Limited evidence for a negative effect of heavy metal pollution on the egg characteristics and reproductive success of great tits (*Parus major*)

Piia Pajunen

Master's Thesis

University of Turku
Department of Biology
26.4.2018

Track: Ecology
Degree programme: Ecology and Evolutionary Biology

Credits: 40 ECTS

Examiners:

1:

2:

Approved:

Grade:

The originality of this thesis has been checked in accordance with the University of Turku quality assurance system using the Turnitin OriginalityCheck service.

TURUN YLIOPISTO

Biologian laitos
Luonnontieteiden ja tekniikan tiedekunta

PAJUNEN, PIIA: Limited evidence for a negative effect of heavy metal pollution on the egg characteristics and reproductive success of great tits (*Parus major*)

Pro gradu -tutkielma, 45 s.
Ekologia
Huhtikuu 2018

Raskasmetalleilla tiedetään olevan haitallisia vaikutuksia lintujen pesimämenestykseen, munan laatuun ja poikasten kehitykseen. Tämän tutkimuksen tarkoituksena oli selvittää, vaikuttavatko raskasmetallit talitiaisten (*Parus major*) munien painoon, kuoren paksuuteen ja painoon, immuunipuolustukseen (lysotsyymi-entsyymi), kilpirauhashormonien (trijodityroniini ja tyroksiini) pitoisuuksiin, pigmentin voimakkuuteen sekä pesinnän onnistumistodennäköisyyteen neljällä saastuneella alueella. Lysotsyymi ja kilpirauhashormonit ovat tärkeitä lintujen poikasten kehitykselle, eikä raskasmetallien vaikutuksesta niiden pitoisuuteen munissa ole aiempaa tietoa.

Aineisto kerättiin keväällä 2016 Suomesta, Belgiasta, Unkarista ja Portugalista. Kustakin maasta kerättiin talitiaisten munia saastuneelta alueelta päästölähteen läheisyydestä ja kauempaa päästölähteestä (puhdas alue). Munien osat eroteltiin ja kilpirauhashormoni- ja lysotsyymipitoisuudet analysoitiin laboratoriossa. Raskasmetallipitoisuudet (As, Cd, Cu, Ni, Pb) analysoitiin poikasten ulostenäytteistä. Pigmenttitäplien koko, sävyn voimakkuus ja jakautuminen arvioitiin visuaalisesti. Tutkimuksessa selvitettiin, vaikuttiko alue (saastunut/puhdas) tai raskasmetallien pitoisuus tutkittuihin munan ominaisuuksiin ja pesinnän onnistumiseen.

Alueella tai raskasmetallipitoisuudella ei havaittu olevan yhteyttä munan ominaisuuksiin tai pesinnän onnistumiseen. Korkeimmat raskasmetallipitoisuudet olivat saastuneilla alueilla Suomessa ja Belgiassa. Unkarissa pigmentin jakautuminen oli aggregoituneempaa saastuneella kuin puhtaalla alueella. Kokonaan epäonnistuneiden pesintöjen osuus vaihteli maiden välillä.

Tulokset viittaavat siihen, että raskasmetallipitoisuudet ovat laskeneet alueilla eivätkä vaikuta oleellisesti tutkittuihin ominaisuuksiin. Tuloksista ei voi luotettavasti päätellä raskasmetallien yhteyttä pesintöjen epäonnistumiseen, sillä siihen saattoivat vaikuttaa monet muut tekijät, kuten ravinnon saatavuus ja munan laatu, joita ei otettu huomioon. Alentuneiden raskasmetallipitoisuuksien ja ympäristöolojen ja lisääntymismenestyksen vuosittaisen vaihtelun vuoksi on suositeltavaa, että raskasmetallien yhteyttä etenkin pigmentteihin, kilpirauhashormoneihin ja lysotsyymiin tutkitaan lisää pitkäkestoisilla tutkimuksilla muilla voimakkaasti saastuneilla alueilla.

Asiasanat: *Parus major*, kilpirauhashormonit, lysotsyymi, raskasmetallit, saasteet

UNIVERSITY OF TURKU

Department of Biology
Faculty of Science and Engineering

PAJUNEN, PIIA: Limited evidence for a negative effect of heavy metal pollution on the egg characteristics and reproductive success of great tits (*Parus major*)

Master's thesis, 45 pp
Ecology
April 2018

Heavy metals are known to have negative effects on the reproduction, egg formation and offspring development of birds. This study aimed to investigate whether heavy metals affect egg mass, eggshell mass and thickness, pigment patterns, immune defence (lysozyme enzyme) and thyroid hormones (triiodothyronine and thyroxine) in eggs of great tits (*Parus major*) and fledging probability in four pollutes areas. Lysozyme and thyroid hormones are essential for the development of avian offspring, and there is no previous knowledge of how heavy metals affect their concentration in eggs.

The material was collected in spring 2016 from Finland, Belgium, Hungary and Portugal. Eggs were collected from a polluted zone near a pollution source and further away from it (unpolluted zone) in each country. The eggs were dissected and thyroid hormone and lysozyme concentrations were analysed in a laboratory. Heavy metal (As, Cd, Cu, Ni, Pb) concentrations were analysed from nestlings' faecal samples. The size, intensity and distribution of pigment spots were estimated visually. The effect of zone (polluted/unpolluted) and heavy metals on the egg characteristics and fledging probability were tested with statistical analyses.

No association was found between zone or heavy metals and the egg characteristics or fledging probability. The highest heavy metal concentrations were in the polluted zones of Finland and Belgium. In Hungary, eggshell pigmentation was more aggregated in the polluted compared to the unpolluted zone. The proportion of failed nests varied between countries.

The results indicate that heavy metal pollution has decreased in the study areas and is not an important factor for the studied egg characteristics and fledging probability. It is uncertain to what extent heavy metal pollution contributed to the failure of nests, as many other factors, like food availability and egg quality, may have had a bigger impact. Due to the decreased level of pollution and annual variation in environmental conditions and breeding success, it is recommended that the effects of heavy metals especially on pigment patterns, thyroid hormones and lysozyme are further studied with long-term studies on other heavily polluted areas.

Key words: *Parus major*, thyroid hormones, lysozyme, heavy metals, pollution

Contents

1 INTRODUCTION

1.1 Pollution in the environment and its effects on species

As urbanization and industrialization have expanded in the past centuries, the amount of anthropogenic pollution has become an increasingly important factor altering natural environments (Grimm et al. 2008). The release of chemicals and other toxicants into the environment has detrimental and often long-lasting impacts on individual organisms, as well as on entire ecosystems (Fleeger et al. 2003). The sources of pollution include for example agriculture, industry, transportation and waste treatment (Nagajyoti et al. 2010). Even single point sources, like factories, can have widespread effects as pollutants can be transported long distances in the air or water (Grimm et al. 2008).

One significant group of pollutants are heavy metals, reviewed by Nagajyoti et al. (2010). Heavy metals include arsenic (As), cadmium (Cd), cobalt Co), chromium (Cr), copper (Cu), iron (Fe), lead (Pb), manganese (Mn), molybdenum (Mo), nickel (Ni), silver (Ag), zinc (Zn), the platinum-group metals and some other metals. Heavy metals occur naturally in the environment, but their quantities have increased due to human activities. Some heavy metals are essential for the physiology of organisms in small amounts (Cu, Zn, Fe, Mn, Mo, Ni, Co), and become toxic when their supply exceeds the tolerance of the organism. Heavy metals are typically needed as cofactors of certain enzymes.

Heavy metals are mainly found in the soil and in aquatic ecosystems and to a smaller extent in the atmosphere (Nagajyoti et al. 2010). Anthropogenic heavy metals may occur in their elemental form or as components of compounds. Many factors, such as the form of the metal, soil acidity and temperature, affect the behaviour and availability of heavy metals in the environment. Heavy metals can enter plants through roots and leaves, after which they can move up in the food chain and bioaccumulate in organisms in higher trophic levels.

The effects of pollution on birds were first acknowledged in the 1960's, when DDT-caused (dichlorodiphenyltrichloroethane) eggshell thinning and the subsequent population declines were observed in raptors (Pain et al. 1999). Later it was noticed that birds are sensitive to heavy metals and respond noticeably to changes in heavy metal contamination (Eeva & Lehikoinen 1996). Heavy metals have negative effects on the eggshells (Eeva & Lehikoinen 1995), fledgling development (Janssens et al. 2003b) and reproductive success (Eeva & Lehikoinen 1995, Espin et al. 2016a, Janssens et al. 2003a)

of birds. Therefore, birds may be used as indicators of heavy metal pollution (Eeva & Lehikoinen 1996). Especially small passerine birds, which accumulate metals mostly through their insect diet, are found to be useful in monitoring pollution (Dauwe et al. 2000, Eeva & Lehikoinen 1996). Birds can excrete heavy metals in faeces and feathers, which can be used as non-invasive means to study heavy metal exposure (Dauwe et al. 1999). Metals can also be stored in the body or excreted in eggs (female birds) (Dauwe et al. 1999).

1.2 Maternal effects and pollution

Maternal effects are effects that the mother's phenotype has on the offspring's phenotype (Marshall & Uller 2007, Ruuskanen et al. 2016d). The effects can be both prenatal and postnatal and include genetic and nongenetic effects. The genetic effects are caused by genetic variance among females for instance in the investment in postnatal care (Ruuskanen et al. 2016d). Nongenetic effects can arise from the female's physiological condition and nutritional state (Espin et al. 2016b). An important prenatal effect in birds is the female's allocation of resources to the eggs, including maternal hormones, immune factors and antioxidants (Espin et al. 2016b, Ruuskanen et al. 2016d). The allocation of these resources is possibly adaptive and may affect the fitness of the offspring (Marshall & Uller 2007, Ruuskanen et al. 2016d). However, the production of some resources, such as immune factors, is costly for the female, which may lead to trade-offs between the female and the offspring (Ruuskanen et al. 2016d).

Environmental factors, such as pollution, can affect the female's physiology. For example, heavy metals increase the level of corticosterone (Baos et al. 2006) and reactive oxygen species (Koivula & Eeva 2010) and decrease the concentration of haemoglobin and haematocrit (Geens et al. 2010). Heavy metal pollution can also alter food availability and thereby affect the nutritional state of the female (Espin et al. 2016b). Changes in the female's physiology affect the amount of resources it can allocate into eggs, so the effects of pollution are transferred to the eggs through maternal effects (Espin et al. 2016b). The amount of resources in the eggs may also affect the nestlings by effects on growth and fledging success (Espin et al. 2016b).

1.3 Egg resources and eggshell traits

Avian eggs contain essential nutrients for the growth of the embryo, as well as several other components. Steroid and thyroid hormones, antioxidants and immune factors are some of the resources found in eggs (Espin et al. 2016b, Ruuskanen et al. 2016d). Eggshell traits then again include for example eggshell thickness and pigment patterns (distribution, intensity, spot size). The egg components studied in this study include thyroid hormones, lysozyme enzyme, eggshell thickness and pigment patterns (discussed in detail below).

1.3.1 Thyroid hormones and their functions in birds

Thyroid hormones (THs) are amine hormones found in all vertebrate taxa. They include several hormones, of which L-thyroxine (T4) and 3,5,3′-triiodothyronine (T3) are among the most important. T3 is the biologically active form of the hormone, whereas T4 functions as a prohormone (McNabb 2007). The production and release of THs is regulated by the hypothalamic-pituitary-thyroid (HPT) axis, described in detail by McNabb (2007). THs are produced in the thyroid glands by iodination of thyroglobulin. The release of THs from the thyroid glands is controlled by the hypothalamus, which releases thyrotropin-releasing hormone (TRH). TRH stimulates the anterior pituitary to produce and release thyrotropin (TSH), which then stimulates the thyroid gland to release thyroid hormones.

Most of the stored THs in the thyroid glands is T4, which comprises about 97 % of the thyroidal hormone content in birds (McNabb 2007). T3 is mostly formed outside the thyroid glands from T4 by deiodinase enzymes. In line with the active role of T3, thyroid receptors in tissues have a high affinity for T3 and a much lower affinity for T4. The binding of T3 to thyroid receptors initiates the transcription of specific genes, leading to the hormone-mediated responses of T3, for example in the differentiation of tissues during development.

The main effects of THs in vertebrates are related to metabolism, thermogenesis and development. THs are known to increase basal metabolic rate in a variety of taxa, such as rodents (Li et al. 2010) and humans (Kim 2008). During development, THs have some crucial effects on growth and differentiation of certain tissue types. THs are especially important for normal development of the nervous system, for example in the brain development of rats and humans (Howdeshell 2002).

Although much of the knowledge of THs is based on studies on non-avian vertebrates, it has recently been discovered that THs are important in birds as well and have similar effects as in mammals. In fact, the function of the HPT axis is very similar in birds as in mammals and avian thyroid receptors are nearly identical to those of mammals' (McNabb 2006).

As in mammals, THs are essential for growth, tissue differentiation, metabolism and thermoregulation in birds (Chastel et al. 2003, McNabb 2007). THs are especially important for the development of neural, skeletal and muscle tissues (McNabb 2007). The effects on growth are based on interactions between the HPT axis and the growth axis: the HPT axis regulates the release of growth hormone, which in turn has some effects on T3 release (McNabb 2007). A normal function of the HPT axis is required for growth, and both hypo- and hyperthyroidism have been found to decrease growth (McNabb 2007).

In addition, THs have important effects on reproduction in birds. They regulate the onset of puberty, gonadal maturation and reproductive activity in female birds (McNabb 2007). Experimental inhibition of THs decreases egg laying, egg weight, shell thickness and ovarian weight in poultry (McNabb 2007). THs also affect the seasonality of reproduction by reacting to day length: the level of THs declines in the early reproductive season when day length is increasing, and rises at the end of the season, when the bird becomes refractory to long days and reproduction ends (McNabb 2007).

The development of the HPT axis differs fundamentally between different groups of birds, reviewed by McNabb (2006). Precocial birds are well developed at hatching and have their own thermoregulation. The function of the HPT axis and the production of THs start before hatching. There is a perihatch peak in TH levels and THs stimulate hatching (McNabb 2007). After hatching, the level of THs decreases to the level of adult birds (McNabb 2006). In altricial birds, then again, the HPT axis starts to function only after hatching and the offspring depend on their mother's heat production and care for about the first two weeks (McNabb 2006). The level of THs in altricial birds increases steadily after hatching and there is no perihatch peak (McNabb 2006). The length of the HPT axis development differs between altricial species, but generally TH levels are increasing during the first 2−3 weeks before thyroid function is fully developed (McNabb 2006).

Most studies on birds have been conducted on precocial birds, such as galliformes, and have focused on TH concentration in the plasma of embryos, nestlings or adult birds

(McNabb 2006). However, avian eggs also contain maternal THs, which are transferred from the mother into the yolk (Ruuskanen et al. 2016b). Egg THs are less studied than plasma THs, and their function is especially poorly known in altricial birds. However, two recent studies have investigated the effects of experimentally increased egg TH (T3 & T4) concentration within the physiological range in two altricial bird species. In the rock pigeon (*Columba livia*), increased THs were found to improve hatching success (Hsu et al. 2017). In the great tit (*Parus major*), elevated egg THs affected offspring growth in a sex-specific way: increased TH level induced growth in male chicks but decreased it in female chicks (Ruuskanen et al. 2016b).

The results indicate that maternal THs in the eggs are important for altricial birds, even though the offspring's own TH production starts only after hatching (Ruuskanen et al. 2016b). Moreover, another recent study has reported natural variation in egg TH levels within clutches of great tits (Ruuskanen et al. 2016a). The level of T4 increased over the laying sequence, which may be a way to adapt offspring phenotype (Ruuskanen et al. 2016a). However, the mechanisms by which the female might adjust the amount of THs it allocates into each egg are unknown, and the increase of T4 along laying sequence could merely reflect the TH level in the mother's circulation (Ruuskanen et al. 2016a).

Thyroid hormone function may be disrupted by anthropogenic effects, such as pollution. Indications of pollution's detrimental effect on THs date back to the 1980's, when thyroid gland hypertrophy was observed in herring gulls (*Larus argentatus*) from the Great Lakes, where the level of PHAHs (polyhalogenated aromatic hydrocarbons), such as PCBs (polychlorinated biphenyls), were high (McNabb & Fox 2003). Since then, more than a hundred synthetic chemicals that interfere with different aspects of TH function have been identified (Howdeshell 2002). The effect of these chemicals on THs is negative, i.e. they reduce the amount of circulating THs or otherwise disrupt TH function (Howdeshell 2002).

Apart from synthetic chemicals, also naturally occurring heavy metals may interfere with THs, when their concentrations are high due to anthropogenic contamination. For example, in tree swallow (*Tachycineta bicolor*) nestlings, plasma T3 and T4 levels were lower in mercury (Hg) contaminated sites than in control sites, suggesting that mercury may disrupt TH function (Wada et al. 2009). Studies on other vertebrates support the idea of heavy-metal-induced TH disruption: in mice, cadmium (Cd) decreases the concentrations of T3 and T4 in blood serum (Gupta & Kar 1998) and in zebrafish, lead (Pb) has the same effect in larval tissues (Miao et al. 2015).

Although the above examples suggest a negative impact of heavy metals' on circulating THs, information about the effects of metals on egg THs is still lacking. Considering the newly-discovered functions of egg THs (Hsu et al. 2017, Ruuskanen et al. 2016b), it is important to understand how the possible effects of heavy metals on the mother's circulating THs are manifested in the TH concentration in eggs.

1.3.2 Egg lysozyme

Lysozymes are a family of enzymes that are a part of antibacterial immune defence in microbes, plants and animals (Masschalck & Michiels 2003). Lysozyme breaks down peptidoglycan, which is the main component of bacterial cell walls, and causes cell lysis. Gram-positive bacteria have multiple layers of peptidoglycan in their cells walls, whereas gram-negative bacteria usually have only one layer of peptidoglycan, on top of which there is an outer membrane. The outer membrane protects gram-negative bacteria from lysozyme, and gram-positive bacteria that lack it are more sensitive to lysozyme.

Lysozyme is an important part of the immune defence in birds' eggs (Saino et al. 2002). It is transferred from the female to the albumen of developing eggs, where it forms about 3.5 % of the total protein content (Javůrková et al. 2015). Lysozyme enhances both physical and chemical defence mechanisms in the egg and likely has an important role in the early immune system (Saino et al. 2002). Lysozyme may also be transferred from the albumen to the yolk and into the developing embryo (Saino et al. 2002). In accordance with this, a significant positive correlation of plasma lysozyme concentration between nestlings and the mother, as well as a correlation between albumen and nestling plasma lysozyme, has been observed in barn swallows (*Hirundo rustica*) in the first few days after hatching (Saino et al. 2002).

However, contradicting results also exist: several studies have not been able to find lysozyme in embryonic tissues, nor a correlation between albumen and nestling plasma lysozyme concentration (Javůrková et al. 2015). The observed correlation between albumen and nestling plasma lysozyme in some studies has been proposed to occur because hatchlings swallow the albumen, including its lysozyme, not because lysozyme is transferred from the albumen to the embryo (D'Alba et al. 2010). Nevertheless, it is agreed that lysozyme most likely forms an important part of the offspring's innate immune system, as they do not produce their own antibodies at birth and depend on maternal immune factors (Chatelain et al. 2016). In fact, lysozyme concentration is relatively high in young nestlings and decreases rapidly during the nestling phase as

maternal lysozyme is either metabolised or diluted in the increasing blood volume, further highlighting the role of maternal lysozyme in the very early stages of life (Killpack et al. 2013).

Apart from the immune system, egg lysozyme may have some important effects on offspring phenotype. Lysozyme seems to have some growth suppressing effects in nestlings, as lysozyme concentration has been found to correlate negatively with tarsus length (Javůrková et al. 2015, Killpack et al. 2013). Lysozyme concentration in the mother, then again, might affect the hatchability of eggs. However, the related literature is limited and somewhat equivocal. Some studies have found a positive correlation between lysozyme concentration in the female's plasma and hatching success (Saino et al. 2002), while some have not (Javůrková et al. 2015).

One interesting issue is whether egg lysozyme can be used to adapt offspring phenotype to the environment. As lysozyme likely has important effects on growth and innate immune system, females might utilize adaptive allocation of lysozyme to modify offspring phenotype. Lysozyme is likely a costly protein for the female, as the level of lysozyme decreases along the reproductive season (Bonisoli-Alquati et al. 2010) and is negatively correlated with clutch size (Saino et al. 2002). Hence, females might be limited in the amount of lysozyme they can produce. It is also known that the lysozyme concentration of eggs has both an environmental and an inheritable basis, which makes the question of possible adaptive causes of variation in lysozyme concentration relevant (Cucco et al. 2006).

Possible adaptive allocation within clutches has been observed in relation to laying order (Saino et al. 2002). Lysozyme concentration decreased with laying order in barn swallows, which was suggested to indicate a higher investment of lysozyme in earlier-laid eggs that are more exposed to microbes, because they are not incubated immediately after laying (Saino et al. 2002). However, this idea has not been supported by other studies (D'Alba et al. 2010, Bonisoli-Alquati et al. 2010). Lysozyme could also be allocated for example in relation to male-attractiveness (D'Alba et al. 2010) or predation risk (Morosinotto et al. 2013). Yet, it is not known whether differential allocation of lysozyme affects offspring fitness (D'Alba et al. 2010). It is also unclear whether females can regulate the transport of lysozyme to the eggs in the first place, or if it is based only on diffusion (D'Alba et al. 2010).

Recently, it has been recognized that heavy metal pollution may affect immune factors, like lysozyme. However, the effects are complex and not well known. Some heavy metals (e.g. Pb, Cd) are immunosuppressive and expected to reduce the level of immune factors, while some heavy metals (e.g. Zn, Cu, Fe) are necessary for the immune system and may increase the level of immune factors (Chatelain et al. 2016). An experimental study on feral pigeons (*Columba livia*) has shown this to hold true for at least lead (Pb) and zinc (Zn) (Chatelain et al. 2016). Females that were exposed to lead, laid eggs with reduced transfer of specific antibodies (anti-KLH antibodies), whereas females exposed to zinc laid eggs with higher levels of lysozyme (Chatelain et al. 2016). In addition, a high level of lead in pigeon feathers has been observed to correlate positively with the abundance of blood pathogens, while zinc had the opposite pattern (Gasparini et al. 2014). These studies demonstrate the need to study the relationship of pollution and immune factors to understand the effects that pollution may have on the immune system of natural populations living in polluted areas.

1.3.3 Structure and thickness of eggshells

Eggshells have multiple important functions: they provide protection from the environment, prevent infections, control gas exchange and so water loss from the egg, and provide calcium for the skeletal mineralization of the embryo (Reynolds et al. 2004). Therefore, sufficient eggshell thickness is vital for normal development of the embryo. The negative effects of eggshell thinning on reproduction have been known since 1960's, when DDT-caused eggshell thinning was found to decrease reproductive success in raptors (Pain et al. 1999). Also heavy metal pollution is known to affect shell thickness and reproduction negatively in birds (Eeva & Lehikoinen 1995).

A major component of the eggshell is calcium, which comprises 98 % of the dry mass of the eggshell (Reynolds et al. 2004). Passerine birds get their calcium from their diet, and because they cannot store calcium efficiently, a daily intake of calcium during reproduction is required for the formation of eggshells (Espin et al. 2016a). The primary source of calcium for great tits is land snail shells (Graveland et al. 1994). Calcium deficiency due to a decline in land snail abundance has been proven to cause eggshell thinning and reduced reproductive success in the great tit (Graveland et al. 1994).

Calcium deficiency may also enhance the effects of heavy metals, as calcium and heavy metals use the same binding proteins (Dauwe et al. 2006). When calcium is low, heavy

metals bind to the calcium-binding proteins and their absorbtion increases (Dauwe et al. 2006). Calcium availability therefore affects the responses to heavy metal pollution. This may be seen as different responses of bird species that vary in their ability to find calcium-rich foods. Studies on great tits and pied flycatchers breeding near a copper smelter have revealed that eggshell thickness and reproductive success of pied flycatchers have decreased considerably more than in great tits, which is likely because great tits obtain more calcium from their diet (Eeva & Lehikoinen 2004).

1.3.4 Function of eggshell pigments

Eggshell pigmentation is a common characteristic of all passerine birds (Gosler et al. 2005). Pigmented eggs are found in certain other avian orders as well, such as Falconiformes and Galliformes, but most non-passerine birds have white, unpigmented eggs (Jagannath et al. 2008). The pigments are based on either biliverdin (blueish pigment) or protoporphyrin (reddish pigment). Biliverdin usually forms an even ground colour on the egg, while protoporphyrin creates a speckled pattern (Jagannath et al. 2008). In the great tit, the pigment is based on protoporphyrin, which is distributed in spots of varying size, intensity and abundance (Gosler et al. 2005). Often, there is a ring of intense pigmentation in the broad end of the egg, called the corona ring (Gosler et al. 2005).

Multiple hypotheses have been presented for the function of eggshell pigments, reviewed by Gosler et al. (2005). Pigments may be used in camouflaging the eggs from predators, mimicking the host's eggs in brood parasitism or in signalling female condition. Although some evidence for these hypotheses exists for non-passerine species, they do not account for the pigment patterns in passerine birds. The great tit usually covers its eggs, which contradicts the hypotheses of a signalling or camouflaging function. In addition, the great tit is not parasitized by the European cuckoo (*Cuculus canorus*), making the brood parasitism hypothesis unsuitable for the species.

A more likely hypothesis for the function of protoporphyrin is linked to shell structure (Gosler et al. 2005). Pigment patterns have been found to correlate with shell thickness in the great tit, thin areas being the most pigmented and the intensity of the spots increasing with decreasing thickness. This has been suggested to indicate that pigments are used in strengthening the thin areas of the eggshell. Therefore, factors that are linked to shell thickness may also correlate with pigmentation. In fact, calcium availability in the breeding area, which positively affects shell thickness, correlated negatively with

pigment spotting pattern. In areas with low calcium availability, shells were thinner than in other areas and had more intensively pigmented eggs.

In addition, certain pollutants that cause eggshell-thinning may have similar effects on pigment patterns. In the Eurasian sparrowhawk (*Accipiter nisus*), eggs that had reduced shell thickness due to DDE (dichlorodiphenyldichloroethylene) contamination, had more protoporphyrin, which suggests that protoporphyrin might compensate for the thinning effect of DDE (Jagannath et al. 2008). As heavy metals can also reduce shell thickness (Eeva & Lehikoinen 1995), they could be predicted to increase the amount of protoporphyrin. A study on a natural population of great tits found that copper was positively associated with pigment aggregation, but no other differences in eggshell spotting pattern were observed (Hargitai et al. 2016a). Aggregation was suggested to occur, because pigments are used to strengthen the broad end of the egg (Hargitai et al. 2016a).

1.4 Study questions and hypotheses

This study aimed to find out whether heavy metal pollution affects certain egg characteristics (TH and lysozyme concentration, pigment patterns, shell thickness, shell dry weight and egg mass) and fledging probability in great tits in polluted areas in Finland, Belgium, Hungary and Portugal. These study areas were chosen for this study, because they have been used in similar studies previously and effects of heavy metals on eggs and fledglings have been reported in all but the study area in Portugal (Finland; Eeva et al. 2009, Belgium; Janssens et al. 2003b Hungary; Hargitai et al. 2016a, Portugal; Costa et al. 2011a). Using several study areas offers the possibility to study the issue of heavy metals' effects on birds and their eggs in a broad scale. The hypothesis is that the level of heavy metal pollution affects the studied parameters, which can be seen as differences in the parameters between polluted and reference areas.

TH concentration and eggshell thickness are expected to be affected negatively by heavy metal pollution, based on previous literature (Eeva & Lehikoinen 1995, Wada et al. 2009). Fledgling number is also expected to decrease with pollution (Eeva & Lehikoinen 1995, Espin et al. 2016a, Janssens et al. 2003a). Pigment intensity, aggregation and spot size are expected to increase with pollution, as pigments are possibly used to compensate for the thinning effect of heavy metals (Gosler et al. 2005, Hargitai et al. 2016a). No single

hypothesis can be made for the concentration of lysozyme, as different heavy metals are expected to have opposite effects on lysozyme (Chatelain et al. 2016).

2 MATERIALS & METHODS

2.1 Study material

The study material consists of in total 150 great tit eggs, of which 49 are from Belgium, 33 from Finland, 32 from Portugal and 36 from Hungary. About half of the eggs in each country are from the polluted zone and half from the reference zone (Belgium: 25+24; Finland: 17+16; Portugal: 17+15; Hungary: 15+21 polluted zone eggs and reference zone eggs).

2.2 Study species

The great tit is a common passerine bird in Europe, parts of Asia and North Africa (Drent et al. 2003). The great tit lives in many kinds of habitats, but it favours deciduous forests over coniferous (Van Balen 1973). The breeding densities of great tits are significantly higher in deciduous forests (Van Balen 1973). The difference is likely caused by a higher amount of caterpillars in deciduous forests compared to coniferous forests (Van Balen 1973).

Great tits are territorial in the breeding season, males throughout the year (Drent et al. 2003). Great tits nest in both natural nesting holes and in artificial nest boxes (Drent et al. 2003). Clutch size varies from roughly eight to eleven eggs and the incubation period lasts from 12 to 15 days (Van Balen 1973). Females typically lay their eggs in April, but there is annual variation in the mean laying date (Van Balen 1973). The timing of breeding is adjusted to the timing of a peak in caterpillar abundance, using temperature as a cue (Ruuskanen et al. 2016c). A small proportion of females also lay a second clutch later in the breeding season (Van Balen 1973).

The main food of great tits are caterpillars and spiders (Graveland et al. 1994). These foods, however, do not cover for the increased calcium need during reproduction (Graveland et al. 1994). Calcium for eggshell formation is mainly derived from eating land snail shells (Graveland et al. 1994). The great tit also benefits from food supplementation (Ruuskanen et al. 2016a).

Great tits have been used commonly in studies, because they are easy to study (Dauwe et al. 1999). The species is common and often lives in populations of high densities (Dauwe et al. 1999). The fact that great tits nest in nest boxes makes them a convenient study species (Dauwe et al. 1999). The great tit is also a suitable bioindicator species for heavy metal contamination, because, in addition to the above-mentioned qualities, they are high in the food chain and so accumulate heavy metals, which can be analysed from faeces, feathers and eggshells (Dauwe et al. 1999).

2.3 Data collection

The data was collected in spring 2016 from four countries: Finland (Harjavalta), Belgium (Antwerp), Hungary (Budapest) and Portugal (Figueira da Foz). In each country, data was collected from one or more sites in a pollution zone near a pollution source, and from one or more sites in a reference zone further away from the pollution source. The pollution sources were a copper-nickel smelter in Finland, a non-ferrous metallurgic factory in Belgium, a paper and pulp mill industrial area in Portugal and the city of Budapest in Hungary (see details below). Each site has a stable great tit population and nest box studies have been conducted in the areas previously, so these sites were optimal for this study.

Great tit nest boxes were checked to keep track of nest building. When egg laying started, the laying date was recorded and the nests were checked daily. Newly laid eggs were marked with a felt-tip pen to follow the laying sequence. 4^{th} eggs in the laying sequence were collected from the nests on the day of laying and replaced with dummy eggs. The eggs were transported in small plastic bags inside plastic jars with cotton in the bottom and top of the jar to avoid breakage. Also, a paper label with the egg's unique identification code was placed in the jar. The code contained an abbreviation for country, nest code, position in the laying sequence and date of collection.

The eggs were weighed on the day of collection (precision 0.01 g), and length and width of the eggs were measured (precision 0.01 mm). The eggs were put back in the jars with the cotton and paper label and frozen at −20°C. Nests were continued to be checked after laying to record clutch size, hatching date, brood size, and fledgling number. Also, nestlings' faeces were collected for metal analysis. The eggs were transported frozen to Turku, Finland for later analyses.

2.4 Study areas

2.4.1 Harjavalta, Finland

The town of Harjavalta is located in southwestern Finland (61°20′ N, 22°10′ E). A copper-nickel smelter was established in Harjavalta in the 1940's and is still functioning. In the early years, the smelter was found to cause a loss of forest and ground vegetation by releasing SO_2 in the air. In the 1990's, soil observations around the smelter showed acidification and leaching of nutrients. Since then, most of the SO_2 has been used to produce sulphuric acid (Eeva & Lehikoinen 1995). Significant amounts of heavy metals have been released in the air and remain common in the surrounding area due to both current and long-term deposition. The most common heavy metals in the area are copper, nickel, lead, cadmium, zinc and arsenic (Espin et al. 2016a, Espin et al. 2016b). Heavy metals reach background levels at a distance of 5–10 km from the smelter (Eeva & Lehikoinen 1995).

Studies on passerine birds have shown clear effects of the pollutants on egg characteristics and breeding success. For example, decreased eggshell thickness has been observed in pied flycatchers in the polluted area (Eeva & Lehikoinen 2004). In great tits, a decrease in clutch size and hatching success has been observed near the pollution source (Eeva et al. 2009). However, some studies report no effect of distance from the smelter on breeding success in great tits (Eeva et al. 1995). Interestingly, such studies have still found strong effects on the pied flycatcher (*Ficedula hypoleuca*), which suggests that the species is likely more susceptible to the pollutants than the great tit (Eeva et al. 1995, Eeva & Lehikoinen 2004). Indeed, higher concentrations of accumulated arsenic, lead and cadmium in the liver have been measured from the pied flycatcher compared to the great tit (Berglund et al. 2011).

The different responses are most likely due to differences in the diet of the two species. The pied flycatcher's diet includes insects from the ground layer and the species is susceptible to direct effects of the pollutants at the egg stage, whereas the great tit depends mostly on caterpillars, which are scarce later in the nestling period (Eeva et al. 1997). The breeding success of the pied flycatcher in the polluted area has been found to depend on calcium availability (Eeva & Lehikoinen 2004). Breeding success was the lowest in territories with low calcium availability (measured as number of snail shells in nests) and high heavy metal concentrations. The feaces of great tit nestlings had four times higher levels of calcium than flycatcher feaces, which indicates that the foraging behaviour of

the great tit enables it to acquire greater amounts of calcium and thus avoid some of the negative effects of the pollutants (Eeva & Lehikoinen 2004).

2.4.2 Antwerp, Belgium

The study site in Belgium is located south of Antwerp in the region of Flanders. The polluted area is near a non-ferrous metallurgic factory in Hoboken, where extremely high levels of heavy metals have been observed (Dauwe et al. 1999). The high local pollution level is caused by emissions from dust of ore piles, which are carried by wind (Dauwe et al. 1999). Most common heavy metals in the area are lead, cadmium, arsenic, copper, and zinc, which form a decreasing pollution gradient away from the factory (Janssens et al. 2001). Their levels measured from great tit feathers are among the highest found in birds (Janssens et al. 2001).

Four specific study sites eastwards from the factory along the pollution gradient have been used in several great tit studies (Janssens et al. 2001, Geens et al. 2010). Each site has 30 to 50 nest boxes occupied by a population of great tits (Geens et al. 2010). The first site (Union Minière, UM) is close to the factory (0–350 m) and has high lead and cadmium levels (Janssens et al. 2001). The second site (Fort 8, F8) is 400-600 m, the third one (Fort 7, F7) 2500 m and the fourth one (Universitaire Instelling Antwerpen, UIA) 4000 m from the factory.

Research in the last decades has provided clear evidence for a pollution gradient away from the factory. Concentrations of silver, arsenic, cadmium, cobalt, copper, mercury, nickel, lead, selenium and zinc in great tit feathers have been found to decrease along the pollution gradient (Janssens et al. 2001). The concentrations were up to 40 times higher near the factory than further away from it. Excrements of great tit nestlings have higher concentrations of many heavy metals near the factory, as well (Janssons et al. 2003b). Measurements on great tit blood show a clear gradient for cadmium and lead (Geens et al.2010). Some studies have indicated that lead is one of the most important pollutants in the area, based on its especially high concentrations (Dauwe et al. 2000, Geens et al. 2010).

The observed effects of pollutant exposure in the area are diverse, including a decrease in reproductive success, reduced nestling health, and changes in haematological parameters. In a three-year study, a significant decrease in hatching and fledging success was found in great tits (Janssens et al. 2003a). Also, more females interrupted their laying near the factory than females further away. A study on the pollutants' health effects discovered

growth abnormalities in legs of great tit nestlings, reduced body mass and condition and delayed fledging (Janssens et al. 2003b). Haematological parameters, such as haemoglobin concentration, haematocrit and corpuscular volume, were lower near the pollution source and were negatively correlated with blood lead concentration (Geens et al. 2010).

2.4.3 Figueira da Foz, Portugal

The pollution source in Portugal is a paper and pulp mill industrial complex in Figuiera da Foz. The polluted zone is located in the National Pine Forest of Urso, 1 km south of the industrial complex (MU; 40°02′ N, 8°52′ W). The reference zone is located 20 km north of the pollution zone in the National Pine Forest of Quiaios, included in the Natura 2000 site "Dunas de Mira" (MQ; 40°14′ N, 8°47′ W) (Costa et al. 2012). Prevailing north-northwest winds in the area prevent emissions from reaching MQ, and the area is considered to be free from direct influence of industrial pollution (Costa et al. 2011a). Both areas are dominated by even-aged pine trees (80–90 years) and have sandy soils (Costa et al. 2012)

In previous studies, significantly higher levels of mercury have been measured from great tit feathers (Costa et al. 2011b) and excrements (Costa et al. 2012) in MU than MQ. In the latter study, the excrements were analysed for nine metals/metalloids (As, Hg, Pb, Ni, Cu, Cd, Zn, Se, Ca), of which mercury (Hg) and arsenic (As) alone differed significantly between the study sites, MU having higher levels of mercury and MQ higher levels of arsenic. The source of mercury emissions is uncertain, whereas arsenic is likely from pesticides and herbicides used in agriculture fields around MQ. The lack of significant differences in the levels of other metals indicates that the area around the paper and pulp mill complex is not heavily polluted by heavy metals (Costa et al. 2012). However, the paper-and-pulp mill is known to emit certain air pollutants, such as hydrogen sulphide (H_2S) and nitrogen oxides (Costa et al. 2011a, Costa et al. 2012).

In accordance with the relatively low level of metal pollution in the polluted zone (MU), no decrease in breeding performance has been observed in great tits. In fact, studies have reported a higher breeding success in MU compared to MQ (Costa et al. 2011a; Costa et al. 2012). Costa et al. (2011a) found that great tits started egg laying on average 10 days earlier and produced more fledglings in the first breeding attempt in MU than in MQ and the average clutch size was larger in the first and second breeding attempts in MU (Costa et al. 2011a). A larger clutch size and higher fledgling number were also observed in a second study (Costa et al. 2012).

Measurements of food availability by the frass-fall method have shown a higher caterpillar biomass in MU compared to MQ, which is the most likely cause for the difference in breeding success (Costa et al. 2011a; Costa et al. 2012). Caterpillars are an important food source for great tits and food availability strongly affects breeding performance (Costa et al. 2012).

2.4.4 Budapest, Hungary

The polluted area in Hungary is an urban park (Arboretum of Buda) in Budapest (47° 28′N, 19° 02′E). The reference zone is a sessile oak (*Quercus petraea*) dominated woodland in the Pilis Mountains (47° 43′N, 19° 01′E). Nest boxes were situated about 700–1500 m from the nearest road in the reference zone and 10–70 m in the urban park. The soil in the reference zone is acidic and has a low level of calcium, making land snails rare in the area. In the urban park, soil is neutral and has a higher level of calcium (Hargitai et al. 2016a).

The most important pollutants in the soil of the urban park include arsenic, copper, nickel, lead and zinc. Higher levels of lead, copper and zinc have been measured from great tit eggshells in the urban park compared to the reference zone (Hargitai et al. 2016a).

So far, no decreases in reproductive success have been found in the urban park compared to the reference zone. However, Hargitai et al. (2016a) discovered that great tit eggs in the urban area had lower levels of three antioxidants, yolk lutein, retinol and selenium. The antioxidant levels correlated with the lead concentration of the eggshell, but not with copper or zinc concentrations. Also, pigment aggregation was positively associated with copper concentration. Other pigment patterns, egg volume or eggshell thickness were not related to the copper, lead and zinc concentrations of the eggshell. Eggshells were thinner in the reference zone, which was suggested to result from the lower availability of calcium in the woodland compared to the urban park.

2.5 Methods

2.5.1 Metal analysis

Faecal samples were collected from nestlings of 7–9 days age, placed into Eppendorf tubes and frozen at −20°C. Samples of the same nest were combined to analyse within-brood metal concentrations. Samples were dried for 72 h at 45 °C and analysed at the University of Murcia (Spain).

Before the analysis, the faecal samples were put in digestion tubes with 4 ml of HNO_3 (70%) and 1 ml of H_2O_2 (33%) (Espin et al. 2016a). After that, the samples were heated in a microwave and diluted to ultrapure water. The accuracy of the analysis was tested beforehand by determining the recovery of metals in a reference material (TORT-2, lobster hepatopancreas, National Research Council Canada). The recoveries of the metals from 15 replicates of the reference material were between 74 and 120 %. A coefficient of variation (CV) was calculated to estimate repeatability and it was under 20 %.

An inductively coupled plasma optical emission spectrometer (ICP-OES) was used to analyse the concentrations of aluminium (Al), arsenic (As), cadmium (Cd), copper (Cu), nickel (Ni), lead (Pb) and zinc (Zn) with a quantification limit of 0.01 ppm. Also calcium (Ca) was analysed (quantification limit 1 ppm), as it is known to affect eggshell formation and reproductive success (Espin et al. 2016a). Metal and calcium concentrations were expressed as pg/mg dry sample weight (d.w.). Average water content in faeces was 77.9 ± 6.7 % in Finland and 78.1 ± 5.2 %, in Belgium.

2.5.2 Dissection

The eggs were kept frozen until dissection. One egg at a time was dissected to prevent the yolk from melting. The dissection was done by carefully separating the shell into two halves and scraping the albumen off the yolk with tweezers and a small knife (Fig. 1). Each component was weighed and frozen. Later, the shells were dried in 60 °C for 24 hours. After that, they were weighed to get the eggshell dry weight.

Figure 1. Dissection of frozen great tits' eggs with tweezers and a knife.

2.5.3 Eggshell thickness

Eggshell thickness was measured with a micro meter (Fig. 2) after drying the shells (Morales et al. 2013). The micro meter measured thickness with precision of 0.01 mm. Three small pieces from different parts of the shell were measured and thickness was calculated as the average of the three values. Shell membranes were left in the shells and included in the measurements. A coefficient of variation (CV %) was calculated for each egg from the tree measurements. The average CV % was 6.76 %.

Figure 2. Micro meter used in measuring eggshell thickness.

2.5.4 Pigment patterns

Before dissection, a photograph was taken on one side of each egg. The photos were taken on a colourpanel next to the colour red to be able to estimate the intensity of the pigment. Each photo was taken in the same place and height, with the same lighting.

Pigment patterns were analysed from the photographs using the method by Gosler et al. (2005) (Fig. 3). Pigment intensity (1 for palest, 3 for darkest), distribution (1 for aggregated, 3 for even) and spot size (1 for smallest, 3 for largest) were estimated in 0.5 increments. The pigment components (intensity, distribution and spot size) were analysed in three rounds, changing the order of the photographs between rounds. This way each egg had three replicates of each pigment component and the value of the component was calculated as the average of the replicates. All eggs were analysed by the same observer. A coefficient of variation (CV %) was calculated for every component of each egg to assess repeatability. The average CV % was 6.08 % for intensity, 4.21 % for distribution and 5.43 % for spot size.

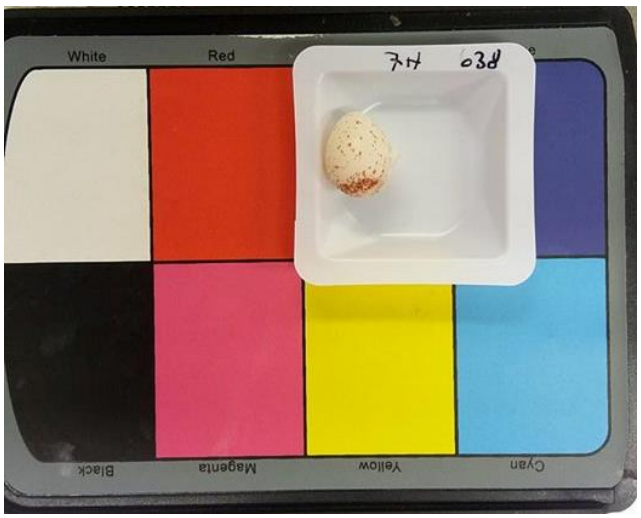

Figure 3. Visual estimation of pigment intensity, distribution and spot size in great tits' eggs. Example of an aggregated (average score 1) and intensive (average score 3) pigmentation with smaller than medium spot size (average score 1.67).

2.5.5 Lysozyme analysis

Lysozyme activity was measured from the albumen samples as the rate at which lysozyme breaks down cell walls of the bacteria *Micorococcus lysodeikticus*, following methods by Ruuskanen et al. (2011). Both the albumen samples and the bacterial suspension were diluted in a phosphate buffer. The buffer was prepared beforehand by mixing two phosphate buffers: disodium phosphate (Na_2HPO_4) and potassium dihydrogen phosphate (KH_2PO_4). The molarity of both buffers was 1/15 M. The final buffer was prepared by mixing the two buffers until reaching a pH-value of 6.2.

The bacterial suspension was prepared on the day of the analysis. All samples were analysed during the same day and with the same suspension to avoid any biases due to possible differences in the quality of the suspension. 100 ml of suspension (0.5 mg/ml) was made by dissolving 50 mg of dried *Micorococcus lysodeikticus* cell walls to 100 ml of the final buffer. The suspension was mixed and allowed to incubate for at least 30 minutes before the analysis.

The albumen samples were kept in ice and diluted to the buffer in a ratio of 1:500. 1996 µl of the buffer was pipetted in 2 ml Eppendorf and 4 µl of albumen sample (thoroughly vortexed) was added to the tubes.

The analysis was performed with Perkin Elmer EnVision 2103 multilabel plate reader. The albumen samples were divided in five plates of 96 wells. Each plate had 30 samples with three replicates each. The remaining six wells had two control wells (always from the same albumen sample), two with only buffer and suspension and two with only

suspension. The samples were evenly distributed and each plate had samples from all polluted and reference zones. 100 µl of the samples were pipetted into three wells in the plate in the predetermined order, again vortexing the samples before pipetting. Right before starting the analysis, the bacterial suspension was vortexed well and 100 µl of suspension was pipetted into the wells. The plate was analysed in the plate reader for 15 minutes at 1-minute intervals, and the plate was shaken for 15 seconds before each measurement.

Before analysing the samples, the procedure had been tested with three albumen samples to determine a suitable dilution ratio for the analysis. The samples were tested for 30 minutes, with ratios of 1:100 and 1:500 of albumen and phosphate buffer. Based on the lysozyme activity curves, the 1:500 ratio was chosen for the analysis, as the 1:100 ratio was too concentrated and yielded a strong, rapid decrease in the beginning of the curve and would not have been appropriate for measuring lysozyme activity. Also, a 15-minute analysis was found sufficient, because it included the linear part from which the activity can be measured.

An absorbance graph was drawn for each well, where the y-axis is the absorbance and the x-axis is time (min). Lysozyme activity was calculated from the linear part of the graph, where absorbance decreases steadily. In this study, the linear part was between 1 and 8 minutes (points 2−9). Lysozyme activity was calculated by dividing the change in absorbance (absorbance at point 2 minus absorbance at point 9) by the number of measuring points (8), multiplied by thousand ((Δ Absorbance/ number of points) *1000 = lysozyme activity). The lysozyme activity of a sample was calculated as the average of the three replicates of that sample. A coefficient of variation (CV %) was calculated for each sample and the average CV % was 4.016 %.

2.5.6 Thyroid hormone analysis

Hormone extraction

Hormone extraction was performed as described in Ruuskanen et al. (2016). Before the extraction, yolk samples were evenly distributed into 7 groups. Necessary reagents were prepared beforehand: a resin for the second part of the extraction (Dowex AG 1-X2 Resin, BIO RAD 140-1251), pH solutions of pH 3, 4 and 7 from sodium acetate, acetic acid of

three concentrations (1 %, 35 %, 70 %), $CaCl_2$ (0.05 %), chloroform ($CHCl_3$)/methanol solution in ratio 2:1 and chloroform/methanol/$CaCl_2$ solution in ratio 3:49:48.

The samples were extracted one group at a time. The frozen yolk samples were prepared by first homogenizing them. 1.5 ml of methanol was put on each sample Eppendorf. The samples were homogenized for 1 minute in a Qiagen homogenizer with the help of metal beads, after which the bead was removed. The homogenized liquid was poured into a glass tube. 0.5 ml of methanol was pipetted in the Eppendorf tubes and the tubes were vortexed to get all the yolk in, and the liquid was poured into the same glass tube. The samples were now in 2 ml of methanol in the glass tubes.

Next, 60µl $13C^{12}$- labelled T4 (Larodan AB Sweden, 0.001pmol/µl in 99 % methanol) was added to each glass tube. By analysing the amount of the labelled hormone and calculating its loss in per cent after the extraction, the amount of lost thyroid hormones (i.e. recovery) can be calculated by assuming that the loss of thyroid hormones in per cent is the same as the loss of the labelled hormone.

First, a liquid-liquid extraction was performed. To initiate the separation of lipids and proteins in the samples, 3 ml chloroform was added to the tubes. Lipids dissolve in chloroform, whereas proteins, as well as thyroid hormones (amine hormones), dissolve in methanol. The tubes were vortexed for 15 s, after which they were centrifuged for 15 min (3000 rpm, 4°C, Eppendorf 5810R centrifuge). Solid compounds were separated as a pellet and the supernatant, including the hormones, was decanted to another glass tube. Some hormones were still left in the tubes, so the samples were re-extracted: 2 ml of chloroform-methanol mixture (2:1) was added and the samples were centrifuged again for 15 minutes. This time the pellet was slightly scattered, so the supernatant was carefully pipetted and added to the second glass tube.

Next, $CaCl_2$ (0.05 %) was pipetted to the tubes, according to the weight of each yolk sample (Fig. 4). The tubes were shaken three times and centrifuged for 10 minutes (2000 rpm, 4 °C). After this, the two phases were separated in the tubes, the methanol phase on top of the chloroform phase. A mark at the liquid's surface was drawn in each tube for later purpose.

In the second part of extraction (solid phase extraction), the hormone samples were further purified and concentrated using ion-exchange resin. Iodine ions in the thyroid hormones were bound to a resin (Dowex AG 1-X2) by ion exchange with acetate ions. First, the resin columns were prepared to syringes by pipetting 1.3 ml resin and placing a

piece of filter paper in the bottom of the syringes (Fig. 5). The columns were washed five times with 2 ml of purified water. Also, 2 ml of a pH 7 solution was run through the columns twice. The upper methanol phase was then pipetted to the columns from the tubes with the separated phases. To increase extraction efficiency, chloroform/methanol/$CaCl_2$ was added to the tubes at the level of the mark and centrifuging was repeated. Again, the upper phase was pipetted to the columns.

After the iodine ions were bound to the resin columns, the samples were purified with sequential washes of the resin columns. Finally, the ion exchange reaction was reversed so that the iodine ions were released and they could be collected for the hormone analysis. This was achieved by washing the columns several times to change the pH gradually and by increasing the concentration of acetate ions. 2 ml of pH7, ethanol, pH7, pH4, pH3, acetic acid 1 % and acetic acid 35 % were pipetted in the columns in the above order. After that, 0.5 ml of acetic acid 70% was added twice. To collect the hormones, 2 ml Eppendorf tubes were placed under the columns and 0.5 ml of acetic acid 70 % was run through the columns another four times. The Eppendorf tubes were then put in a vacuum centrifuge overnight for the acetic acid to evaporate from the tubes, leaving the hormones left. The dry samples were kept at –20 °C until hormone analysis.

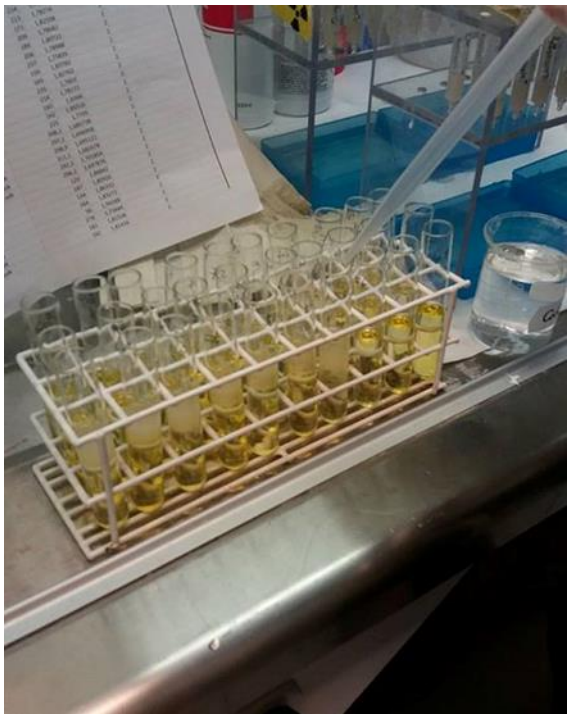

Figure 4. Pipetting of $CaCl_2$ into Eppendorf tubes during liquid-liquid extraction of thyroid hormones from great tits' yolk samples.

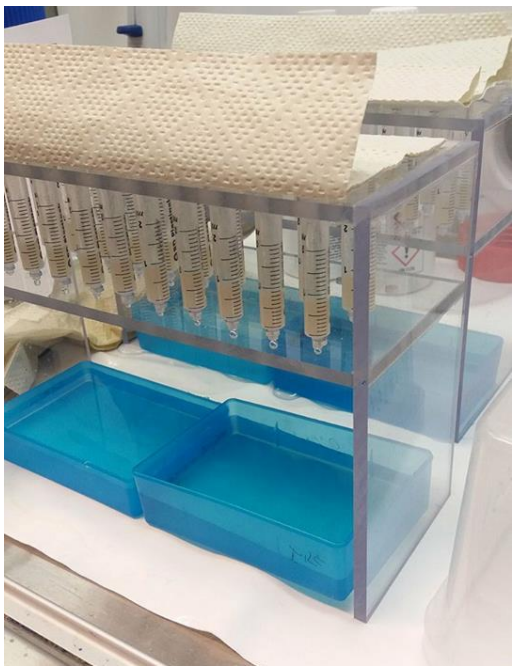

Figure 5. Syringes used in solid phase extraction of thyroid hormones from great tits' yolk samples.

Hormone analysis

To quantify the small concentrations of T4 and T3 in the hormone samples, a liquid chromatography−mass spectrometry (LC-MS) method was developed in this study. A triple quadrupole mass spectrometer (TSQ Vantage, Thermo Scientific, San Jose, CA) was used to analyse the samples. For the chromatographic separation of hormones, a nanoflow HPLC system Easy-nLC (Thermo Scientific) was applied. The quantification limits were 10.6 amol for T4 and 17.9 amol for T3.

Before the analysis, the dry samples were diluted in ammonium (NH_3). Internal standards $^{13}C_6$-T_3 and $^{13}C_6$-T_4 (labelled forms of the hormones) were added to each sample to identify the THs. T3 and T4 have the same retention times as their labelled forms (T3 with $^{13}C_6$-T_3 and T4 with $^{13}C_6$-T_4) and they could be identified from the peaks in their labelled forms.

To measure extraction efficiency, an additional internal standard, $^{13}C_{12}$-T_4, was added to each sample. The recovery of T4 was calculated from the recovery of $^{13}C_{12}$-T_4. The recovery of T3 was calculated utilizing the correlation between the recoveries of T4 and T3, as a $^{13}C_{12}$-isotope was not available for T3. For that, a correction factor for the recovery of T3 in each sample was calculated using average recovery ratios of $^{13}C_6$-T_3 and $^{13}C_6$-T_4 ($^{13}C_6$-T_3 recovery/$^{13}C_6$-T_4 recovery) as an estimate of T3 and T4 recovery ratio. The mean coefficient of variation was < 10 %.

2.5.7 Statistical analyses

All statistical analyses were performed with SAS statistical software, ver. 9.4 using Enterprise Guide 7.1. Mixed linear models (proc MIXED) were used to test the effects of zone (polluted/unpolluted) and the level of heavy metal pollution on the egg components (T3, T4, lysozyme, pigment intensity, distribution and spot size, eggshell thickness and dry mass, egg fresh mass) (N=150 eggs) and generalized linear mixed models (proc GLIMMIX) were used to test the effects on fledgling number.

For the heavy metals (As, Cd, Cu, Ni, Pb), a principal components analysis was performed to reduce the number of possibly correlated explanatory factors (correlated metal concentrations) by combining them into a new factor that reflects the total level of the metal pollution. The first principal component (PC1) explained 60.5 % of the variation in the heavy metal data (Eigenvector 3.024), so it described heavy metal pollution well and was kept in the models as an explanatory factor. The concentrations of arsenic (As), cadmium (Cd), copper (Cu), nickel (Ni) and lead (Pb) correlated positively with the PC1 (Eigenvectors As 0.47, Cd 0.29, Cu 0.45, Ni 0.47 and Pb 0.51). Aluminium and zinc were not included in the PC1, but their between-population differences were tested, as aluminium is known to negatively affect eggshell structure and growth (Eeva & Lehikoinen 1995) and zinc can affect the concentration of lysozyme (Chatelain et al. 2016). The between-population differences of calcium were also tested, as calcium is known to affect eggshell formation and reproductive success (Espin et al. 2016a).

Before the analyses, some variables were modified to make them more suitable for the models. Laying date was changed into a numeric value (1=April 1st) and standardized by subtracting the population's average from each egg's value. This way each egg was compared to its own population instead of the whole data. One outlier was removed from the lysozyme data. Fledgling number was changed into a binomial variable (0=nest failed, 1=at least one fledgling, hereafter fledging probability) that describes whether the nest produced any fledglings, because the data contained a high proportion of failed nests.

In the models that tested the effect of zone on the response variables, zone, country, the interaction of zone and country (i.e. population), laying date and clutch size were used as explanatory variables. Laying date and clutch size were added in the models as they can affect egg quality, laying date by affecting the amount of resources available for the female and clutch size by influencing their allocation between the offspring. The interaction of country and zone was included, because the degree of differences, for example in heavy metal concentrations, between the two populations of each country can

vary. When the interaction or country was significant, Tukey's test was used to test which populations or countries differed significantly from each other. Country and the interaction were removed from the models where neither of them was significant.

The normality of the variables was tested from the residuals of the models. Country was set as a random intercept in the models where the interaction and country were removed. In the lysozyme models, plate was also set as a random intercept. Country was set as a random intercept, because the observations in a country are not independent of each other. The plates in the lysozyme analysis are also not independent, because the plates were analysed separately and an error in the analysis of a plate would affect the results of all the plate's samples. In the fledging probability and eggshell thickness models, calcium was added as an explanatory variable, as calcium is known to affect reproductive success and shell thickness (Espin et al. 2016a).

The effect of PC1 on the response variables was tested using similar models as above, but without any interactions. Country was set as a random intercept. The differences of PC1 between populations, as well as differences in individual metal concentrations (Al, As, Ca, Cd, Cu, Ni, Pb, Zn), were tested with Tukey's test, using a linear model. Before testing the between-population differences in metal concentrations, all concentrations were log transformed and values below the detection limit were replaced with $0.05/\sqrt{2}$ to improve the distributions.

3 RESULTS

3.1 Between-population differences in metal concentrations

The polluted zones in all countries seemed to have higher faecal concentrations of heavy metals compared to the reference zones, based on PC1 (Fig 6). However, the only significant difference in PC1 was between the polluted zone of Finland, which had the highest average PC1 value, and all the other populations (all Tukey test t-values >3.38, all p-values <0.001). The average concentrations of the PC1 heavy metals (As, Cd, Cu, Ni, Pb) are presented in Table 1. The concentrations of nickel and copper were significantly higher in the polluted zone of Finland compared to all other populations (nickel: all Tukey test t-values >6.89, all p-values <0.0001, copper: all Tukey test t-values >2.13, p-values <0.035). The second highest concentration of copper was in the polluted zone of Portugal (PtP vs. all but FiP and PtU: Tukey test t-values >2.42, p-values

<0.0163). The polluted zone of Hungary had significantly higher concentration of nickel than the unpolluted zones in Belgium and Hungary and both populations in Portugal (HuP vs. BeU, HuU, PtU, PtP: Tukey test t-values: >2.80, p-values <0.0058).

The polluted zone of Belgium had significantly higher concentrations of cadmium and lead than the other populations (cadmium: all Tukey test t-values >3.01, all p-values <0.0031, lead: all Tukey test t-values >9.22, all p-values <0.0001) and higher concentration of arsenic than all but the polluted zone of Finland (BeP vs. all but FiP: Tukey test t-values >8.12, p-values <0.0001). The polluted zone of Finland had the second highest concentration of arsenic (FiP vs. all but BeP; Tukey test t-values >5.82, p-values <0.0001) and cadmium (FiP vs. all but BeP: Tukey test t-values >3.32, p-values <0.0012).

The average faecal concentrations of the other metals (Al, Zn) and Ca are presented in Table 2. The concentration of aluminium was significantly the highest in the unpolluted zone of Hungary compared to all other populations (all Tukey test t-values >2.01, all p-values <0.0455). The significantly highest concentration of zinc was in the unpolluted zone of Portugal, which had a significantly higher concentration than all but the polluted zones of Finland and Portugal (PtU vs. all but FiP and PtP: Tukey test t-values >3.48, p-values <0.0007). The highest concentration of calcium was in the unpolluted zone of Portugal, which differed significantly from the unpolluted zones of Finland and Hungary and both populations in Belgium (PtU vs. FiU, HuU, BeP, BeU: Tukey test t-values >2.92, p-values <0.0041).

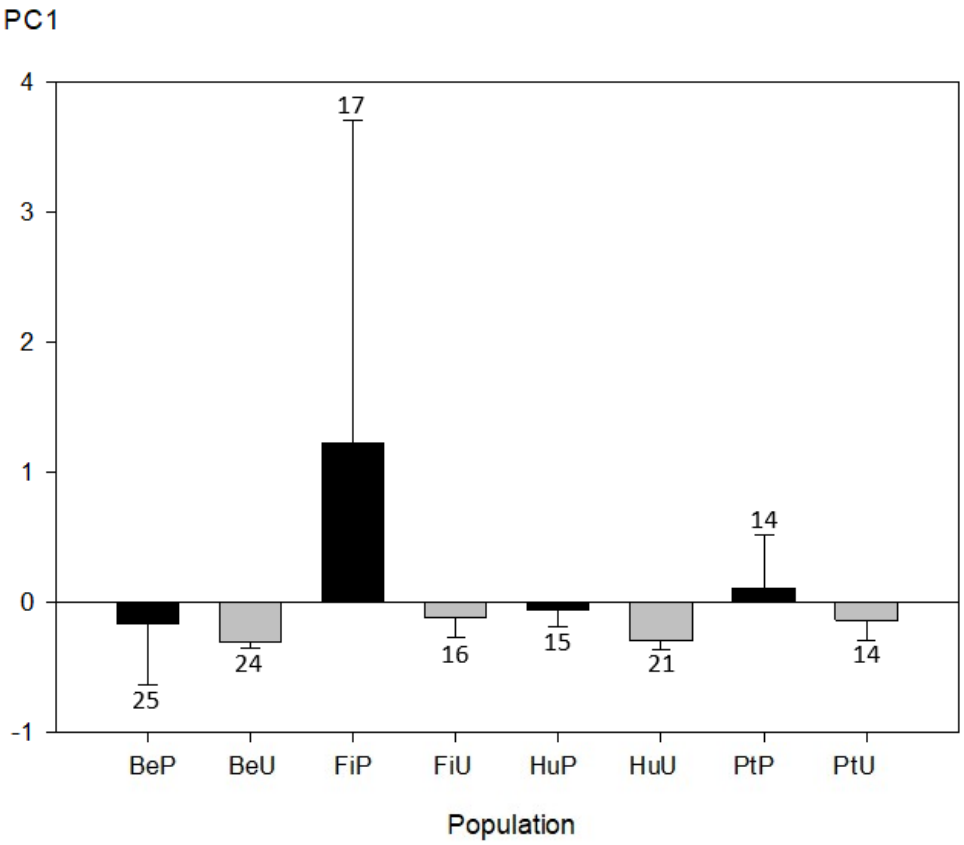

Figure 6. Level of heavy metal pollution in populations of great tits. Average levels and standard deviations of the first principal component describing concentrations of arsenic, cadmium, copper, nickel and lead in faecal samples of nestlings. Number of nests is indicated above the bars. BeP=Belgium polluted zone, BeU=Belgium unpolluted zone, FiP=Finland polluted zone, FiU=Finland unpolluted zone, HuP=Hungary polluted zone, HuU=Hungary unpolluted zone, PtP=Portugal polluted zone and PtU=Portugal unpolluted zone.

Table 1. Average concentrations (mg/kg dry weight) and standard deviations of the PC1 heavy metals (arsenic As, cadmium Cd, copper Cu, nickel Ni and lead Pb) in nestlings' faecal samples in populations of great tits. BeP=Belgium polluted zone, BeU=Belgium unpolluted zone, FiP=Finland polluted zone, FiU=Finland unpolluted zone, HuP=Hungary polluted zone, HuU=Hungary unpolluted zone, PtP=Portugal polluted zone and PtU=Portugal unpolluted zone. N=sample size.

Population	N	As	Cd	Cu	Ni	Pb
BeP	25	18.0±13.7	11.2±10.8	75.0±45.2	3.22±1.48	82.4±72.0
BeU	24	1.44±1.23	1.47±0.80	34.7±10.8	2.18±1.10	7.52±3.85
FiP	17	12.1±13.4	4.46±2.56	194±162	36.7±56.2	38.4±141
FiU	16	0.39±0.37	1.22±1.04	73.8±39.4	3.51±1.70	1.98±1.82
HuP	15	1.15±0.49	0.60±0.14	70.8±26.6	4.72±2.70	5.80±3.09
HuU	21	0.02±0.10	0.85±0.51	36.4±14.5	1.80±1.27	5.06±3.00
PtP	14	0.93±1.27	1.63±0.90	143±97.5	0.39±0.22	0.78±0.50
PtU	14	1.73±1.61	1.97±1.24	89.5±43.9	0.64±0.30	1.09±0.53

Table 2. Average concentrations and standard deviations of aluminium (Al), calcium (Ca) and zinc (Zn) in nestlings' faecal samples in populations of great tits (Al and Zn as mg/kg and Ca as g/100g dry weight). BeP=Belgium polluted zone, BeU=Belgium unpolluted zone, FiP=Finland polluted zone, FiU=Finland unpolluted zone, HuP=Hungary polluted zone, HuU=Hungary unpolluted zone, PtP=Portugal polluted zone and PtU=Portugal unpolluted zone. N=sample size.

Population	N	Al	Ca	Zn
BeP	25	1470±1142	0.632±0.274	299±92.7
BeU	24	2463±1996	0.694±0.535	280±86.8
FiP	17	1974±1444	1.522±0.770	422±153
FiU	16	1690±1911	0.915±0.616	300±110
HuP	15	3616±2048	1.477±0.581	320±90.4
HuU	21	9186±7925	0.568±0.458	231±59.6
PtP	14	292±212	1.359±0.753	511±277
PtU	14	532±523	1.967±1.338	654±425

3.2 The effects of zone, country and PC1 of heavy metals on the egg components and fledging probability

The population averages of each egg component (T3, T4, lysozyme, pigment intensity, distribution and spot size, shell thickness, shell mass, egg mass) and fledging probability are presented in Tables 3 & 4. Neither zone nor PC1 had significant associations with any of the egg components or fledging probability (Tables 5 & 6). The interaction of zone and country was significant for pigment distribution (Table 5, Fig 7). The polluted zone of Hungary had significantly more aggregated pigment distribution (lower score in distribution) than the unpolluted zone (Tukey test t=-3.69, p=0.0003, Fig. 7). In the other countries, there was no significant difference in pigment distribution between the polluted and unpolluted zone. Also, in all pairwise comparisons between the populations, the polluted zone of Hungary had significantly more aggregated pigment spotting (lowest values in distribution) (HuP vs. all populations; Tukey test t-values >2.03, p-values <0.0430, Fig 7).

Between countries, there were significant differences in shell dry mass (Table 5, Fig 8), egg fresh mass (Table 5, Fig 9) and fledging probability (Table 5, Fig 10). Belgium had significantly lighter eggshells than Finland (Tukey test t=-3.20, p=0.0017) and Hungary (Tukey test t=-2.51, p=0.0133). The egg fresh mass also was significantly lower in Belgium than in Finland (Tukey test t=-4.58, p=<0.0001) and Hungary (Tukey test t=-2.89, p=0.0044). The egg fresh mass of Portugal was significantly lower than in Finland

(Tukey test t=-3.34, p=0.0011). The highest fledging probability was in Belgium, which differed significantly from Hungary (Tukey test t=2.62, p=0.0098) and Portugal (Tukey test t=3.25, p=0.0015). Finland had also significantly higher fledging probability than Portugal (Tukey test t=2.02, p=0.0455).

Clutch size had a significant negative association with T3, eggshell thickness and eggshell dry mass (Tables 5 & 6). Standardized laying date had no significant association with any egg component in any model (Tables 5 & 6).

Table 3. Population averages and standard deviations of T3 (triiodothyronine), T4 (thyroxine), lysozyme enzyme, eggshell thickness, eggshell dry mass, egg mass and fledging probability (FB, 0=nest failed, 1=at least one fledgling) in great tits. BeP=Belgium polluted zone, BeU=Belgium unpolluted zone, FiP=Finland polluted zone, FiU=Finland unpolluted zone, HuP=Hungary polluted zone, HuU=Hungary unpolluted zone, PtP=Portugal polluted zone and PtU=Portugal unpolluted zone.

Popu-lation	T4 (pg/mg)	T3 (pg/mg)	Lyso-zyme	Thick-ness (mm)	Shell mass (g)	Egg mass (g)	FB
BeP	1.19± 0.29	0.16± 0.07	7.41± 0.85	0.086± 0.007	0.095± 0.007	1.56± 0.12	0.96± 0.20
BeU	1.30± 0.46	0.17± 0.10	6.75± 1.75	0.084± 0.007	0.099± 0.009	1.59± 0.13	0.79± 0.41
FiP	1.23± 0.29	0.15± 0.08	7.46± 0.73	0.087± 0.009	0.103± 0.008	1.73± 0.15	0.59± 0.51
FiU	1.13± 0.31	0.14± 0.05	7.56± 1.08	0.086± 0.008	0.104± 0.010	1.71± 0.14	0.81± 0.40
HuP	1.12± 0.28	0.15± 0.06	6.91± 0.82	0.084± 0.008	0.102± 0.011	1.66± 0.16	0.40± 0.51
HuU	1.19± 0.40	0.16± 0.07	7.01± 1.02	0.082± 0.006	0.100± 0.008	1.66± 0.12	0.76± 0.44
PtP	1.33± 0.42	0.16± 0.07	6.66± 1.08	0.088± 0.006	0.099± 0.008	1.58± 0.11	0.53± 0.51
PtU	1.24± 0.33	0.17± 0.05	6.81± 1.68	0.087± 0.011	0.104± 0.009	1.66± 0.14	0.47± 0.52

Table 4. Population averages and standard deviations of pigment intensity (1=pale, 3=dark tone), distribution (1=aggregated, 3=even distribution) and spot size (1=small, 3=large spots) in great tits. BeP=Belgium polluted zone, BeU=Belgium unpolluted zone, FiP=Finland polluted zone, FiU=Finland unpolluted zone, HuP=Hungary polluted zone, HuU=Hungary unpolluted zone, PtP=Portugal polluted zone and PtU=Portugal unpolluted zone.

Population	Intensity	Distribution	Spot size
BeP	2.19± 0.50	2.14± 0.61	1.64± 0.56
BeU	1.97± 0.52	2.17± 0.62	1.60± 0.51
FiP	2.07± 0.69	2.21± 0.59	1.65± 0.54
FiU	2.26± 0.50	2.14± 0.62	1.78± 0.40
HuP	2.25± 0.44	1.67± 0.67	1.94± 0.59
HuU	1.88± 0.47	2.43± 0.62	1.66± 0.46
PtP	2.14± 0.35	2.18± 0.56	1.92± 0.49
PtU	2.05± 0.52	2.31± 0.62	1.57± 0.37

Table 5. The effect of zone (polluted/unpolluted), country, zone x country interaction, standardized laying date, clutch size and faecal calcium concentration (g/100g dry weight) on the egg components (thyroxine T4, triiodothyronine T3, pigment intensity, distribution and spot size, eggshell thickness, lysozyme and shell and egg mass) and fledging probability (0=nest failed, 1=at least one fledgling) of great tits in the final models. The analyses were performed with generalized linear mixed models (proc GLIMMIX) for fledging probability and with mixed linear models (proc MIXED) for the egg components. In models without the interaction, country was set as a random intercept. Significant p-values in bold. Degrees of freedom are given in brackets.

Compo-nent	Zone	Country	Zone x Country	Laying date	Clutch size	Ca
T4 (pg/mg)	$F_{(1,137)}$=0.04 p=0.8482	–	–	$F_{(1,137)}$=1.40 p=0.2393	$F_{(1,137)}$=0.76 p=0.3863	–
T3 (pg/mg)	$F_{(1,136)}$=0.73 p=0.3947	–	–	$F_{(1,136)}$=0.01 p=0.9038	$F_{(1,136)}$=5.08 **p=0.0259**	–
Intensity	$F_{(1,143)}$=2.58 p=0.1103	–	–	$F_{(1,143)}$=0.00 p=0.9871	$F_{(1,143)}$=0.51 p=0.4780	–
Distri-bution	$F_{(1,137)}$=3.51 p=0.0633	$F_{(3,137)}$=0.21 p=0.8923	$F_{(3,137)}$=3.61 p=**0.0150**	$F_{(1,137)}$=0.11 p=0.7368	$F_{(1,137)}$=1.02 p=0.3134	–
Spot size	$F_{(1,142)}$=0.69 p=0.4071	–	–	$F_{(1,141)}$=1.09 p=0.2984	$F_{(1,59.5)}$=0.03 p=0.8722	–
Thickness (mm)	$F_{(1,124)}$=2.96 p=0.0881	–	–	$F_{(1,124)}$=2.74 p=0.1001	$F_{(1,124)}$=3.96 **p=0.0487**	$F_{(1,124)}$=0.14 p=0.7126
Lysozyme	$F_{(1,126)}$=0.11 p=0.7354	–	–	$F_{(1,127)}$=1.30 p=0.2567	$F_{(1,110)}$=0.18 p=0.6735	–
Shell dry mass (g)	$F_{(1,139)}$=2.86 p=0.0930	$F_{(3,139)}$=4.21 p=**0.0069**	$F_{(3,139)}$=0.37 p=0.7771	$F_{(1,139)}$=0.12 p=0.7309	$F_{(1,139)}$=8.50 p=**0.0041**	–
Egg mass (g)	$F_{(1,140)}$=1.47 p=0.2274	$F_{(3,140)}$=8.30 p=**<0.0001**	$F_{(3,140)}$=0.48 p=0.6936	$F_{(1,140)}$=0.02 p=0.8904	$F_{(1,140)}$=2.91 p=0.0901	–
Fledging probability	$F_{(1,136)}$=0.12 p=0.7253	$F_{(3,136)}$=3.97 **p=0.0095**	$F_{(3,136)}$=2.05 p=0.1092	$F_{(1,136)}$=3.10 p=0.0806	–	$F_{(1,136)}$=0.69 p=0.4074

Table 6. The effect of PC1 of heavy metals (As, Cd, Cu, Ni and Pb), standardized laying date, clutch size and faecal calcium concentration (g/100g dry weight) on the egg components (thyroxine T4, triiodothyronine T3, pigment intensity, distribution and spot size, eggshell thickness, lysozyme and shell and egg mass) and fledging probability (0=nest failed, 1=at least one fledgling) of great tits. The analyses were performed with generalized linear mixed models (proc GLIMMIX) for fledging probability and with mixed linear models (proc MIXED) for the egg components. Country was set as a random intercept. Significant p-values in bold. Degrees of freedom are given in brackets.

Egg component	**PC1**		**Laying date**	**Clutch size**	**Ca**
	F, p	Estimate ± SE			
T4 (pg/mg)	$F_{(1,133)}$=0.17 p=0.6842	0.0121± 0.0297	$F_{(1,133)}$=1.58 p=0.2103	$F_{(1,133)}$=0.62 p=0.4314	–
T3 (pg/mg)	$F_{(1,132)}$=0.00 p=0.9464	0.0004± 0.0060	$F_{(1,132)}$=0.06 p=0.8123	$F_{(1,132)}$=4.22 **p=0.0418**	–
Intensity	$F_{(1,140)}$=0.02 p=0.8848	0.0063± 0.0432	$F_{(1,140)}$=0.50 p=0.4815	$F_{(1,140)}$=0.24 p=0.6215	–
Distribution	$F_{(1,140)}$=0.11 p=0.7403	-0.0174± 0.0525	$F_{(1,140)}$=0.37 p=0.5446	$F_{(1,140)}$=0.14 p=0.7086	–
Spot size	$F_{(1,114)}$=0.13 p=0.7178	-0.0158± 0.0436	$F_{(1,138)}$=2.22 p=0.1382	$F_{(1,62.5)}$= 0.01 p=0.9052	–
Thickness (mm)	$F_{(1,124)}$=1.29 p=0.2587	0.0007± 0.0006	$F_{(1,124)}$=1.04 p=0.3087	$F_{(1,124)}$=4.89 **p=0.0289**	$F_{(1,124)}$=0.18 p=0.6755
Shell dry mass (g)	$F_{(1,141)}$=0.16 p=0.6914	-0.0003± 0.0008	$F_{(1,138)}$=1.26 p=0.2629	$F_{(1,123)}$=8.62 **p=0.0040**	–
Egg mass (g)	$F_{(1,142)}$=1.25 p=0.2648	-0.0128± 0.0115	$F_{(1,139)}$=0.23 p=0.6359	$F_{(1,140)}$=2.59 p=0.1100	–
Lysozyme	$F_{(1,104)}$=0.09 p=0.7653	-0.0824± 0.2751	$F_{(1,125)}$=2.07 p=0.1531	$F_{(1,97)}$=0.20 p=0.6554	–
Fledging probability	$F_{(1,142)}$=0.09 p=0.7685	-0.0577± 0.1918	$F_{(1,142)}$=3.64 p=0.0585	–	$F_{(1,142)}$=3.71 p=0.0560

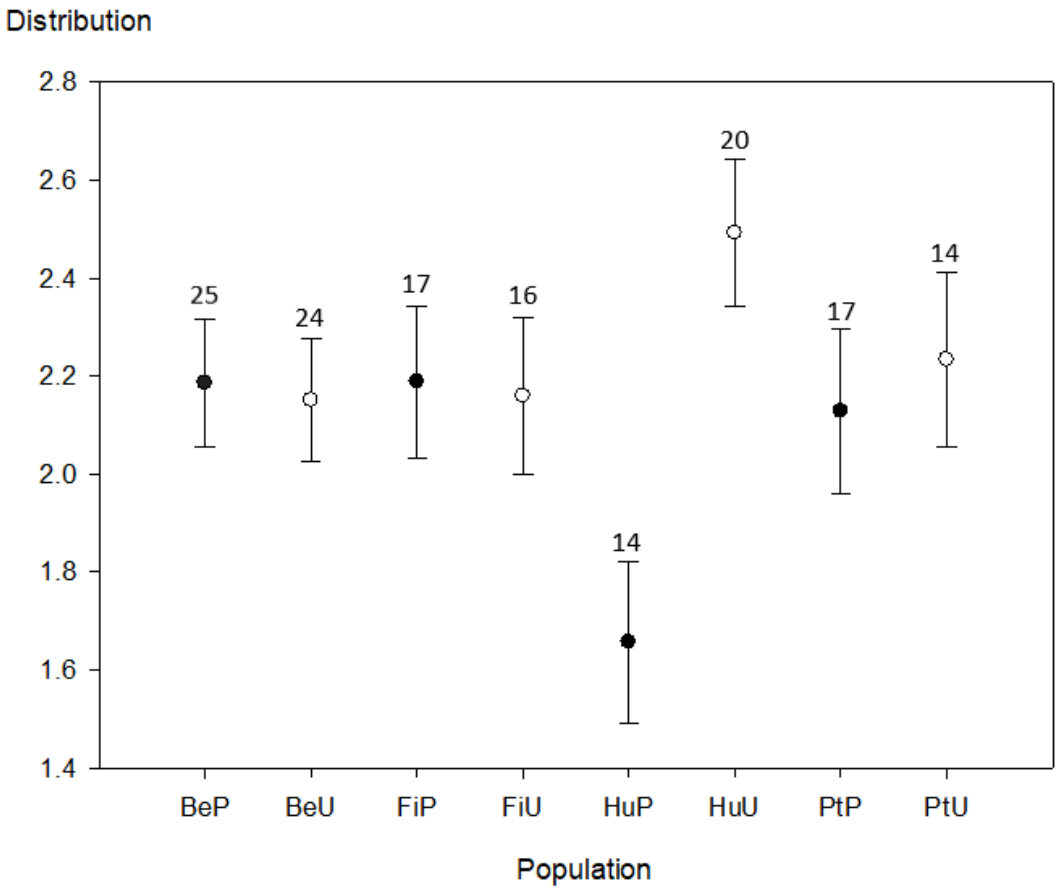

Figure 7. Population differences in pigment distribution (1=aggregated, 3=even distribution) in eggs of great tits. Least square means and standard errors from mixed linear models (proc MIXED) testing the effect of zone, country, zone x country, laying date and clutch size on pigment distribution. Number of eggs is indicated above the bars. BeP=Belgium polluted zone, BeU=Belgium unpolluted zone, FiP=Finland polluted zone, FiU=Finland unpolluted zone, HuP=Hungary polluted zone, HuU=Hungary unpolluted zone, PtP=Portugal polluted zone and PtU=Portugal unpolluted zone.

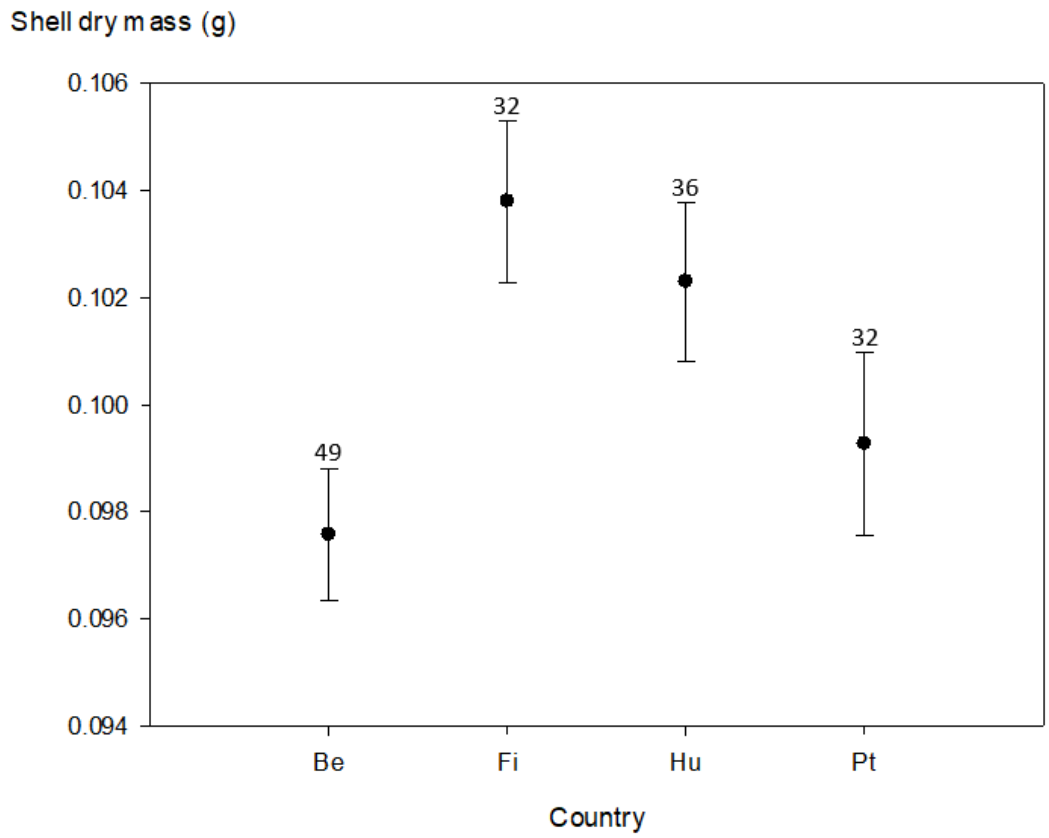

Figure 8. Between-country differences in eggshell dry mass (g) in great tits. Least square means and standard errors from mixed linear models (proc MIXED) testing the effect of zone, country, zone x country, laying date and clutch size on eggshell dry mass. Number of eggs is indicated above the bars. Be=Belgium, Fi=Finland, Hu=Hungary and Pt=Portugal.

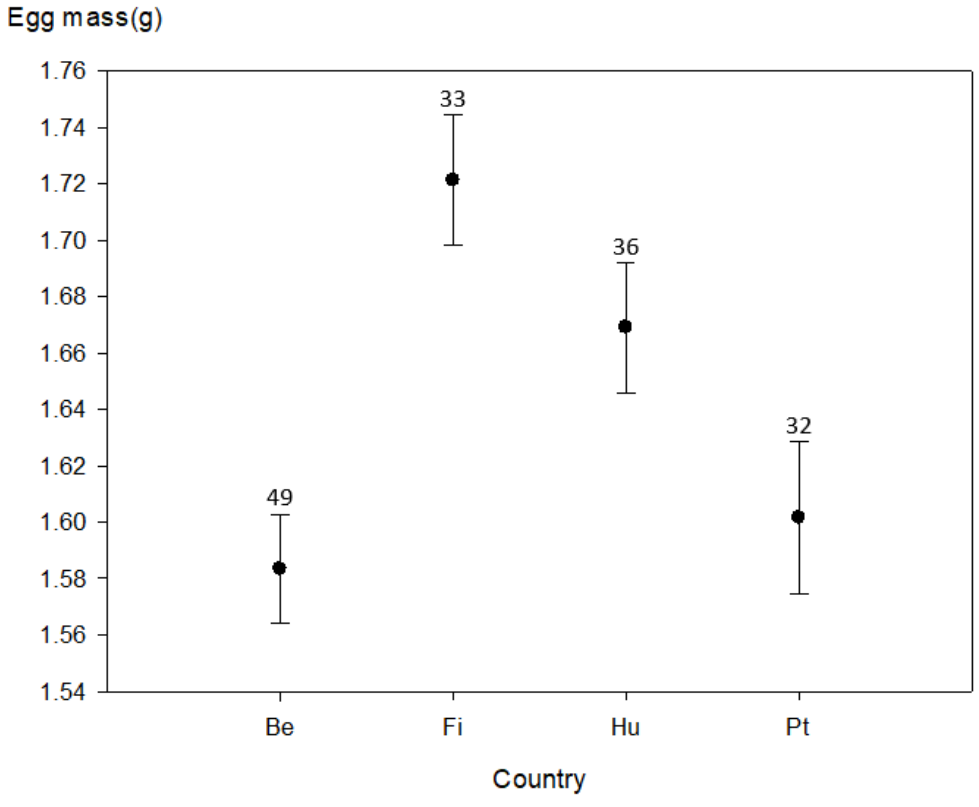

Figure 9. Between-country differences in egg fresh mass (g) in great tits. Least square means and standard errors from mixed linear models (proc MIXED) testing the effect of zone, country, zone x country, laying date and clutch size on egg mass. Number of eggs is indicated above the bars. Be=Belgium, Fi=Finland, Hu=Hungary and Pt=Portugal.

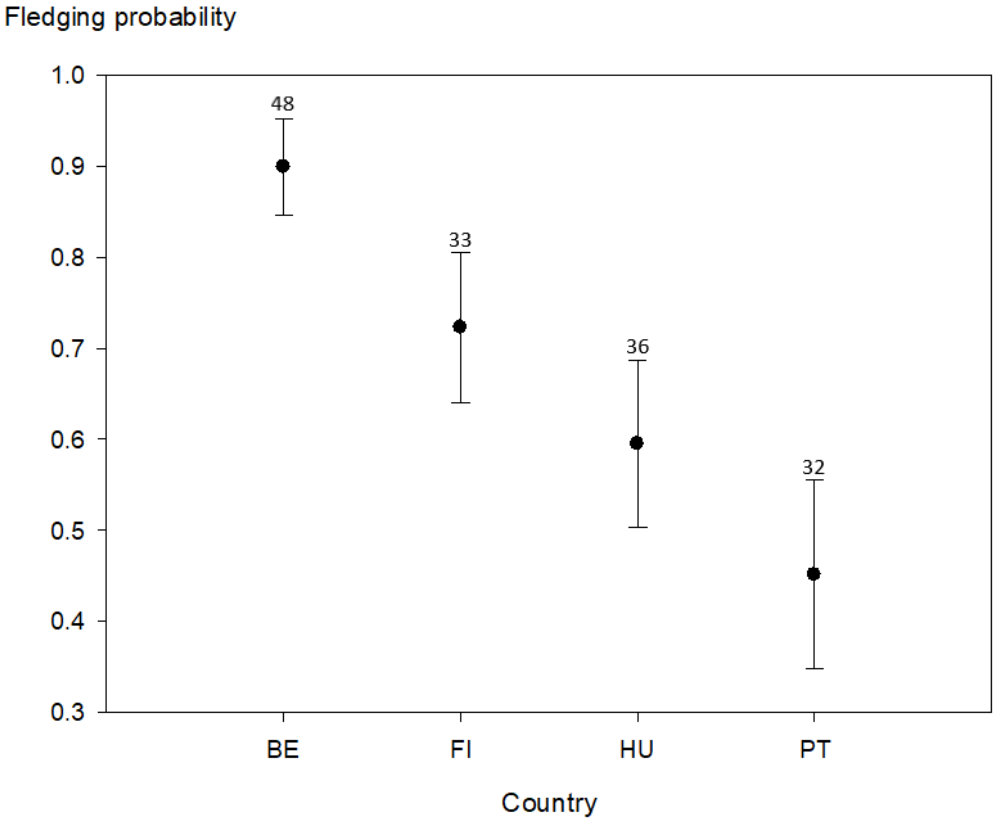

Figure 10. Between-country differences in fledging probability (0=nest failed, 1=at least one fledgling) in nests of great tits. Least square means and standard errors from generalized linear mixed models (proc GLIMMIX) testing the effect of zone, country, zone x country, laying date and faecal calcium concentration on fledging probability. Number of nests is indicated above the bars. BE=Belgium, FI=Finland, HU=Hungary and PT=Portugal.

4 DISCUSSION

4.1 Main results of the study

Zone (polluted/unpolluted) and PC1 of heavy metals (arsenic, cadmium, copper, nickel, lead) had no significant associations with any of the egg components or fledging probability in the study populations, so the hypotheses of this study were not supported. Based on the results, heavy metal pollution had little effect on the egg components or reproductive success of great tits in the study populations. This result was surprising and in contradiction with previous studies in the study areas. The only significant result was

in the interaction of zone and country on pigment distribution, where Hungary differed from other countries.

Clutch size was negatively associated with T3, eggshell thickness and eggshell dry mass. When clutch size is big, the female supposedly has less resources to allocate to each egg compared to a small clutch size, which explains why the concentration of T3, shell thickness and dry mass decreased with increasing clutch size (Pellerin et al. 2016).

4.2 Differences in the egg components

The results indicate that heavy metal pollution is not an important factor for thyroid hormone (T3 & T4) concentration in the study populations. There seemed to be no trend in the concentrations of T3 and T4 between the polluted and unpolluted zones. This is surprising because heavy metals, like lead and cadmium, have been found to affect TH concentration negatively in other vertebrates (in blood serum: Gupta & Kar 1998, in larval tissues: Miao et al. 2015). This study did not, however, investigate the effect of mercury on THs, which has previously been linked with TH disruption in tree swallows (Wada et al. 2009). Also, the TH concentration in the mother's circulation was not measured, and it is unclear to what extent the TH concentration in the yolk reflects the mother's TH concentration. It is possible that there is regulation in the transport of THs from the mother to the eggs, in which case any effect of heavy metals on THs might be undetected when measured only from the eggs.

For lysozyme, different metals were expected to have opposite effects on its concentration. Some metals, like lead and cadmium, are known to suppress immune defence, while zinc is necessary for the immune system and may increase the concentration of lysozyme (Chatelain et al. 2016). However, no association between metal pollution and lysozyme was detected in this study. This might be because the metal concentrations were insufficient to cause any noticeable differences in lysozyme concentration, or because there were other factors that were more important for lysozyme concentration and blurred any possible effects of the metals.

Eggshell pigmentation was expected to be more intense and aggregated in the polluted areas. Pigmentation has been suggested to strengthen the eggshell and more pigment is found in thin areas of the eggshell (Gosler et al. 2005). Since pollution causes eggshell thinning (Eeva & Lehikoinen 1995), which pigments may compensate for, pollution can be expected to correlate positively with pigmentation (Gosler et al. 2005, Hargitai et al.

2016a). A difference in pigment patterns between populations was found only in Hungary, where pigment spotting was more aggregated in the polluted zone than in the unpolluted zone. This is in line with a previous study that was conducted in the same area (Hargitai et al. 2016a). The polluted zone had significantly more aggregated pigment spotting than any other population, but interestingly it did not have the highest concentration of any heavy metal studied. In Hungary, pigment aggregation has been associated with copper concentration (Hargitai et al. 2016a). Therefore, the study populations that had a high copper concentration, especially the polluted zones in Finland and Portugal, would be expected to have highly aggregated pigment spotting if copper affects pigment patterns, but this was not observed. The relationship between copper and pigment patterns might not be straightforward and it is possible that the copper concentration in Hungary correlates with some other pollutant in the urban park that causes pigment aggregation but has not been taken into account.

Zone and PC1 had no effect on egg mass, eggshell dry mass or eggshell thickness, so the differences in shell dry mass and egg mass between countries are likely not due to direct effects of pollution (Hargitai et al 2016a). Indeed, egg volume has not been found to correlate with the level of pollution in previous research (Hargitai et al 2016a). Differences in eggshell dry mass were associated with clutch size, which is likely a better predictor of eggshell dry mass than the level of pollution, because clutch size can affect the allocation of resources into the eggs (Pellerin et al. 2016).

The egg components studied are related to egg quality, which can be affected by multiple environmental factors other than pollution, including food availability, temperature, laying date and breeding density, reviewed by Hargitai et al. (2016b). Food availability is crucial for egg quality, as it affects the amount of resources allocated into the eggs. Temperature and laying date are related to food availability by affecting the abundance of insects. Low temperatures also increase the energy needed for thermoregulation, decreasing the available energy for reproduction. High breeding density decreases the amount of resources per individual, and increased social interactions in high density may interfere with foraging.

In addition to environmental conditions, maternal effects and parental quality are important for egg quality. Females can affect egg quality by adjusting the investment of resources into the eggs (Hargitai et al. 2016b). According to the differential allocation hypothesis, females increase their investment when they mate with high-quality males, which improves egg quality (Hargitai et al. 2008). The condition of the female also

influences its investment in egg quality (Espin et al. 2016b). However, neither environmental conditions nor parental quality were assessed in this study, so it is uncertain which factors most affected the results in the egg components.

4.3 Differences in fledging probability between populations and countries

Although zone and PC1 had no effect on fledging probability, there were some notable differences between countries. Belgium had the lowest proportion of failed nests, and Finland had less failed nests than Portugal. Although the interaction of zone and country (i.e. population) was not significant, the proportion of failed nests differed visibly between the two populations of some countries. In Finland and Hungary the percentage of failed nests was clearly higher in the polluted zone (Finland: 41.2 % vs. 18.8 %, Hungary: 60.0 % vs. 23.8 %), whereas in Belgium the unpolluted zone had more failed nests (4.0 % vs. 25.0 %). In Portugal, there was no clear difference between the populations, although the polluted zone seemed to have slightly less failed nests (47.1 % vs. 53.3 %).

It was surprising that even though the polluted zone of Belgium had the significantly highest concentrations of cadmium, lead and arsenic and the lowest eggshell dry mass and egg mass, it appeared to have the highest fledging probability. This is in contradiction with previous studies that have found a decreased fledging success in the polluted zone (Janssens et al. 2003a). The result indicates that the level of pollution has possibly decreased and no longer has a negative effect on reproductive success. Indeed, the concentrations of arsenic, copper and nickel were at least twice lower in this study compared to an earlier study in the area (Table 1 in Janssens et al. 2003a). The concentrations of cadmium and lead were higher in this study, but the variation was very high, so it is unclear whether their concentrations have decreased as well.

In Finland, fledging probability seemed to be lower in the polluted zone compared to the unpolluted zone. The average concentrations of heavy metals in the polluted zone (Table 2) were slightly higher compared to a study that found decreased hatching success and nestling growth in great tits (Table 3 in Eeva et al. 2009). Considering this, it seems that heavy metal pollution continues to impact the reproduction of great tits negatively in the area. However, the level of heavy metal pollution is known to have decreased in the area (Espin et al. 2016a) and the average concentrations of heavy metals in this study might have been biased by the very high variation in the concentrations. Moreover, since zone

and PC1 had no significant association with fledging probability, the low fledging probability in the polluted zone might well be due to some other environmental factors. A small number of observations per population and random variation in the individuals' fitness, which can be due to annual variation in weather conditions and food availability, may also affect the results of each country.

Similarly to Finland, the fledging probability in Hungary seemed to be lower in the polluted zone compared to the unpolluted zone. Decreased reproductive success in the polluted zone has not been reported in previous research, which suggests that the result of this study is likely not explained by pollution, but possibly by random annual variation in reproductive success. In Portugal, the fact that the polluted zone showed no decrease in fledging probability was not unexpected, given that a previous study in the area found better fledging success in the polluted zone compared to the unpolluted zone (Costa et al. 2011a). The most likely cause is a higher availability of invertebrate food in the polluted zone (Costa et al. 2011a).

Apart from pollution, egg quality can influence fledging probability. Egg quality is important for the survival of offspring (Hargitai et al. 2016b). For example, egg mass is associated with hatching probability and the growth and survival of fledglings (Hargitai et al. 2016b). Egg quality can also influence the amount of care the father provides for the offspring (Hargitai et al. 2016b). The effect of egg quality on fledging probability was not tested for in this study, but it seems that other factors, like the conditions after hatching, might have been more important, since the highest fledging probability was in Belgium, which had the lowest eggshell dry mass and egg mass.

4.4 The level and indirect effects of heavy metal pollution

These results do not necessarily suggest that pollution has no effect on the egg components of great tits generally, since the concentrations of heavy metals have likely decreased in the study areas compared to the levels in earlier studies. The heavy metal concentrations were possibly too low for detecting any effect on the response variables. However, there were significant differences in heavy metal concentrations between the populations. For example, the average concentration of nickel was about tenfold and copper more than double in the polluted zone of Finland compared to the other populations, whereas the average concentrations of cadmium and lead were more than twice higher in the polluted zone of Belgium compared to all other populations. Based on

these clear differences in heavy metal concentrations it could be expected that some differences in the egg components would have been detected if heavy metals affect them, but no differences were observed.

Heavy metals can, however, have indirect effects on the egg components, such as egg mass via food availability. In Belgium, which had lower egg mass than Finland and Hungary (Fig. 9), the polluted zone had the highest concentrations of cadmium, lead and arsenic. These heavy metals may have had negative effects on insect and snail abundance, decreasing food availability (Eeva et al. 1997, Eeva et al. 2010). Food availability limits egg mass, which is a costly resource for the female (Ruuskanen et al. 2016a). The average concentration of calcium was relatively low in Belgium (Table 2), which suggests that the availability of calcium-rich foods was indeed lower than in some other populations. However, food and calcium availability may have varied independently of heavy metal pollution, too. The differences in egg mass in Belgium could also be linked to clutch size (Pellerin et al. 2016), although clutch size was not significantly associated with egg mass.

A low availability of calcium can also increase the negative effects of heavy metals, as heavy metals bind to calcium-binding proteins when calcium is low and their absorbtion increases (Dauwe et al. 2006). The relatively low availability of calcium (Table 2) and high heavy metal pollution in the polluted zone of Belgium could have been expected to decrease fledging probability by negative effects on eggshell quality (Eeva et al. 2009), but on the contrary, fledging probability was high (Table 3). This seems counter-intuitive, but as noted above, many other factors can influence fledging probability. Also, fledging probability only tells whether the nest failed completely or had at least one fledgling, not how many fledglings left the nest.

4.5 Limitations of the methods

The methods and sample sizes of this study may have affected the results. The sample sizes in the populations were rather small, which may have limited the statistical power of the analyses. Variation in the concentrations of individual metals within populations was very high, and errors in the analyses are possible, although all metals were analysed with the same method in the same laboratory. Accuracy was limited in the visual estimation of pigment patterns and in the measuring of eggshell thickness with a micrometer.

4.6 Conclusions

In conclusion, this study found little evidence for a negative effect of heavy metal pollution on the egg components and breeding performance of great tits. It seems that the level of pollution has decreased in the study areas and populations of great tits are doing well even in the vicinity of the pollution sources. This result is positive in the sense that during the last decades, research has shown a variety of detrimental effects of pollution on avian reproduction and egg components in the study areas. However, it must be noted that the results should be interpreted and generalized with caution. For example, although no effects of pollution on THs or lysozyme were found, this might not be the case in all situations. Also, great tits are known to tolerate pollution relatively well, so more sensitive species, like the pied flycatcher, might have different responses to heavy metals (Eeva & Lehikoinen 1995).

It is important to take these aspects into consideration in future studies. Since the level of pollution has decreased in the study areas, other areas with heavier pollution might be more useful in studying the effects of heavy metals on birds. More bird species should be studied to compare between-species differences in responses to heavy metals. Long term studies that take into account the annual variation in weather conditions and ecological factors, like food availability, are recommended.

5 ETHICAL STATEMENT

Ethics were taken into account in planning the data collection. The great tit is a common species and the collected number of eggs (one egg per nest) was small in relation to the population size. Fledglings were unharmed during handling. Permissions for collecting the data were given by local authorities in each country.

6 ACKNOWLEDGEMENTS

I would like to thank everyone in the research groups who participated in collecting and analysing the study material. A huge thanks to my supervisor Suvi Ruuskanen for her valuable advice and help during the process of making this thesis.

7 REFERENCES

Baos R, Blas J, Bortolotti GR, Marchant TA, Hiraldo F (2006) Adrenocortical response to stress and thyroid hormone status in free-living nestling white storks (*Ciconia ciconia*) exposed to heavy metal and arsenic contamination. *Environmental Health Perspectives* 114: 1497–1501.

Bentley GE, Goldsmith AR, Dawson A, Glennie LM, Talbot RT, Sharp PJ (1997) Photorefractoriness in European starlings (*Sturnus vulgaris*) is not dependent upon the long-day-induced rise in plasma thyroxine. *General and Comparative Endocrinology* 107:428–438.

Berglund AMM, Koivula MJ, Eeva T (2011) Species- and age-related variation in metal exposure and accumulation of two passerine bird species. *Environmental Pollution* 159:2368–2374.

Bonisoli-Alquati A, Rubolini D, Romano M, Cucco M, Fasola M, Caprioli M, Saino N (2010) Egg antimicrobials, embryo sex and chick phenotype in the yellow-legged gull. *Behavioral Ecology and Sociobiology* 64:845–855.

Chastel O, Lacroix A, Kersten M (2003) Pre-breeding energy requirements: thyroid hormone, metabolism and the timing of reproduction in house sparrows *Passer domesticus*. *Journal of Avian Biology* 34:298–306.

Chatelain M, Gasparini J, Haussy C, Frantz A (2016) Trace Metals Affect Early Maternal Transfer of Immune Components in the Feral Pigeon. *Physiological and Biochemical Zoology* 89:206–212.

Collin A, Cassy S, Buyse J, Decuypere E, Damon M (2005) Potential involvement of mammalian and avian uncoupling proteins in the thermogenic effect of thyroid hormones. *Domestic Animal Endocrinology* 29:78–87.

Costa RA, Eeva T, Eira C, Vaqueiro J, Vingada JV (2011a) Effects of air pollution from pulp and paper industry on breeding success of Great tit in maritime pine forests. *Ecoscience* 18:115–123.

Costa RA, Petronilho JMS, Soares AMVM, Vingada JV (2011b) The use of passerine feathers to evaluate heavy metal pollution in central Portugal. *Bulletin of Environmental Contamination and Toxicology* 86:352–356.

Costa RA, Eeva T, Eira C, Vaqueiro J, Vingada JV (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* 63:594–600.

Cucco M, Malacarne G, Ottonelli R, Patrone M (2006) Repeatability of cell-mediated and innate immunity, and other fitness-related traits, in the Grey Partridge. *Canadian Journal of Zoology* 84:72–79.

D'Alba L, Shawkey MD, Korsten P, Vedder O, Kingma SA, Komdeur J, Beissinger SR (2010) Differential deposition of antimicrobial proteins in blue tit (Cyanistes caeruleus) clutches by laying order and male attractiveness. *Behavioral Ecology and Sociobiology* 64:1037–1045.

Dauwe T, Bervoets L, Blust R, Pinxten R, Eens M (1999) Are eggshells and egg contents of Great and Blue Tits suitable as indicators of heavy metal pollution? *Belgian Journal of Zoology* 129:439–447.

Dauwe T, Bervoets L, Blust R, Pinxten R, Eens M (2000) Can excrement and feathers of nestling songbirds be used as biomonitors for heavy metal pollution? *Archives of Environmental Contamination and Toxicology* 39:541–546.

Dauwe T, Snoeijs T, Bervoets L, Blust R, Eens M (2006) Calcium availability influences lead accumulation in a passerine bird. *Animal Biology* 56:289–298.

Drent PJ, van Oers K, van Noordwijk AJ (2003) Realized heritability of personalities in the great tit (*Parus major*). *Proceedings of the Royal Society B-Biological Sciences* 270:45–51.

Eeva T, Ahola M, Lehikoinen E (2009) Breeding performance of blue tits (*Cyanistes caeruleus*) and great tits (*Parus major*) in a heavy metal polluted area. *Environmental Pollution* 157:3126–3131.

Eeva T, Lehikoinen E (1995) Egg-shell quality, clutch size and hatching success of the great tit (*Parus major*) and the pied flycatcher (*Ficedula hypoleuca*) in an air-pollution gradient. *Oecologia* 102:312–323.

Eeva T, Lehikoinen E (1996) Growth and mortality of nestling great tits (*Parus major*) and pied flycatchers (*Ficedula hypoleuca*) in a heavy metal pollution gradient. *Oecologia* 108:631–639.

Eeva T, Lehikoinen E (2004) Rich calcium availability diminishes heavy metal toxicity in *Pied Flycatcher*. *Functional Ecology* 18:548–553.

Eeva T, Lehikoinen E, Pohjalainen, T (1997) Pollution-related variation in food supply and breeding success in two hole-nesting passerines. *Ecolocy* 78:1120–1131.

Eeva T, Rainio K, Suominen O (2010) Effects of pollution on land snail abundance, size and diversity as resources for pied flycatcher, *Ficedula hypoleuca*. *Science of the Total Environment* 408:4165–4169.

Espin S, Ruiz S, Sanchez-Virosta P, Eeva T (2016a) Effects of calcium supplementation on growth and biochemistry in two passerine species breeding in a Ca-poor and metal-polluted area. *Environmental Science and Pollution Research* 23:9809–9821.

Espin S, Ruiz S, Sanchez-Virosta P, Salminen JP, Eeva T (2016b) Effects of experimental calcium availability and anthropogenic metal pollution on eggshell characteristics and yolk carotenoid and vitamin levels in two passerine birds. *Chemosphere* 151:189–201.

Fleeger JW, Carman KR, Nisbet RM (2003) Indirect effects of contaminants in aquatic ecosystems. *Science of the Total Environment* 317:207–233.

Geens A, Dauwe T, Bervoets L, Blust R, Eens M (2010) Haematological status of wintering great tits (*Parus major*) along a metal pollution gradient. *Science of the Total Environment* 408:1174–1179.

Graveland J, Vanderwal R, Vanbalen JH, Vannoordwijk AJ (1994) Poor reproduction in forest passerines from decline of snail abundance on acidified soils. *Nature* 368:446–448.

Gosler AG, Higham JP, Reynolds SJ (2005) Why are birds' eggs speckled? *Ecology Letters* 8: 1105–1113.

Grimm NB, Foster D, Groffman P, Grove JM, Hopkinson CS, Nadelhoffer KJ, Pataki DE, Peters DPC (2008) The changing landscape: ecosystem responses to urbanization and pollution across climatic and societal gradients. *Frontiers in Ecology and the Environment* 6:264–272.

Gupta P, Kar A (1998) Role of ascorbic acid in cadmium-induced thyroid dysfunction and lipid peroxidation. *Journal of Applied Toxicology* 18:317–320.

Hargitai R, Herényi M, Török J (2008) Eggshell coloration in relation to male ornamentation, female condition and egg quality in the collared flycatcher *Ficedula albicollis*. *Journal of Avian Biology* 39:413–422.

Hargitai R, Nagy G, Herényi M, Török J (2013) Effects of experimental calcium availability, egg parameters and laying order on Great Tit Parus major eggshell pigment patterns. *Ibis* 155:561–570.

Hargitai R, Nagy G, Nyiri Z, Bervoets L, Eke Z, Eens M, Török J (2016a) Effects of breeding habitat (woodland versus urban) and metal pollution on the egg characteristics of great tits (*Parus major*). *Science of the Total Environment* 544:31–38.

Hargitai R, Herényi M, Nagy G, Nyiri Z, Eke Z, Török J (2016b) Effects of environmental conditions on the egg mass, yolk antioxidant level, eggshell thickness and eggshell spotting patterns of Great Tits (*Parus major*). *Journal of Ornithology* 157:995–1006.

Howdeshell KL (2002) A model of the development of the brain as a construct of the thyroid system. *Environmental Health Perspectives* 110:337–348.

Hsu BY, Dijkstra C, Darras VM, de Vries B, Groothuis TGG (2017) Maternal thyroid hormones enhance hatching success but decrease nestling body mass in the rock pigeon (*Columba livia*). *General and Comparative Endocrinology* 240:174–181.

Jagannath A, Shore RF, Walker LA, Ferns PN, Gosler AG (2008) Eggshell pigmentation indicates pesticide contamination. *Journal of Applied Ecology* 45:133–140.

Janssens E, Dauwe T, Bervoets L, Eens M (2001) Heavy metals and selenium in feathers of great tits (*Parus major*) along a pollution gradient. *Environmental Toxicology and Chemistry* 20:2815–2820.

Janssens E, Dauwe T, Pinxten R, Bervoets L, Blust R, Eens M (2003b) Effects of heavy metal exposure on the condition and health of nestlings of the great tit (*Parus major*), a small songbird species. *Environmental Pollution* 126:267–274.

Janssens E, Dauwe T, Pinxten R, Eens M (2003a) Breeding performance of great tits (*Parus major*) along a gradient of heavy metal pollution. *Environmental Toxicology and Chemistry* 22:1140–1145.

Javůrková V, Krkavcova E, Kreisinger J, Hyrsl P, Hyankova L (2015) Effects of experimentally increased in ovo lysozyme on egg hatchability, chicks complement activity, and phenotype in a precocial bird. *Journal of Experimental Zoology Part a-Ecological Genetics and Physiology* 323:497–505.

Killpack TL, Oguchi Y, Karasov WH (2013) Ontogenetic patterns of constitutive immune parameters in altricial house sparrows. *Journal of Avian Biology* 44:513–520.

Kim B (2008) Thyroid hormone as a determinant of energy expenditure and the basal metabolic rate. *Thyroid* 18:141–144.

Koivula MJ, Eeva T (2010) Metal-related oxidative stress in birds. *Environmental Pollution* 158:2359–2370.

Li YG, Yan ZC, Wang DH (2010) Physiological and biochemical basis of basal metabolic rates in Brandt's voles (*Lasiopodomys brandtii*) and Mongolian gerbils (*Meriones unguiculatus*). *Comparative Biochemistry and Physiology* 157:204–211.

Marshall DJ, Uller T (2007) When is a maternal effect adaptive? *Oikos* 116:1957–1963.

Masschalck B, Michiels CW (2003) Antimicrobial properties of lysozyme in relation to foodborne vegetative bacteria. *Critical Reviews in Microbiology* 29:191–214.

McNabb FMA (2007) The hypothalamic-pituitary-thyroid (HPT) axis in birds and its role in bird development and reproduction. *Critical Reviews in Toxicology* 37:163-193.

McNabb FMA, Fox GA (2003) Avian thyroid development in chemically contaminated environments: is there evidence of alterations in thyroid function and development? *Evolution & Development* 5: 76-82.

McNabb FMA (2006) Avian thyroid development and adaptive plasticity. *General and Comparative Endocrinology* 147:93–101.

Miao W, Zhu BR, Xiao XH, Li Y, Dirbaba NB, Zhou BS, Wu HJ (2015) Effects of titanium dioxide nanoparticles on lead bioconcentration and toxicity on thyroid endocrine system and neuronal development in zebrafish larvae. *Aquatic Toxicology* 161:117–126.

Morales J, Ruuskanen S, Laaksonen T, Eeva T, Mateo R, Belskii E, Ivankina EV, Järvinen A, Kerimov A, Korpimäki E, Krams I, Mänd R, Morosinotto C, Orell M, Qvarnstrom A, Siitari H, Slater FM, Tilgar V, Visser ME, Winkel W, Zang H, Moreno J (2013) Variation in eggshell traits between geographically distant populations of pied flycatchers *Ficedula hypoleuca*. *Journal of Avian Biology* 44:111–120.

Morosinotto C, Ruuskanen S, Thomson RL, Siitari H, Korpimäki E, Laaksonen T (2013) Predation risk affects the levels of maternal immune factors in avian eggs. *Journal of Avian Biology* 44:427–436.

Nagajyoti PC, Lee KD, Sreekanth TVM (2010) Heavy metals, occurrence and toxicity for plants: a review. *Environmental Chemistry Letters* 8:199–216.

Pellerin S, Paquette SR, Pelletier F, Garant D, Belisle M (2016) The trade-off between clutch size and egg mass in tree swallows *Tachycineta bicolor* is modulated by female body mass. *Journal of Avian Biology* 47:500–507.

Power DM, Llewellyn L, Faustino M, Nowell MA, Bjornsson BT, Einarsdottir IE, Canario AVM, Sweeney GE (2001) Thyroid hormones in growth and development of fish. Comparative *Biochemistry and Physiology* 130:447–459.

Reynolds SJ, Mänd R, Tilgar V (2004) Calcium supplementation of breeding birds: directions for future research. *Ibis* 146: 601–614.

Ruuskanen S, Siitari H, Eeva T, Belskii E, Jarvinen A, Kerimov A, Krams I, Moreno J, Morosinotto C, Mänd R, Mostl E, Orell M, Qvarnstrom A, Salminen JP, Slater F, Tilgar V, Visser ME, Winkel W, Zang H, Laaksonen T (2011) Geographical variation in egg mass and egg content in a passerine bird. *PLoS ONE* 6: e25360. https://doi.org/10.1371/journal.pone.0025360.

Ruuskanen S, Darras VM, de Vries B, Visser ME, Groothuis TGG (2016a) Experimental manipulation of food availability leads to short-term intra-clutch adjustment in egg mass but not in yolk androgen or thyroid hormones. *Journal of Avian Biology* 47:36–46.

Ruuskanen S, Darras VM, Visser ME, Groothuis TGG (2016b) Effects of experimentally manipulated yolk thyroid hormone levels on offspring development in a wild bird species. *Hormones and Behavior* 81:38–44.

Ruuskanen S, Groothuis TGG, Schaper SV, Darras VM, de Vries B, Visser ME (2016c) Temperature-induced variation in yolk androgen and thyroid hormone levels in avian eggs. *General and comparative endocrinology* 235:29–37.

Ruuskanen S, Gienapp P, Groothuis TGG, Schaper SV, Darras VM, Pereira C, de Vries B, Visser ME (2016d) Heritable variation in maternally derived yolk androgens, thyroid hormones and immune factors. *Heredity* 117:184–190.

Saino N, Dall'ara P, Martinelli R, Moller AP (2002) Early maternal effects and antibacterial immune factors in the eggs, nestlings and adults of the barn swallow. *Journal of Evolutionary Biology* 15:735–743.

Silva JE (1995) Thyroid hormone control of thermogenesis and energy balance. *Thyroid* 5: 481–492.

Van Balen JH (1973) A comparative study of the breeding ecology of the great tit *Parus major* in different habitats. *Ardea* 61:1–93 https://doi.org/10.5253/arde.v61.p1.

Wada H, Cristol DA, McNabb FMA, Hopkins WA (2009) Suppressed adrenocortical responses and thyroid hormone levels in birds near a mercury-contaminated river. *Environmental Science & Technology* 43:6031–6038.