selected in each study area. Plots were within 2 km of each other. The wooden nest boxes, with a 2.8-cm entrance and cavity measurements of 20 × 15 × 15 cm (height, width, and length, respectively), were placed at an average density of 9/ha at equal distances from each other, resulting in 20–50 nest boxes/plot.

Breeding Parameters

From 2003 until 2010, the breeding parameters of *P. major* were monitored yearly in both areas between February and July. The nest boxes were checked at least once a week to gather information on different breeding parameters (e.g., clutch size, number of fledglings). From this data, breeding success (number of fledglings/numbers of eggs laid) was calculated. On day 15 (±1) posthatch, all nestlings were measured for body mass (g) using a spring scale and marked with an individually numbered aluminium ring.

Measurements of Food Availability

Herbivorous caterpillars and sawfly larvae are important food sources for tits (Gibb 1954, 1960). Therefore, in 2009 and 2010 the amount of herbivorous caterpillars and sawfly larvae in tree foliage was measured by frass-fall method (Southwood 1978). The amount of falling frass is a relative reliable measure of caterpillar biomass in the tree canopy

(Fischbacher et al. 1998). Round plastic funnels (34 cm in diameter) were attached with wire to trunks of pine trees (the dominant trees) at 2-m height. The number of collectors per area during each year was 18 (i.e., 6 collectors/study plot). Sampling took place between April 1 and July 30 for both study years (2009 and 2010). Funnels were placed within 20 m of nest boxes. Under the funnel, there was a container in which frass accumulated during the collection period. Contents were dried and stored in paper bags until the frass was separated from the litter and weighted. Mean values (mg) of frass-fall per trap per day were calculated.

Excrement Sampling

During the 2009 breeding season (February–July), we collected fresh faeces from defecating nestlings at the age of 15 (±1) days. Nestlings were induced to defecate on handling, and excrements were immediately collected in metal-free plastic containers. Samples were air dried before element analysis. A total of 48 nestlings (MQ: $n = 27$, MU: $n = 21$) from 18 nests were sampled (MQ: $n = 10$, MU: $n = 8$), producing 48 individual excrement samples. All samples were analyzed separately, but "nest" was considered a random factor in the statistical analysis (see later text).

Metal Analyses

Dried faeces were weighted and placed in Teflon bombs with 2 ml Supra-pure HNO_3 and 0.5 ml H_2O_2 for microwave digestion (Millestone High Performance Microwave Digestion Unit mls 1200 mega; Millestone, Leutkirch, Germany). The samples were then diluted to 50 ml with deionized water (Elgastat Maxima). Nine elements [arsenic (As), Ca, cadmium (Cd), copper (Cu), Hg, nickel (Ni), lead (Pb), selenium (Se), and zinc (Zn)] were determined with ICP-MS (Elan 6100 DRC; PerkinElmer-Sciex, Boston, MA). The detection limit for most of the metals was approximately $\leq$1 ng/l. Calibration of the instrument was performed using a certified solution (multielement solution 2A; Claritas PPT, Metuchen, NJ) from Spex Certiprep. Certified reference materials (mussel tissue ERM-CE278; skim-milk powder BCR-063R; Ni not included) were used for validation. The mean recoveries (±SE) in five reference samples were as follows: As 90 ± 0.69 %, Ca 86 ± 1.03 %, Cd 89 ± 1.93 %, Cu 103 ± 1.88 %, Hg 94 ± 5.21 %, Pb 96 ± 1.00 %, Se 100 ± 4.24 %, and Zn 81 ± 1.04 %.

Statistics

Statistical analyses were performed using SAS statistical software 9.2 (SAS Institute 2003). Using nestling faeces collected in 2009, differences in metal concentrations between areas (industrial vs. rural) were analyzed with generalized linear models (GLMs; Glimmix procedure in SAS), and "nest" was included as a random factor in the model. Pearson correlation matrix was applied to examine relationships between metal concentrations from great tit faeces and their breeding and condition parameters (clutch size, number of fledglings, and fledgling body mass). The average number of nestlings per brood, corresponding to 18 nests, was used in this analysis to avoid pseudoreplication.

The effect of area (MQ and MU) and year (2003–2010) on breeding and condition parameters (clutch size, number of fledglings, breeding success, and fledgling body mass) was examined using two-way analysis of variance (ANOVA) in which area, year, and area × year were used as independent factors (procedure Mixed in SAS followed by Tukey-Kramer *post hoc* test). Second breeding attempts, as well as replacement, predated, and deserted clutches, were excluded from the data.

Caterpillar biomass were examined using two-way ANOVA in which area (MQ and MU), year (2009 and 2010), and area × year were used as independent factors (procedure Mixed in SAS followed by Tukey-Kramer *post hoc* test). For all analyses, after checking for normality of distributions, values were $\log_{10}$-transformed to normalize distributions and back-transformed for presentation in the tables. The significance level was set at $p < 0.05$.

Results

Trace Metal and Ca Concentrations in Excrement

Average element concentrations (±SD) in nestlings' excrements are listed in Table 1. Faeces collected from the industrial area (MU) presented a greater average concentration of Hg, whereas those from the rural area showed greater concentrations of As (Table 1). There were no significant differences in faecal Ca concentrations between the areas (Table 1).

There were several significant positive correlations between metal concentrations from nestlings, most of them involving Zn and Cu (Table 2). We also found a significantly positive correlation between As and fledgling body mass and between Pb and number of fledglings (Table 2). There was also a significant negative correlation between fledglings body mass and clutch size and a positive correlation between clutch size and number of fledglings (Table 2).

Breeding Success and Nestling Condition

The mean (±SD) clutch size and number of fledglings in MU (7.25 ± 1.41 and 5.12 ± 1.86, respectively) were

Table 1 Comparison between trace metal and Ca concentrations [mean ± SD (ppm)] in excrements of great tit nestlings at sites near a pulp mill (MU) ($n = 21$ nestlings) and at reference sites (MQ) ($n = 27$ nestlings) using GLMs

Metal	MQ	MU	*df*	*F*	*p*
As	7.09 ± 15.39	1.26 ± 2.04	1.45	4.23	0.046
Hg	0.22 ± 0.09	0.35 ± 0.26	1.44	7.57	0.009
Pb	0.86 ± 0.63	1.10 ± 1.09	1.45	1.01	0.32
Ni	1.21 ± 0.78	1.20 ± 1.09	1.45	0.15	0.70
Cu	81.6 ± 48.2	98.5 ± 58.6	1.45	1.99	0.17
Cd	1.58 ± 1.77	1.08 ± 0.88	1.45	0.93	0.34
Zn	409.8 ± 279.6	391.7 ± 220.9	1.45	0.03	0.86
Se	0.46 ± 0.23	0.55 ± 0.35	1.45	0.00	0.96
Ca	12,121 ± 10,564	11,312 ± 12,526	1.45	0.02	0.88

"Nest" was used as a random factor in the analyses

significantly both greater than in MQ (5.86 ± 1.43 and 3.70 ± 1.58, respectively; Table 3). No significant yearly variation was observed for either parameter, although a marginally significant effect on fledgling number is indicative of some yearly variation (Table 3). There were no significant differences in breeding success (MQ = 0.65 ± 0.25; MU = 0.71 ± 0.24; Table 3) or fledgling body masses (age of 15 days) between areas or years (MQ = 15.06 ± 1.95 g; MU = 14.79 ± 1.89 g; Table 3).

Food Availability

The overall average (±SD) abundance of frass-fall biomass was two times greater in MU than in MQ (MQ = 1.34 ± 1.62 mg/trap/d; MU = 2.81 ± 1.28 mg/trap/d, $F_{1.44} = 31.88$, $p < 0.0001$). There was no significant variation between the two sampled years ($F_{1.44} = 1.56$, $p = 0.22$), but there was a significant interaction effect between area and year ($F_{1.44} = 5.14$, $p = 0.03$). The interaction was due to the higher caterpillar abundance in MQ in the year 2010 compared with 2009 (Tukey-Kramer *post hoc* comparison, $p = 0.02$).

Discussion

The results suggest that the area around the pulp mill is not heavily polluted by metals and does not cause reproductive problems for the breeding population of *P. major*. In fact, considering the nine metals/metalloids evaluated from nestling faeces, only Hg and As showed differences between the study areas. In the industrial area (MU), we found significantly higher levels of Hg in tit faeces indicating that tits are more exposed to Hg in the industrial area than in the rural area. Nonetheless, great tits in MU laid more eggs and produced more fledglings than those in the rural area (MQ).

An earlier study on the same areas performed in 2003 had already showed greater levels of Hg in feathers of nestling great tits in the industrial area (MU; Costa et al. 2011b), even though no Hg emissions were indicated on the report given by the paper-and-pulp facilities in 2004 (EPER 2009). Therefore, the origin of Hg cannot be traced, and the levels detected in MU could be a result of a past long-term emission, i.e., when environmental concerns were not considered a priority and there was a lack of regulations for these kinds of emissions. Alternatively, current emissions from accessory processes in factories, namely, paper and pulp mills, such as electricity production and residue incineration (European Commission 2001), are also a possibility. In fact, although factories' gaseous effluents may not contain Hg, metal emissions from combustion of organic matter or fuel are possible, thus allowing

Table 2 Pearson correlation matrix for faecal metal concentrations and offspring characteristics of great tits in 2009 ($n = 18$)

	As	Pb	Ni	Cu	Cd	Zn	Se	Hg	Fledgling body mass	Clutch size	No. of fledglings
Ca	−0.44	−0.15	−0.02	−0.35	−0.01	−0.37	−0.005	−0.21	−0.17	0.24	−0.10
As		0.24	0.21	0.23	−0.31	−0.05	−0.15	−0.08	0.47*	−0.27	0.05
Pb			0.46	0.59**	0.38	0.53*	0.30	0.17	−0.008	0.45	0.55*
Ni				0.41	0.30	0.55*	0.08	0.42	0.31	−0.09	0.13
Cu					0.59*	0.81***	0.58**	0.57*	−0.03	0.07	0.20
Cd						0.73***	0.67**	0.15	−0.09	−0.006	−0.0001
Zn							0.47	0.44	−0.06	−0.004	0.11
Se								0.29	−0.18	0.07	−0.12
Hg									−0.18	0.19	0.12
Fledgling body mass										−0.54*	0.17
Clutch size											0.65*

* $p < 0.05$, ** $p < 0.01$, *** $p = 0.0002$

Table 3 Two-way ANOVA showing the effects of area (industrial vs. rural) and year (2003–2010) on clutch size, number of fledglings, breeding success, and fledgling body mass of great tits first breeding attempt

	Num *df*	Den *df*	*F*	*p*
Clutch size				
Area	1	169	31.23	<0.0001
Year	7	169	0.48	0.85
Area × year	7	169	0.85	0.55
No. of fledglings				
Area	1	169	13.20	0.0004
Year	7	169	1.82	0.09
Area × year	7	169	0.56	0.79
Breeding success				
Area	1	169	1.06	0.31
Year	7	169	1.66	0.12
Area × year	7	169	0.49	0.84
Fledgling body mass				
Area	1	155	0.11	0.74
Year	7	155	1.88	0.08
Area × year	7	155	0.47	0.86

for the occurrence of production processes that still release Hg. Nevertheless, the breeding performance of great tits was better in the industrial area, with more eggs and more fledglings per nest. The higher amount of caterpillar biomass in MU is a likely reason for the higher productivity in this area because food availability is one of the most important factors affecting birds' breeding performance (Lack 1964; Martin 1987).

Abiotic factors, such as air pollution, can affect insects and their subsequent fecundity and survival by way of their effects on plant quality (Leather and Awmack 1998; Heliövaara and Väisänen 1990; Eeva et al. 1997). Although the past emissions from the factory are not well known, atmospheric pollutants, such as nitrogen oxides and carbon dioxide, have been reported by the pulp mill (EPER 2009), and these pollutants are known to influence insect performance by way of their effects on host plant quality (Dohmen et al. 1984; Watt et al. 1995). In fact, increases in insect populations have been recorded as a response to exposed foliage, which appears more nutritious to insects (see Pimentel 1993).

In the rural area, we found greater values of As (although marginally significantly), which can be accounted for by the presence of agriculture fields in the surroundings of MQ where pesticides and herbicides are used. Few experiments have been performed to determine the threshold levels of As at which adverse effects occur in birds (Eisler 1988; Neff 1997; Burger et al. 2007). In our study, concentrations of As were positively correlated with fledgling body mass, which in turn did not vary between the areas, indicating that exposure to this element may be a lesser concern for great tits in the rural area.

In the present study, Ca values in feces were within the range of those reported by other investigators (Eeva et al. 2009; Berglund et al. 2011). There were no significant differences in excreted Ca concentrations between rural and industrial areas, indicating that Ca availability should not account for the differences detected in the breeding success between study areas.

There were several significant positive correlations between metal concentrations from nestlings, most of them involving Zn and Cu, which are metabolically regulated essential micronutrients. The several positive significant relationships detected may suggest similar regulation and detoxification processes. In fact, one of the detoxification mechanisms possibly explaining parallel metal accumulation or excretion is the potential binding of several metals, such as Zn, Cu, and Cd, to metallothioneins (e.g., Scheuhammer 1987; Stewart et al. 1994).

Overall trace metal levels in our study areas (both MQ and MU) can be considered lower or within the range of metal levels reported in other European studies. With respect to Hg, we recorded 0.35 ppm in the polluted site, whereas the only other available data on great tit faeces ranged from 0.07 to 11 ppm (see Dauwe et al. 2004; Janssens et al. 2003) and were recorded for the same study area in Belgium.

In studies performed by Dauwe et al. (2000, 2004), Eeva et al. (2009), and Janssens et al. (2003) breeding success was decreased in metal-polluted areas. Although those study sites have industrial activity that typically emits metals (metallurgy, smelters), the contamination levels in our study area were similar, especially for Cu, Hg, and Zn. In addition, breeding parameters (clutch size, number of fledglings, fledgling body mass) in our study were generally lower than values reported in many other studies in Europe, including those performed in coniferous forests (Lemel 1989; Tilgar et al. 1999; Mänd et al. 2005; Mägi et al. 2009). These differences may be related with the generally unproductive environment (sandy soils) and low caterpillar numbers of the southern coastal maritime habitat of our study area. Our study suggests that the great tit breeding performance was not directly affected by metal pollution. However, it seems to have been indirectly affected by pollution through the modulation of food availability, which emphasises the importance of quantifying food resources in pollution-impact studies using wild bird populations.

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6.3 Paper III

Assessing heavy metal pollution using great tits (*Parus major*): feathers and excrements from nestlings and adults.

Rute A. Costa, Tapio Eeva, Catarina Eira, Jorge Vaqueiro and José V. Vingada (*in press*) Assessing heavy metal pollution using great tits (*Parus major*): feathers and excrements from nestlings and adults. *Environmental Monitoring and Assessment* DOI 10.1007/s10661-012-2949-6.

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Assessing heavy metal pollution using Great Tits (*Parus major*): feathers and excrements from nestlings and adults

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Abstract Passerine species have been increasingly used as bioindicators of metal bioaccumulation especially by taking benefit of non-invasive procedures, such as collecting feathers and excrements. In 2009, metal (As, Cd, Cu, Hg, Ni, Pb, Se and Zn) concentrations were determined in feathers and excrements of nestling and adult female great tits (*Parus major*) in industrial (a paper mill) and rural sites in maritime pine forests on the west coast of Portugal. The aim of this study was to compare the levels of metals between the areas but also between sampling methods (feather vs. excrement) and age classes (nestling vs. adult). Although excrements and feathers of nestling great tits showed different concentrations, similar patterns of accumulation were detected in both study areas. There was a significantly higher concentration of mercury in the industrial area and significantly higher concentrations of arsenic in the rural area in both sample types. Metal levels in adult females had quite different results when compared to nestlings, and only nickel presented significantly higher levels near the paper mill. Since metal levels showed a consistent pattern in feathers and excrements of nestling great tits, we conclude that both represent good and non-invasive methods for the evaluation of these elements in polluted areas.

Keywords Biomonitoring · Heavy metal pollution · Feathers and excrements · Great tit nestlings

Introduction

Pollutants have continuously been introduced into ecosystems as a consequence of urbanisation and industrial processes. Metals are globally distributed, and persistent pollutants with bioaccumulation potential being important to monitor their possible effects on wildlife. Non-invasive procedures, such as collecting feathers and excrements, have been successfully used in biomonitoring studies focusing on heavy metal pollution (Denneman and Douben 1993; Hahn et al. 1993). Birds excrete elements into growing feathers (Burger 1993) and can also eliminate metals through

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excrements or by depositing them in the uropygial gland and salt gland (Burger and Gochfeld 1985). Females can also excrete metals in their eggs and eggshells (Burger 1994). However, each matrix has potential problems as regards to biomonitoring, such as external contamination in feathers or the tissue-specific mechanisms that regulate excretion (Dauwe et al. 2004).

The pulp and paper industry is known for the emission of malodorous sulphurous air pollutants such as hydrogen sulphide (H_2S), methyl mercaptan (CH_3SH), and methyl sulphides, but the available information concerning the effects of these pollutants on wildlife is still sparse (Haahtela et al. 1992; Soimasuoa et al. 1995; Harris and Elliott 2000; Isaksson et al. 2005).

Insectivorous Great Tits, *Parus major*, are potentially good biomonitors of heavy metal pollution because they are ubiquitous and abundant and, sometimes, the only forest passerine species available in reasonable densities in polluted areas. They readily nest in man-made nest boxes, and so breeding populations can easily be monitored. In 2009, we measured the concentrations of heavy metals and calcium levels in feathers and excrements of *P. major* adult females and nestlings in an industrial environment around a pulp and paper mill and in a rural control environment, both located in a coastal area of Portugal.

The objective of this study was to compare heavy metal levels between the two study areas, industrial and rural, while comparing the performance of the two non-invasive procedures (feathers and excrement sampling) to assess heavy metal contamination. Although previous studies showed low correlations between both methods (Morrissey et al. 2005; Berglund et al. 2011), these comparisons may be important for understanding the excretion processes and toxicity of metals.

Material and methods

Study area

The present work is part of a long-term monitoring study on the effects of air pollution on wild birds, which has been ongoing since 2003 (Costa et al. 2011a). The study has been carried out at multiple study plots in two maritime pine (*Pinus pinaster*) forest areas located in Figueira da Foz (Portugal). Both areas share the same altitude (±50 m asl) and average temperature, both presenting a sandy soil and including 70–80-year-old pine plantations dominated by *P. pinaster* interspersed by some *Pinus pinea* patches, with a tree density varying from 666 to 1,066 individuals/ha. The shrub layer is dominated by *Myrica faya*, *Halimium halimifolium*, *Cytisus scoparius*, *Ulex* spp., *Cistus* spp. and *Acacia* spp.

One area is located in the National Pine Forest of Quiaios (MQ), a 6,000-ha forested area bordered by agriculture fields without a direct influence of industrial pollution, included in the Natura 2000 site "Dunas de Mira" (40°14′ N, 8°47′ W). Because of the north–northwest prevalent winds in this area, MQ is not exposed to any emissions from the pulp mill complexes. The second area is located in the National Pine Forest of Urso (MU), a 9,000-ha forested area sited less than 1 km to the south of a paper and pulp mill industrial complex (40°02′ N, 8°52′ W). The mill produces bleached kraft pulp using an elemental chlorine-free method, and the officially reported air emissions include carbon monoxide, nitrous oxide, nitrogen oxides and PM10 particulates (EPER 2009). Although no metals were presented in the mill report, previous studies comparing the same study areas detected higher mercury concentrations in feathers of both nestling (Costa et al. 2011b) and adult Great Tits (Norte et al. 2010) at the industrial area. Q1 Three homogeneous even aged plots (trees aged 70–80 years) were selected in each study area. Plots were within 2 km of each other. Nest boxes were placed at an average density of 9/ha, at equal distances from each other, resulting in 20 to 50 nest boxes per plot.

Feather and excrement sampling

In the summer of 2009, we collected fresh faeces from defecating nestlings at the age of 15 (±1) days. Nestlings were induced to defecate upon handling, and excrements were immediately collected in metal-free plastic containers. With respect to excrements, a total of 48 samples (MQ 27, MU 21) were produced by different nestlings from 18 nests (MQ 10, MU 8). With respect to feathers, a total of 70 samples (MQ 33, MU 37) from different nestlings from 12 nests (MQ 6, MU 6) and 13 samples (MQ 7, MU 6) from different adult females from 13 nests (MQ 7, MU 6) were collected. Only the two outermost tail feathers were collected from adult females and from nestlings at the age of 15 (±1) days. Nestling tail feathers measure 25–28 mm (Orell 1983), and adult female tail feathers measure ca.

58 mm (Cramp and Perrins 1993). Feathers were stored in sterile, metal-free plastic Eppendorf tubes and maintained at −20 °C until analysis. All samples were analysed separately, but nest was considered as a random factor in the statistical analysis.

Metal analyses

Prior to analysis, feathers were washed vigorously in deionized water alternated with 1 mol/l acetone to remove any external contamination and dried at room temperature for 48 h. Feather samples were weighed (range 5–80 mg), and excrement samples were dried in an oven at 50 °C for 72 h and weighed. All samples were then digested in Teflon bombs by adding 2 ml of suprapure HNO_3 and 0.5 ml of H_2O_2 and placed in a microwave system (Anton Paar, Multiwave 3000, Graz, Austria). The samples were then diluted to 50 ml with deionized water (Elgastat Maxima). Eight elements (As, Cd, Cu, Hg, Ni, Pb, Se and Zn) were determined with ICP-MS (Elan 6100 DRC, PerkinElmer-Sciex, Boston, USA). The detection limits for most of the metals were around 1 ng/l and below. The calibration of the instrument was done with a certified solution (Ultra Scientific, multi-element solution IMS-102, N. Kingstown, RI, USA) from LGC Promochem. Certified reference materials (mussel tissue ERM-CE278; skim milk powder BCR-063R; Ni not included) were used for method validation. The mean recoveries (±SE) in reference samples were as follows: As 90±0.69 %, Cd 89±1.93 %, Cu 103±1.88 %, Hg 94±5.21 %, Pb 96±1.00 %, Se 100±4.24 % and Zn 81±1.04 %.

Statistics

Statistical analyses were performed with SAS statistical software 9.2 (SAS Institute 2003). Means and standard deviations are given in the tables. Differences in metal concentration in feathers and excrements of nestling Great Tits between areas (industrial vs. rural) were assessed with generalised linear models (GLIMMIX procedure in SAS) where nest was included as a random factor to account for the non-independence of the measurements in nestlings from the same brood. Pearson correlations were applied to examine relationships between metal concentrations in feathers and excrements from the same nestling, and therefore, only 30 animals were included in this analysis. Metal concentrations in feathers of adult females were compared between areas (industrial vs. rural) using a *t* test for unequal variances. Comparison between feathers of nestlings and females was made using generalised linear models (GLIMMIX procedure in SAS) where nest was included as a random factor. For all analysis, after checking for normality of distributions, some variables were $\log_{10}$ transformed to normalise distributions for the analyses and back transformed for presentation in the tables. The significance level was set at $p<0.05$.

Results

Metal concentrations in nestling feathers and excrements

Mean metal concentrations in Great Tit nestlings' excrements presented significantly higher levels of mercury in the industrial area (MU) and a marginally significantly higher level of arsenic in the rural area (MQ) (Table 1). There were no significant differences concerning the remaining analysed metals in both areas (Table 1).

Mean metal concentrations in Great Tit nestlings' feathers presented significantly higher levels of mercury and selenium in the industrial area (MU) and significantly higher levels of arsenic in the rural area (MQ) (Table 1). There were no significant differences concerning the remaining analysed metals in both areas (Table 1). The comparison of metal levels between excreta and feathers revealed that arsenic and selenium levels were significantly positively correlated (Table 1).

Metal concentrations in feathers of adult females

Mean metal concentrations in adult female feathers presented significantly higher levels of nickel in the industrial area (MU) (Table 2). There were no significant differences between the concentrations of the remaining metals in feathers from both areas (Table 2).

Comparison of metal concentrations between nestling and adult feathers

All metals, with the exception of mercury, nickel and selenium, presented significant differences between the concentration levels found in feathers of nestlings and those of adult female Great Tits (Table 3). Copper and zinc levels were higher in nestlings, while arsenic, lead and cadmium levels were higher in adult females (Table 3).

Table 1 Comparison of heavy metal concentrations (in parts per million) in excrements (n=48) and feathers (n=70) of Great Tit nestlings at a site near a pulp mill (MU) and at a reference site (MQ) using generalised linear models (mean±standard deviation) and Pearson correlation matrix of metal levels in excreta and feathers of Great Tit nestlings (n=30)

	Excrements				Feathers				Excrements/feathers
	MQ	MU	*F*	*p*	MQ	MU	*F*	*p*	*r*
As	7.09±15.39	1.26±2.04	4.23	0.046	0.35±0.48	0.19±0.10	4.51	0.04	0.82*
Hg	0.22±0.09	0.35±0.26	7.57	0.009	0.41±0.18	0.45±0.07	7.11	0.01	0.14
Pb	0.86±0.63	1.10±1.09	1.31	0.32	1.27±0.41	1.29±0.43	0.86	0.36	0.10
Ni	1.21±0.78	1.20±1.09	0.15	0.70	2.38±0.92	2.24±0.43	0.05	0.82	−0.05
Cu	81.6±48.2	98.5±58.6	1.99	0.17	8.29±1.75	9.11±3.67	1.01	0.32	0.01
Cd	1.58±1.77	1.08±0.88	0.93	0.34	0.03±0.01	0.03±0.01	2.37	0.13	−0.24
Zn	409.8±279.6	391.7±220.9	0.03	0.86	112.9±5.65	111.0±4.31	2.63	0.11	0.36
Se	0.46±0.23	0.55±0.35	0.00	0.96	0.92±0.20	1.07±0.20	9.18	0.004	0.50**

*p<0.0001; **p=0.005

Discussion

In agreement with our previous study (Costa et al. 2011b), we found a significantly higher concentration of mercury in the industrial area MU and a significantly higher concentration of arsenic in the rural area MQ in both excrement and feathers of Great Tit nestlings. Although metal concentrations differ between sample types (excrement vs. feather), they all showed an equal tendency in both study areas, i.e. an increase in a metal concentration in nestling excrements is accompanied by an increase of the same metal concentration in nestling feathers in each study area.

Nestling feathers also presented significantly higher levels of selenium in the industrial area (MU), which is possibly related with the above-mentioned higher amount of mercury detected in this study area. In fact, the formation of Se–Hg complexes as a detoxification process (e.g. Thompson 1996) may be the source for the higher levels of selenium detected in feathers collected from the industrial area.

The use of feathers and excrements has been a common tool to observe environmental metal pollution, especially because they are non-injurious and non-invasive to birds (Burger 1993; Eens et al. 1999; Dauwe et al. 2000; Janssens et al. 2002; Bianchi et al. 2008). Feathers reflect the amount of metals present in the blood at the time of feather growth, either from current dietary sources or from mobilisation of metals from internal organs (Burger 1993), while excrements represent the unabsorbed remnants of multiple food items, often at higher concentrations than the diet

Table 2 Comparison of heavy metal concentrations (in parts per million) in feathers of female adult Great Tit at a site near a pulp mill (MU) (n=6) and at a reference site (MQ) (n=7) using a t test (mean±standard deviation)

	MQ	MU	*t*	*p*
As	0.98±1.16	0.48±0.22	0.90	0.40
Hg	0.39±0.12	0.65±0.66	−0.77	0.47
Pb	2.49±1.31	8.86±16.0	−0.99	0.36
Ni	1.66±0.15	2.20±0.39	−3.23	0.02
Cu	5.72±0.82	7.11±2.55	−1.30	0.24
Cd	0.11±0.04	0.10±0.03	0.37	0.72
Zn	101.6±11.8	104.2±12.7	−0.37	0.72
Se	0.90±0.18	0.97±0.26	−0.55	0.39

Table 3 Comparison of heavy metal concentrations (in parts per million) in feathers of female adult (n=13) and nestling (n=70) Great Tits at the study area (MQ+MU) using generalised linear models (mean±standard deviation)

	Adult feathers	Nestling feathers	*F*	*p*
As	0.75±0.87	0.26±0.34	22.56	<0.0001
Hg	0.51±0.46	0.43±0.13	0.06	0.81
Pb	5.43±10.9	1.28±0.42	47.52	<0.0001
Ni	1.91±0.39	2.30±0.70	3.24	0.08
Cu	6.36±1.89	8.72±2.94	16.29	0.0001
Cd	0.10±0.03	0.03±0.01	118.9	<0.0001
Zn	102.8±11.7	111.9±5.05	27.84	<0.0001
Se	0.93±0.46	1.00±0.22	1.13	0.29

items, and so they can provide a non-destructive and quantifiable means of monitoring the food chain contamination by trace metals (Spahn and Sherry 1999; Morrissey et al. 2005).

In several studies (Morrissey et al. 2005; Berglund et al. 2011), the correlation among metal levels between feathers or internal tissues and excrements was low, indicating no clear pattern with respect to metal excretion. This is in agreement with our study, where only arsenic and selenium showed a positive correlation. However, feathers and excrements partly indicate a temporally different exposure, which likely weakens the correlations. Also, the small difference in values of some metal concentrations could reduce the probability of a significant correlation.

Feather metal levels in adult female Great Tits showed some different results compared to nestlings. However, the only significantly higher levels were detected for nickel in MU, perhaps due to the low number of adult samples (MQ, $n=7$; MU, $n=6$), which weakens the power for statistical comparisons. However, the patterns of higher arsenic in MQ and higher levels of mercury in the industrial area (MU) were the same as those found in nestlings, although differences were not significant. One cannot rule out external contamination and differences in metabolism, since some elements like nickel can be regulated by homeostatic control in nestlings (Nyholm 1995; Dauwe et al. 2004; Berglund et al. 2011).

When we compared contaminant levels of adult and nestling feathers, we expected levels of metals to be higher in adults than in nestlings, especially because adults have had longer time to acquire and bioaccumulate contaminants (Burger et al. 2009). However, we found significantly higher levels of zinc and copper in nestlings, and only arsenic, lead and cadmium were significantly higher in adults. Interestingly, the metals are here divided by their redox activity Q2 (Koivula et al. 2011). Copper is a redox active metal, while arsenic, lead and cadmium are redox inactive. Adult and nestling feathers present a slightly different composition because, at the time of sample collection, adult feathers are completely formed, while in 15-day-old nestlings, feathers are still growing and have an active blood circulation supporting their growth (Burger and Gochfeld 1992). Once the feather has reached its full size, the blood supply is no longer needed, and the vessels shrivel up. The redox inactive metals have strong affinity to the sulfhydryl groups of keratin (a key protein in feather), and this is probably why we found higher levels of these metals in adult feathers. Nestling feathers, on the other hand, include blood vessels and blood that might contain more copper and zinc than pure keratin.

Since mercury and arsenic levels showed a consistent pattern in feathers and excrements of nestling Great Tits, we may well say that these substrates present a good method for the evaluation of these elements in the study areas. Arsenic is a toxic non-essential element that readily bioaccumulates. Several reported values for arsenic in the feathers and excrements of Great Tit are higher than the levels found in the present study (Janssens et al. 2003; Dauwe et al. 2004; Eeva et al. 2006, 2009), and no toxic effect was found to be directly related to arsenic values. Mercury is considered to be very toxic for wild animals, and at higher levels of contamination, it can adversely affect birds by reducing their fecundity, growth and body length (Eisler 1987). Although the values found in the present study were within the ranges previously reported for mercury levels in feathers and excrements of Great Tit inhabiting metal-contaminated sites (Janssens et al. 2002, 2003), we found no direct adverse effects on the breeding biology of the Great Tit (Costa et al. 2011a). Nevertheless, considering the well-known hazardous effect of these elements on the environment, the regular monitoring of the study areas is essential.

In conclusion, nestlings seem a more optimal choice for the evaluation of local pollution, since we can sample data from a defined area and time period. Furthermore, because the nestlings stay within the nest boxes, there is a very limited possibility of external airborne deposition from industrial sources, which can happen to adults. In addition, we found that nestlings' feathers show a different metal profile, which is partly due to the fact that their feathers are still growing and show a different tissue composition in comparison to fully grown feathers. Therefore, the developmental stage of feathers is important to consider when such results are interpreted.

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6.4 Paper IV

Passerines breeding in nest-boxes: Long-term study in coastal pine stands, central Portugal.

Rute A. Costa, Tapio Eeva, Catarina Eira, Jorge Vaqueiro and José V. Vingada (*under review*) Passerines breeding in nest-boxes: Long-term study in coastal pine stands, central Portugal. *Ornis Fennica*.

De: ornis.fennica@birdlife.fi

Assunto: **Re: Reviewed manuscript - Ornis Fennica OF 3412 decision**

Data: 20 de Agosto de 2012 07:41:41 WEST

Para: Rute Costa <rutealexandra@gmail.com>

Dear Dr. Costa,

many thanks for the resubmission. I will deal with these files shortly. In case another reviewing is required the final decision should be made within 2-3 months.

Best regards,
Matti Koivula, editor-in-chief
Ornis Fennica Finnish Forest Research Institute
PO Box 18, FI-01301 Vantaa
FINLAND

On 2012-08-17 11:20, Rute Costa wrote:

Dear Editor,

We hereby submit a reviewed version of the ms entitled "Hole-nesting bird species breeding in pine stands: Long-term study in central Portugal" by Costa et al., now with a new title: "Passerines breeding in nest-boxes: Long-term study in coastal pine stands, central Portugal". All the changes suggested by the reviewers were considered and the ms was re-written accordingly.

We hope that documents were properly presented and we look forward to hearing from you.

Best wishes,

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De: ornis.fennica@birdlife.fi
Assunto: **Ornis Fennica OF 3412 decision**
Data: 5 de Julho de 2012 08:14:06 WEST
Para: Rute Costa rutealexandra@gmail.com

Dear Dr. Costa,

I have now received comments from two referees, and an opinion from a subject editor, on your cavity-nester study. These all agree in that the general framework is of great interest and would fit to Ornis Fennica scope, and the studied breeding parameters are nicely described for the focal populations, but they also point out certain shortcomings that regrettably prevent me from accepting your manuscript for publication in its present form. These are largely related to focus, clarity and lack of consideration of other studies relevant for your aims. The ms nevertheless has great potential to become an useful piece of work; hence I am willing to reconsider a thoroughly revised manuscript.

Best regards,
--
Matti J. Koivula, editor-in-chief
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Assunto: **Re: Manuscript submission**

Data: 2 de Maio de 2012 13:57:53 WEST

Para: Rute Costa rutealexandra@gmail.com

Dear Dr. Costa,

thank you for the submission. Please allow us 2-3 months to complete the reviewing. In all future correspondence please cite the ms number OF 3412.

Best regards,
Matti Koivula, editor-in-chief

On 2012-05-02 14:04, Rute Costa wrote:
Dear Editor,

We hereby submit the ms entitled “Hole-nesting bird species breeding in pine stands: Long-term study in central Portugal” by Costa et al., for publication in “Ornis Fennica”, if you consider adequate.

We confirm that our manuscript is not under consideration for publication elsewhere and will not be submitted elsewhere while under review by Ornis Fennica.

We hope that documents were properly presented and we look forward to hearing from you.
Best wishes,
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Passerines breeding in nest-boxes: Long-term study in coastal pine stands, central Portugal.

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Abstract

The breeding biology of several hole-nesting bird species was studied in a coastal maritime pine forest habitat, in the central coast of Portugal, from 2003 until 2011, using nest-boxes. The studied species were the Short-toed Treecreeper (*Certhia brachydactyla*), the Coal Tit (*Periparus ater)*, the Blue Tit (*Cyanistes caeruleus*), the Crested Tit (*Lophophanes cristatus)* and the Great Tit *(Parus major)*. The measured breeding parameters included laying date, clutch size, number of fledglings, breeding success (number of fledglings/numbers of eggs laid), and body mass of fledglings. During two years food (caterpillars) availability was also measured by the frass-fall method. Interspecific comparisons revealed significant differences for several parameters. Great Tits produce the largest clutch size, accompanied by the lowest breeding success. We conclude that coastal maritime pine forests are characterized by low offspring numbers (especially for Great and Blue Tit) but also by relatively low yearly variation in breeding parameters. We argue the possible relation between both characters and a constantly low food availability in a relatively barren habitat with minor temperature fluctuations due to the moderating maritime influence.

Key words: Breeding biology; clutch size, hole-nesting birds; marine pine stand habitat.

1. Introduction

Short-toed Treecreepers (*Certhia brachydactyla*), Coal Tits (*Periparus ater)*, Blue Tits (*Cyanistes caeruleus*), Crested Tits (*Lophophanes cristatus)* and Great Tits *(Parus major)* are small hole-nesting insectivorous passerines which are relatively common and some information on their breeding biology in the Mediterranean area is already available (e.g. Barba et al. 1990, 1994, Gildelgado et al. 1992, Maicas & Haeger 1996, 2004, Belda et al. 1998, Encabo et al. 2002, Patten 2007, Sanz et al. 2010). However, studies at coastal areas with coniferous habitats are scarce and most data refers to Great Tits (Fidalgo 1990, Pimentel & Nilsson 2007, Costa et al. 2011) with only one study presenting data on Coal Tits (Fidalgo 1990).

The absence of natural holes in coastal pine forests (Fidalgo 1988) is one of the limiting factors in reproduction of hole-nesting birds. The lack of natural cavities is often overcome by the use of nest-boxes, becoming a critical resource for hole-nesting species during the breeding season (Maícas & Haeger 2004). Nest-boxes have shown to be advantageous in many hole-nesting bird studies (e.g. Sanz 1998, Visser et al. 2003, Both et al. 2004, Griffith et al. 2008). They allow for individual handling and multiple captures and they may also contribute to larger sample sizes thus facilitating data analyses (Lambrecths et al. 2010).

Food availability is another determinant factor in breeding success (Naef-Daenzer et al. 2001, Atiénzar et al. 2010). It is known that caterpillar availability is usually lower and shows a later peak in coniferous than in broad-leaved forests (Sanz et al. 2010, Burger et al. 2012), which may require specific adaptations by the hole-nesting insectivorous passerine populations breeding in southern Europe.

With respect to coastal pine forests in Portugal, they are particularly characterized by a relatively low fluctuation in temperatures due to the moderating maritime influence (Almeida 1997, Ferreira 2000).

The main objective of our long-term study was to increase the knowledge on the breeding biology of the 5 passerine species, relate it with food availability and temperature and compare breeding parameters in southern coastal pine forests to those elsewhere in Europe.

2. Study area and methods

The study was carried out in the National Pine Forest of Quiaios (MQ), located in Figueira da Foz, in the central coast of Portugal, from 2003 until 2011. MQ (40º14'N 8º47'W) is a 6,000 ha forested area included in the Natura 2000 site "Dunas de Mira" (PTCON055). The area presents sandy soils and includes 70 – 80 year-old pine plantations dominated by Maritime pines *Pinus pinaster* interspersed by some Stone pines *Pinus pinea* patches, with a pine density varying between 600 and 1250 individuals per ha (average 875 individuals per ha) and pine tree DBH between 22 and 41 cm (average 32 cm). The shrub layer is dominated by Bayberry *Myrica faya*, Yellow Sunrose *Halimium halimifolium*, Common Broom *Cytisus scoparius*, Gorse *Ulex* spp, Rock Rose *Cistus* spp and *Acacia* spp.

Three homogeneous even aged plots (trees aged 70–80 years) were selected. Plots (5.6 ha) were within 2 km of each other. Nest-boxes were placed at an average density of 9 nest-boxes/ha, at equal distances from each other (40 m), resulting in 20 to 50 nest-boxes per plot (2003-2005: 108 boxes, 2006: 80 boxes, 2007-2008: 600 boxes, 2009-2011: 150 boxes). The wooden nest-boxes had a 28 mm entrance hole and cavity measurements of 20 x 15 x 15 cm (height, width, length), and were initially used for a study on Great Tits (Costa et al. 2011). Old nest material was removed every year before the start of the breeding season.

The breeding population was monitored during 9 years (2003-2011), between February and July. Nest-boxes were checked at least once a week in order to gather information about occupation rate, laying date, clutch size (number of eggs laid per nest), number of fledglings and breeding success (number of fledglings/numbers of eggs laid). In order to estimate the average laying date of the 1st egg, dates were converted into running numbers, day 1 being the 1st of March. On day 15 (± 1) post-hatching, all nestlings were measured for their body mass (g), and marked with an individually numbered aluminium ring.

Average daily temperatures were obtained from the Meteorological Station in Figueira da Foz (40º 8'N 8º 48'W), near the study area, collected by Instituto de Meteorologia Portugal, for all the study years, in order to examine the temperature fluctuation over the breeding period.

In 2009 and 2010 we also monitor the food availability in tree foliage during the birds' breeding period by the frass-fall method (Southwood 1978). Round plastic funnels

(34 cm in diameter) were attached with wire to trunks of pine trees (the dominant trees) at a 2-meter height. The number of collectors per area in each year was 18 (i.e. 6 collectors /study plot). Under the funnel there was a container where the frass accumulated during the collection period. Contents were dried in an oven (Thermodry Ray-DO 50) at 35–40 °C for 24 hours and stored in paper bags until the frass was separated from the litter and weighted. Mean values (mg) of frass-fall per day per m^2 were calculated. Sampling took place between the 1st of April and the 17th of June with an average sampling interval of 9 days.

2.1 Statistical analyses

Statistical analyses were performed with SAS statistical software 9.2 (SAS Institute 2003). Differences in breeding parameters (laying date, clutch size, fledgling number, fledgling body mass and breeding success) between study years and between the five study species were examined by using Kruskal-Wallis Test (NPAR1WAY procedure in SAS), followed by Dunn's Multiple Pairwise Comparison Test. Average nestling body mass per brood was used in the analyses to avoid pseudoreplication. Second breeding attempts, as well as replacement, predated and deserted clutches (n=138) were excluded from the data. Differences in caterpillar biomass were evaluated between study years and collecting dates using a general linear mixed model (Glimmix procedure in SAS) and collector was included as a random factor in the model (the Kenward-Roger option was used to control the number of DFs). Average monthly temperature from the breeding period (February, March and April) was compared between study years using a generalized linear model (Glimmix procedure in SAS). For all analyses the significance level was set at $p<0.05$.

3. Results

Average laying date was significantly different between species (Table 1), with Blue Tit being the latest, and Coal Tit and Crested Tit being the earliest species to lay the eggs (Fig. 1). Average clutch size was also significantly different between species, with Great Tit having the larger clutches and Short-toed Treecreeper the smallest (Table 1). No significant differences were detected in average fledgling number (Table 1). Breeding success was significantly different, with Great Tit having the lower values compared to the other species (Table 1). Average fledglings body mass was significantly different between species, with Great Tit having the heavier fledglings and Coal Tit the lightest (Table 1).

There were no significant differences between the nine study years in clutch size, fledgling number, fledglings' body mass and breeding success in any study species (Table 2). Great Tit laying dates in 2009 (late breeding onset) and also in 2011 (early breeding onset) were significantly different from the laying dates recorded in the remaining study years (Dunn's post-hoc comparison, $p<0.05$).

The temperature values recorded over the breeding period (February, March and April) were similar between all study years ($F_{8,18}=0.24$, $p=0.98$).

In our study frass was constituted by sawfly larvae and herbivorous caterpillars. The overall average (±SE) frass-fall biomass had no significant variation between the sampled years ($F_{1,82}=0.24$, $p=0.63$). However, there was a significant variation in frass-fall between the collecting dates ($F_{10,89}=2.01$, $p=0.04$) but no significant interaction effect between year and date ($F_{3,83}=1.54$, $p=0.21$) (Fig.2).

4. Discussion

Our study area (coastal maritime pine forest) is characterized by low offspring numbers (especially for Great and Blue Tit) and also by a relatively low yearly variation in breeding parameters (see table 3). In fact, when compared to other studies made in Portugal (Fidalgo 1988, 1990, Pimentel & Nilsson 2007) also in pine stands, in our study area the Great Tit has a remarkably lower fledgling number and breeding success. The low food availability in our study area compared to other European studies (Mägi et al. 2009, Navarro 2010) and the need for an higher amount of food (especially caterpillar larvae) considering Great Tits larger body size, most likely led to the lower breeding success.

As expected, almost all breeding parameters presented significant differences between study species since their morphology, phenology, feeding strategies and diets are also quite different (Cramp & Perrins 1993). However, the similarity of clutch sizes and fledgling numbers between species was a surprising finding, since species like the Blue Tit are known by their large clutches in some northern populations (Svensson & Nilsson 1995, Dauwe et al. 2005, Blondel et al. 2006). Also when comparing to southern populations our clutch sizes and fledgling numbers are lower than those reported in Spanish regions (Belda et al. 1998, Atienzar et al. 2010, Sanz et al. 2010). Unfortunately, data is scarce or not available for some species, e.g. Short-toed Treecreeper, and therefore some comparisons are difficult (see table 3).

In our study area we verified that clutch size, number of fledglings, breeding success and fledglings' body mass were similar between study years (2003 to 2011) for all species and we also verified that food availability was quite low in the area (2009 and 2010). Although food availability was assessed during 2 seasons only, it is reasonable to assume similar results for the remaining years since the breeding success of all study species was consistent throughout the years.

Although other factors may be involved in the determination of food availability, one of the most important characteristics of our coastal zones is the lack of extreme temperatures (Almeida 1997, Ferreira 2000) and therefore the low temperature variation recorded during springtime in the study area may account for constant food availability.

Only Great Tits laying dates differed between study years. However, yearly variation in laying dates seems not to influence the other breeding parameters, since breeding values were similar throughout the study years. It seems that Great Tit breeding parameters are closely dependent upon caterpillar phenology (which shows temporal variation).

The breeding parameter values are remarkably lower in our study than in many other studies made in European coniferous forests (Lemel 1989, Tilgar et al. 2002, Mänd et al. 2005, Mägi et al. 2009). However, most of these studies were performed at higher latitudes, in Central and North Europe, where average daily temperatures are considerably lower and more variable than at the Iberian Atlantic coast. Breeding conditions may be much more challenging in southern habitats because, apart from daytime variations, other constraints such as poor food supply and climatic factors (high temperature and low water supply) may be strongly limiting their reproductive output (Sanz et al. 2000).

Overall, this study reports very low clutch sizes for Great and Blue Tit, and low breeding success for the Great Tit, without major annual differences, suggesting that low food availability is the major cause for low breeding performance. In the future, prey specific diet and food availability should be further evaluated in order to confirm their role in nest-box passerines' breeding biology in coastal pine forests.

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Table 1 – Comparison of laying date, clutch size, number of fledglings, breeding success (number of fledglings/number of laid eggs) and fledglings' body mass (± standard deviation) between studied species (Kruskal-Wallis Test, DF and sample sizes in brackets). Significant differences among species using a Dunn's multiple-comparisons test are shown by letters (A and B). Pooled data from 2003 to 2011.

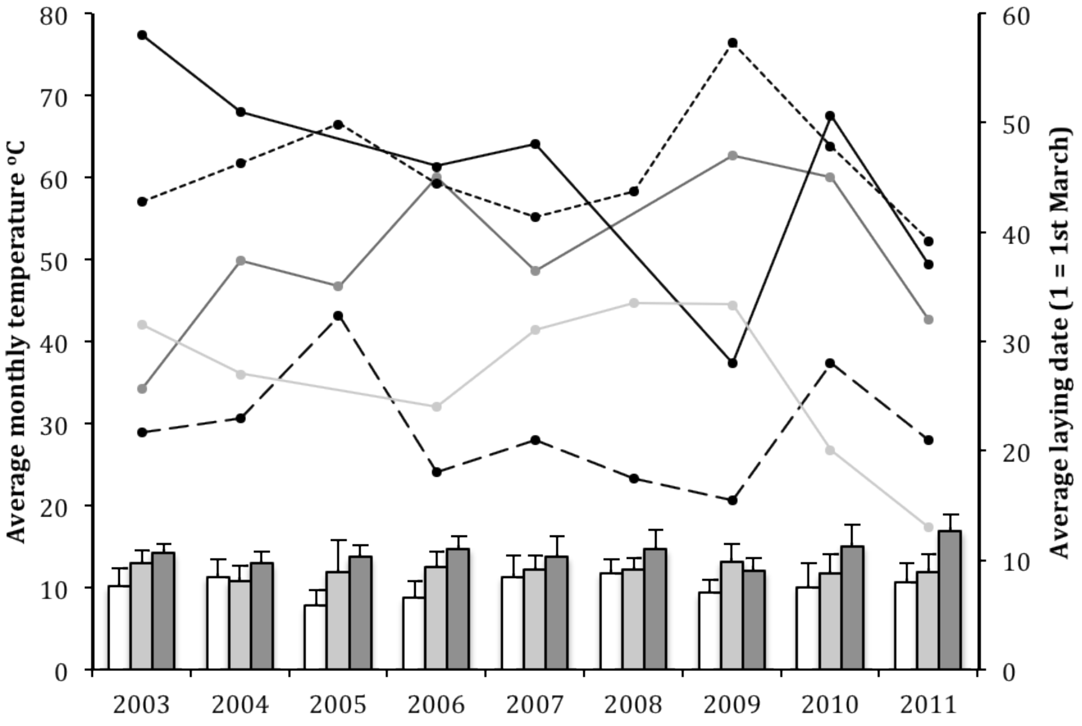
	P. major	*P. ater*	*L. cristatus*	*C. caeruleus*	*C. brachydactyla*	X^2	p
Laying date	45.3 ± 11.1^{A} (4,120)	23.0 ± 8.25^{B} (4,20)	27.7 ± 9.41^{B} (4,16)	46.7 ± 9.38^{A} (4,9)	36.6 ± 10.1^{AB} (4,17)	67.40	<0.0001
Clutch size	5.87 ± 1.41^{A} (4,120)	5.75 ± 0.85^{A} (4,20)	5.38 ± 1.09^{AB} (4,16)	5.78 ± 0.67^{AB} (4,9)	4.59 ± 0.87^{B} (4,17)	18.47	0.001
Number of fledglings	3.74 ± 1.57 (4,120)	4.45 ± 1.54 (4,20)	4.63 ± 1.26 (4,16)	4.00 ± 1.22 (4,9)	3.76 ± 1.15 (4,17)	7.81	0.10
Breeding success	0.65 ± 0.24^{A} (4,120)	0.77 ± 0.25^{AB} (4,20)	0.86 ± 0.15^{B} (4,16)	0.70 ± 0.24^{AB} (4,9)	0.83 ± 0.21^{AB} (4,17)	17.87	0.001
Body mass	15.4 ± 1.77^{A} (4,113)	8.07 ± 0.53^{B} (4,14)	11.0 ± 0.94^{B} (4,11)	9.07 ± 0.57^{B} (4,7)	8.40± 0.70^{B} (4,12)	95.63	<0.0001

Table 2 – Comparison of annual laying date, clutch size, number of fledglings, breeding success (number of fledglings/number of laid eggs) and fledglings body mass of *P. major*, *P. ater*, *L. cristatus*, *C. caeruleus* and *C. brachydactyla* between study years (2003 – 2011) (Kruskal-Wallis test, DF and sample sizes in brackets).

	P. major		*P. ater*		*L. cristatus*		*C.caeruleus*		*C.brachydactyla*	
	X^2	p	X^2	p	X^2	p	X^2	p	X^2	p
Laying date	19.5 (8, 120)	0.01	7.34 (8, 20)	0.50	7.28 (7, 16)	0.40	6.63 (6, 9)	0.36	8.07 (7, 17)	0.33
Clutch size	6.53 (8, 120)	0.59	7.61 (8, 20)	0.47	11.4 (7, 16)	0.12	6.22 (6, 9)	0.40	7.80 (7, 17)	0.35
Number of fledglings	5.31 (8, 120)	0.72	10.1 (8, 20)	0.26	10.9 (7, 16)	0.14	4.75 (6, 9)	0.58	7.06 (7, 17)	0.42
Breeding success	5.66 (8, 120)	0.69	10.9 (8, 20)	0.21	9.33 (7, 16)	0.23	4.01 (6, 9)	0.68	9.00 (7, 17)	0.25
Body mass	6.73 (8, 113)	0.57	8.30 (8, 14)	0.31	8.60 (7, 11)	0.20	5.04 (6, 7)	0.41	3.78 (7, 12)	0.71

Table 3 – Breeding parameters observed in *Certhia brachydactyla*, *Periparus ater*, *Cyanistes caeruleus*, *Lophophanes cristatus* and *Parus major* in several European study areas. Ref., references: 1, Martins 1999; 2, Sanz et al. 2010; 3, Fidalgo 1988; 4, Maicas and Haeger 2004; 5, Atienzar et al. 2009; 6, Lambrechts et al. 2008; 7, Belda et al. 1998; 8, Magi et al. 2009; 9, Gienapp and Visser 2006; 10, Pimentel and Nilsson 2007

Species	**Study period**	**Type of forest**	**Country**	**Clutch size**	**Fledgling Number**	**Laying date**	**Breeding success**	**Fledglings body mass**	**Ref.**
C. caeruleus	1999	Oak	Portugal	6.9±0.9	6.0±2.7	16 Apr	0.68	8.9±0.4	1
	2005	Pine	Spain	6.69±1.24	-	1 May	0.85±0.20	-	2
	2003-2011	Pine	Portugal (Quiaios)	5.78±0.67	4.00±1.22	17 Apr	0.70±0.24	9.07±0.57	Present study
L. cristatus	1987	Pine	Portugal	5.67±0.52	-	24 Mar	0.56	10.7±1.3	3
	1989-1991	Pine	Spain	5.1±0.1	4.1±0.2	-	0.64	10.50±0.13	4
	2005-2007	Pine	Spain	5.1±0.2	4.4±0.2	17 Apr	-	11.50±0.2	5
	2003-2011	Pine	Portugal (Quiaios)	5.38±1.09	4.63±1.26	29 Mar	0.86±0.15	11.0±0.94	Present study
P. ater	1987	Pine	Portugal	6.17±0.88	-	16 Mar	0.85	8.2±0.6	3
	2003-2011	Pine	Portugal (Quiaios)	5.75±0.85	4.45±1.54	24 Mar	0.77±0.25	8.07±0.53	Present study
C. brachydactyla	2003-2011	Pine	Portugal (Quiaios)	4.59±0.87	3.76±1.15	7 Apr	0.83±0.21	8.40±0.70	Present study
P. major	1985-1997	Holm oak	France	8.27±1.22	6.44±1.88	14 May	0.78±0.23	15.85±0.23	6
	1987	Pine	Portugal	6.92±1.10	-	18 Apr	0.39	14.3±2.2	3
	1991-2004	Downy oak	France	9.35±1.34	7.93±2.31	4 Apr	0.85±0.21	17.10±1.34	6
	1992-1995	Pine	Spain	6.38±0.31	-	4 May	-	-	7
	1999	Oak	Portugal	7.1±0.9	5.8±2.2	12 Apr	0.46	16.9±0.9	1
	1999-2002	Pine	Estonia	10.2±1.4	7.5	-	-	17.10±2.00	8
	2001-2003	Mixed	Holand	-	-	16 Apr	-	-	9
	2001-2004	Pine	Portugal (Lisbon)	8.3	6.1	-	-	-	10
	2001-2004	Pine	Portugal (Leiria)	7.4	5.4	-	-	-	10
	2005	Pine	Spain	6.25±2.30	-	21 Apr	0.85±0.25	-	2
	2005-2007	Pine	Spain	6.16±0.36	5.09±0.73	1 May	-	15.47±0.55	5
	2003-2011	Pine	Portugal (Quiaios)	5.87±1.41	3.74±1.57	15 Apr	0.65±0.24	15.4±1.77	Present study



Study years

Fig. 1- Average monthly temperatures (°C) during the breeding season (white bars – February; light grey bars – March; dark grey bars – April) and average laying date for all species (black line – Blue Tit; small dash line – Great Tit; light grey line – Crested Tit; dark grey line – Short-toed Treecreeper; long dash line - Coal Tit), for all study years (2003-2011).

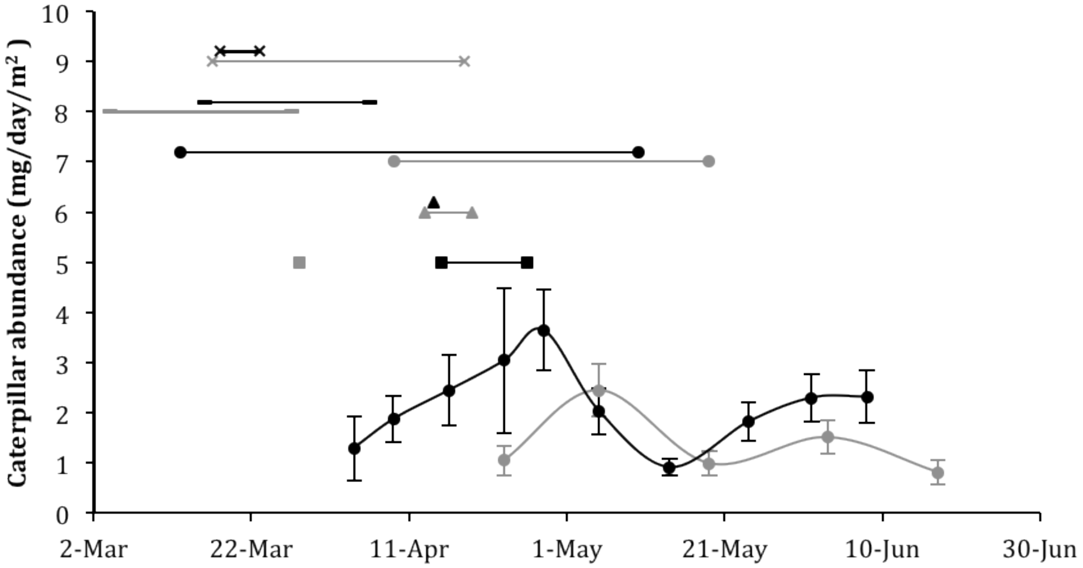


Fig. 2- Temporal variation in frass production (mg/day/m^2±SE) of insect larvae in pine (*Pinus* sp.) canopy in the study area during two study years (2009 and 2010) and laying periods (time between first and last laying date) for all species: squares – Blue Tit; circles – Great Tit; crosses – Crested Tit; triangles – Short-toed Treecreeper; stripe - Coal Tit. Grey line denotes the year 2009 and black line the year 2010.

6.5 Paper V

Great tit as a bioindicator of mercury contamination from the paper and pulp industry.

Rute A. Costa, Tapio Eeva, Catarina Eira, Jorge Vaqueiro, Patricia Medina and José V. Vingada (*submitted*) Mercury contamination from the paper and pulp industry. *Chemistry and Ecology*.

Great tit as a bioindcator of mercury contamination from the paper and pulp industry

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Abstract

Passerine species have been increasingly used as bioindicators of metal bioaccumulation especially by taking benefit of non-destructive indicators of bird exposure, such as collecting feathers, faeces or blood. Starting in 2003 mercury concentrations were determined in the feathers, faeces and blood of nestling great tits (*Parus major*) in industrial (a paper mill) and rural sites in maritime pine forests on the west coast of Portugal. The aim of this study was to compare the level of mercury between the areas and also between sampling years. Breeding biology was also monitored in order to detect contamination effects on breeding performance and health status of Great tits.

Feathers showed a significantly higher concentration of mercury in the industrial area in all study years, but it had significantly lower levels in the year 2010, showing a significantly annual decrease of contamination levels. Blood analyses presented a significantly annual decrease but no difference between areas, and faeces analyses provided no difference between years or areas. We found no direct influence of pollution in Great tit nestling's health status or Great tit's breeding performance.

Key words: Mercury pollution; nestling's Great tit; breeding performance.

1. Introduction

Metals are commonly present in the environment because of natural occurrence and/or human activities, such as industries or agriculture. Metal pollution represents a threat to ecosystems and is responsible for numerous pathologies or impaired survival in wild species [1].

Insectivorous Great tits, *Parus major*, have been successfully used in biomonitoring studies [2,3,4,5] and are potentially good biomonitors for heavy metal because they are ubiquitous and abundant [6,7], and sometimes the only forest passerine species available in reasonable densities in polluted areas. They readily nest in manmade nest boxes and so breeding populations can easily be monitored. Also, nestlings are very easy to manipulate and very insensitive for human disturbances.

Non-invasive procedures, such as collecting feathers and faeces, have been effectively used in studies focusing on heavy metal pollution [4,6,7,8]. Birds excrete elements into growing feathers [9] and can also excrete metals through excrements or by depositing them in the uropygial gland and salt gland [10]. Females can also excrete metals in their eggs and eggshells [11], and metal levels in eggs reflect the levels in the female during egg formation [12]. However, these procedures have potential problems like external contamination in feathers or the mechanism that regulates excretion [8]. Blood sampling, although a more intrusive method, has also been widely used [13,14,15] and it is considered to have no major long-term adverse effects on wild adult or developing birds [16].

Different sampling type is important because it covers several different conditions since feathers reflect the amount of metals present in the blood at the time of feather growth, either from current dietary sources or from mobilization of metals from internal organs [9], faeces represent the unabsorbed remnants of multiple food items [17,18], and metal levels in blood reflect the input of metals through immediate (less than 1 week) dietary intake [19].

We followed the breeding success of *P. major* in an industrial environment around a pulp and paper mill and in a rural control environment in a coastal region of Portugal during 9 years. In several years we measured the concentrations of mercury in feathers (2003, 2009, 2010), faeces (2008, 2009, 2010) and blood (2010, 2011) of nestling *P. major*.

The objective of this study was to compare mercury levels between the two study areas, industrial and rural, and between study years. We were also interested in assessing if contamination levels produce an effect on breeding performance and health status of Great tits.

2. Material and methods

2.1 Study area

The present work is part of a long-term monitoring study on the effects of air pollution on wild birds, which has been on-going since 2003 [20]. The study has been carried out at multiple study plots in two maritime pine (*Pinus pinaster*) forest areas located in Figueira da Foz (Portugal). One area is located in the National Pine Forest of Quiaios (MQ), a 6,000 ha forested area bordered by agriculture fields without a direct influence of industrial pollution, included in the Natura 2000 site "Dunas de Mira" (40º14'N, 8º47'W). The second area is located in the National Pine Forest of Urso (MU), a 9,000 ha forested area sited less than 1km to the south of a paper and pulp mill industrial complex (40º02'N, 8º52'W). The mill produces bleached kraft pulp using an elemental chlorine-free method and reported air emissions include carbon monoxide, sulfur oxide, hydrogen sulphide, nitrogen oxides and PM10 particulates – all values below the legal limit [21]. In 2008 and 2009 the mill went through a process of development with increased production capacity and also technology improvement. MU is approximately 20 km to the south of MQ and both areas share the same altitude (±50 m asl) and average temperature, both presenting a sandy soil and including 70 – 80 year-old pine plantations dominated by *Pinus pinaster* interspersed by some *Pinus pinea* patches. Pine density in MU varied between 675 to 1300 individuals per ha (average 1000 individuals per ha) and pine tree DBH between 15 and 26 cm (average 19 cm). Pine density in MQ varied between 600 to 1250 individuals per ha (average 875 individuals per ha) and pine tree DBH between 22 and 41 cm (average 32 cm). The shrub layer is dominated by *Myrica faya*, *Halimium halimifolium*, *Cytisus scoparius*, *Ulex* spp, *Cistus* spp and *Acacia* spp. Three homogeneous even aged plots (trees aged 70–80 years) were selected in each study area. Plots were within 2 km of each other. The wooden nest boxes, with a 2.8 cm entrance and cavity measurements of 20 x 15 x 15 cm (height, width, length), were placed at an average density of 9/ha, at equal distances from each other, resulting in 20 to 50 nest boxes per plot.

2.2 Breeding parameters

From 2003 to 2011 the breeding parameters of *P. major* were monitored in both areas, between February and July. The nest boxes were checked at least once a week in order to gather information on different breeding parameters (laying date, clutch size, number of fledglings). On day 15 (±1) post-hatching all nestlings were measured for their body mass (g) using a spring scale and marked with an individually numbered aluminium ring.

2.3 Feather, faeces, blood and soil sampling

In summer of 2008, 2009 and 2010 we collected fresh faeces from defecating nestlings at the age of 15 (±1) days. Nestlings were induced to defecate upon handling and excrements were immediately collected in metal-free plastic containers, samples were air dried before the element analysis. In total, 85 (MQ=55, MU=30) nestlings from 39 (MQ=25, MU=14) nests were sampled.

In 2003, 2009 and 2010 the two outermost tail feathers were collected from nestlings at the age of 15 (±1) days. Feathers were stored in sterile, metal-free plastic Eppendorf tubes and maintained at -20 ºC until analysis. In total, 136 (MQ=77, MU=59) nestlings from 62 (MQ=38, MU=24) nests were sampled.

In the summer of 2010 and 2011 we also collected blood samples from the brachial vein of tits nestlings and the blood was transferred into a heparinized microhaematocrit capillary and frozen at -25ºC until analysis for metal concentrations. In total, 58 (MQ=44, MU=14) nestlings from 44 (MQ=33, MU=11) nests were sampled.

In 2010 the blood was also used to make thin film smears to measure haematological parameters (MQ=30, MU=5). Smears were air-dried, fixed in absolute methanol, and stained with azure–eosin. The proportion of different types of leukocytes was assessed by examining 100 leucocytes under 1000× magnification. Estimates of the total white blood cell count (WBC) were obtained by counting the number of leukocytes per approximately 10,000 erythrocytes. Differential leukocyte counts were obtained by multiplying their proportions with WBC [22]. In 2011 the capillary tubes were centrifuged at 6 000 rpm for 15 min and haematocrits were estimated immediately after centrifugation (MQ=46, MU=14). The haematocrit was measured as the part of the capillary tube occupied by erythrocytes and was expressed as a percentage of whole-blood volume [23, 24].

In 2010 soil samples were also collected in MQ (n=15 samples) and MU (n=15) to assess variation in Hg soil pollution.

2.4 Metal analyses

Prior to analysis, feathers were washed vigorously in deionized water alternated with 1 mol/l acetone to remove any external contamination, and dried at room temperature for 48h. Feather and blood samples were weighed and faeces and soil samples were dried in a oven at 50˚C for 72 h and weighed. Feather, faeces and soil samples were then digested in Teflon bombs by adding 2 ml of Supra-pure HNO_3 and 0.5 ml of H_2O_2 and placed in a microwave system (Anton Paar, Multiwave 3000, Graz, Austria). Blood samples were digested by adding of 4 ml of Supra-pure HNO_3 and 0.6 ml of H_2O_2. After digestion, samples were then diluted to 50 ml with deionized water (Elgastat Maxima). Mercury was determined with ICP-MS (Elan 6100 DRC, PerkinElmer-Sciex, Boston, USA). The calibration of the instrument was done with a certified solution (Ultra Scientific, multielement solution IMS-102, N. Kingstown, RI, USA) from LGC Promochem. Blanks and certified reference materials (mussel tissue ERM-CE278; skim milk powder BCR-063R) were used for method validation. Recovered concentrations of the certified samples were within 10% of the certified values, which is an acceptable margin. Mercury concentrations were expressed as mg/kg in blood.

2.5 Statistics

Statistical analyses were performed with SAS statistical software 9.2 [25]. Differences in mercury concentration between areas (industrial versus rural) and year were examined by using general linear models (procedure Glimmix in SAS, followed by Tukey-Kramer post-hoc test) where area, year and area × year were used as independent factors and nest was included as a random factor in the models.

The effect of area and year on breeding and condition parameters (clutch size, fledgling number and laying date) were examined by using general linear models (procedure Glimmix in SAS, followed by Tukey-Kramer post-hoc test) where area, year and area × year were used as independent factors. Second breeding attempts, as well as replacement, predated and deserted clutches were excluded from the data.

Health status indices (WBC, H/L, haematocrit) were compared between areas using generalized linear models (Glimmix procedure in SAS) where nest was included as a random factor.

For all analyses, after checking for normality of distributions, values were $\log_{10}$-transformed to normalize distributions and back-transformed for presentation in the tables. The significance level was set at $P<0.05$.

3. Results

3.1 Metal concentrations in nestling blood, feathers, faeces and soil

Feathers collected from the industrial area (MU) presented a significantly higher concentration of mercury than the rural area (MQ) and also a significant variation among years, with lower values in 2010 compared to 2003 (table 1). There was also a significant interaction between area and year, especially due to the high values of 2003 and the low values of 2010.

Mercury values in faeces samples were slightly higher in MU, however they did not present any significant variations among areas or years (table 1).

Mercury values in blood samples did not presented significant variation among areas, but presented a significant variation among years, with lower values in 2011 compared to 2010 (table 1).

All 15 soil samples collected from MU presented values of mercury below detection limit. In MQ from the 15 samples only 2 were above the limit of detection, so it was impossible to make any statistical comparison.

3.2 Breeding success and nestling condition

Laying date was significantly different between areas (fig.1), with Great tit starting egg laying on average 11 days earlier in the industrial area. There was a significant variation in timing among years and also an interaction effect between area and year on the laying date (Table 2). The interaction was due to the fact that in 2006, 2007, 2008 and 2010 the date of breeding onset was similar in both areas, while in the remaining years breeding onset occurred earlier in MU than in MQ (Tukey-Kramer post-hoc comparison, $p<0.05$).

Average clutch size was significantly larger in MU than in MQ (fig.1), and no significant differences were detected in average clutch sizes among years (Table 2).

Average fledgling number was significantly larger in MU than in MQ (fig.1). There was also a significant variation between sampling years (Table 2).

There were no significant differences among estimates of the total white blood cell count, H/L ratio and haematocrit level (PCV) for both study areas (see table 3).

4. Discussion

In agreement with our previous study [26] we found a significantly higher concentration of mercury in the industrial area MU in feathers of Great tit nestlings. However, faeces and blood samples did not follow the same pattern, since no significant differences were found between study areas. Mercury is known to easily attach to feather keratin thus making excretion through feathers the probable main mercury excretion pathway in birds. In an experimental study made on seabirds by Lewis and Furness [27] 49% of the administered mercury was accumulated in the feathers of chicks regardless of the administered dose. Since the levels found in our study areas are low, the significantly higher concentration of mercury in feathers of Great tit nestlings from the industrial area MU corroborates the postulated role of feathers as the main mercury excretion pathway in birds.

Although the pulp and paper mill does not report any release of mercury, paper and board mills can release low concentrations of heavy metals originated mainly from energy generation (steam and electricity). Also mercury can be released during the process of incineration of different types of RCF residues (Recovered Cellulose Fiber) [28].

Although we were able to record significantly lower Hg concentrations in 2010 and 2011 particularly in nestlings' blood samples, the cause for this the decrease is difficult to pinpoint. In fact, levels in MQ may vary according to the undetermined agriculture-related chemicals used in the fields bordering the study area. In turn, in 2008 and 2009 the paper mill located in MU suffered some production technology improvements, which probably led to cleaner technology. The very low levels of mercury in the soil in 2010 apparently confirm the low mercury contamination in the study area, although soil characteristics (sandy highly permeable) must be taken into consideration since most substances may be leached away from the upper substrate levels.

Despite the decrease in mercury values in the last two study years there were no major differences in breeding parameters, thus corroborating the overall low levels of mercury contamination in the study areas. Although the values found in the present study were within the ranges previously reported for mercury levels in feathers and excrements of Great tit inhabiting metal contaminated sites [7, 31] where impaired breeding success was recorded, our study verifies just the opposite. In fact, birds breeding in the industrial area MU had better breeding performance than those breeding in the rural (control) area MQ. Moreover, the low variability of clutch size and number of fledglings throughout the years illustrates the irrelevance of mercury levels in the study areas.

Even though nestlings are considered to be more sensitive to pollution than adults [32, 33], nestling health indicators were similar in both study areas, revealing no detrimental effects of pulp mill pollution on nestlings' physiology.

The remaining question is why do Great tits present a better breeding performance (more eggs and more fledglings) in the industrial area. In a study made by Costa et al. [34] in the same study areas, caterpillar availability and the overall average abundance of frass-fall biomass was two times higher in MU than in MQ. This was the only explanation found for the better performance of tits from the industrial area MU. Regardless of the higher values of mercury in MU, food availability seems to be the major force driving breeding performance.

Acknowledgements

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Table 1 - Two-way ANOVA showing the effects of area (industrial vs. rural) and year (2003 – 2011) on the levels of Hg on feathers, faeces and blood samples of Great tit.

	MQ	MU	d.f.	F	p
Feathers					
Area	0.37±0.39	0.55±0.37	1,127	17.6	<0.0001
Year			2,127	21.6	<0.0001
Area * year			2,127	9.84	0.0001
Faeces					
Area	0.26±0.16	0.36±0.29	1,74	3.72	0.06
Year			2,74	2.97	0.06
Area * year			2,74	0.11	0.89
Blood					
Area	0.15±0.09	0.12±0.05	1,51	1.39	0.24
Year			1,51	6.62	0.01
Area * year			1,51	1.63	0.21

Table 2 - Two-way ANOVA showing the effects of area (industrial vs. rural) and year (2003 – 2011) on laying date, clutch size and number of fledglings of Great tit.

	Laying date			Clutch size		Number of fledglings	
	d.f.	F	p	F	p	F	p
Area	1, 197	46.7	<0.0001	40.8	<0.0001	25.7	<0.0001
Year	8, 197	3.63	0.0006	0.63	0.75	2.16	0.03
Area * year	8, 197	2.38	0.02	0.82	0.58	0.67	0.72

Table 3 - Average ±SD values for White blood cell counts, H/L ratios and haematocrits (PCV) obtained from the bood samples collected from tits nestling in MQ and MU. ANOVA showing the effects of area (industrial vs. rural) on nestlings' blood parameters from both areas.

	MQ	MU	d.f.	F	p
WBC	2.54±1.59	2.54±2.51	1,32	0.00	0.98
H/L ratio	0.24±0.28	018±0.11	1,25	0.01	0.98
PCV	48.35±6.30	50.01±5.34	1,57	1.61	0.21

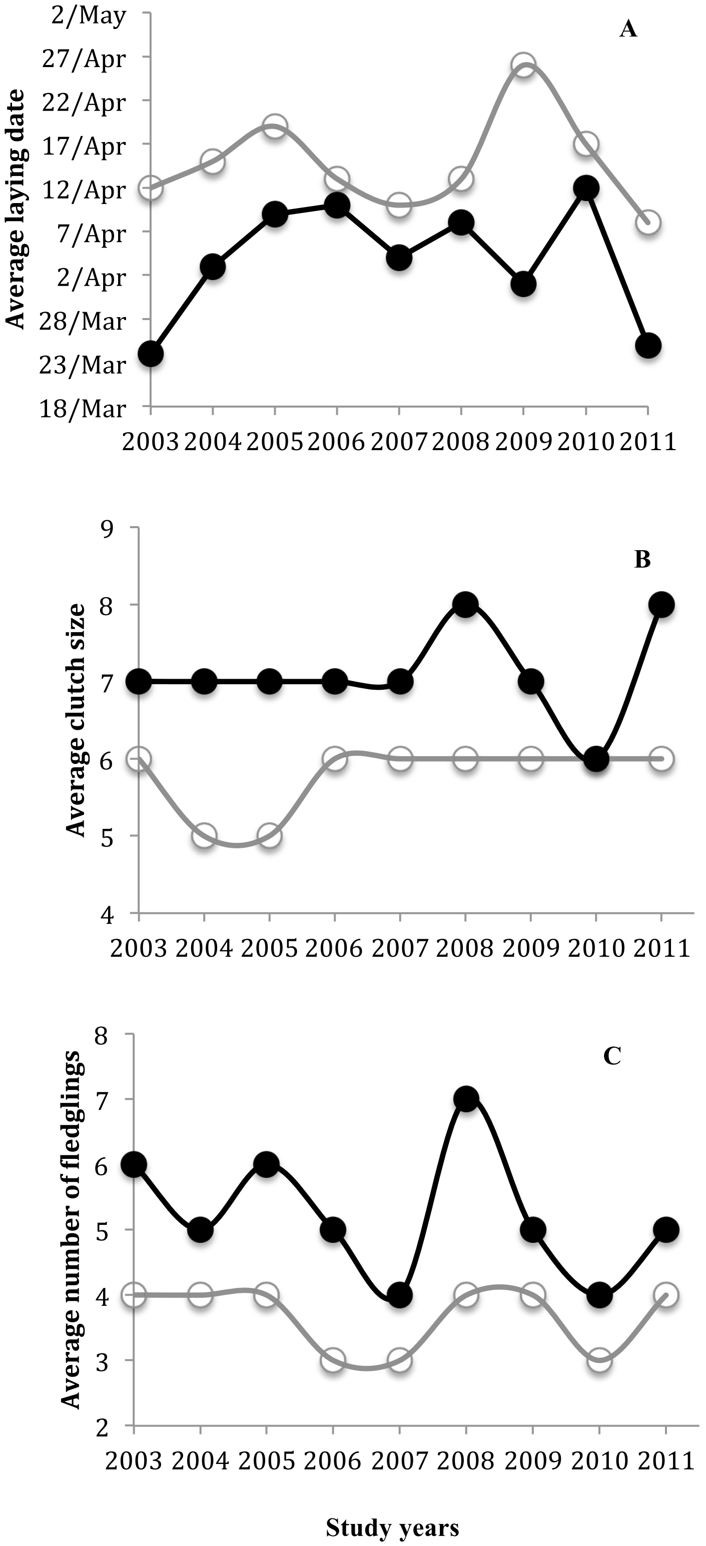

Fig.1- Annual average laying date of the first egg (A), clutch size (B) and number of fledglings (C) of Great tit during the study years in industrial - MU (grey circles) and rural - MQ (empty circles) area.