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Vitamin profiles in two free-living passerine birds under a metal pollution gradient – A calcium supplementation experiment

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ABSTRACT

Vitamin and carotenoid deficiency may impair development in free-living vertebrates, because of the importance of these micronutrients to growth, antioxidant defense and calcium regulation. Micronutrient and calcium insufficiency can be intensified by metal pollution which can interfere with nutrient homeostasis or indirectly reduce food availability. Furthermore, absorption of dietary heavy metals is dependent on food calcium and vitamin levels. We investigated the effect of calcium on plasma vitamin and carotenoid profiles and how these affected growth and survival in two passerine birds with different calcium turnover living along a metal pollution gradient. Vitamins (A, D_3 and E) and carotenoids were quantified from blood plasma of great tit (*Parus major*) and pied flycatcher (*Ficedula hypoleuca*) nestlings. Metal concentrations in soil and in feces from the same nestlings were used to assess the exposure to air pollution. Additionally, we examined the vitamin level variation between developmental stages (eggs and nestlings within the same brood). Our results showed that generally higher concentrations of vitamins and carotenoids circulate in blood of great tits than in pied flycatchers. In general, birds inhabiting the polluted zone presented lower concentrations of the studied micronutrients. Calcium supplementation and metal pollution decreased vitamin A concentration in pied flycatcher, but not in great tit, while vitamin A affected growth and survival in great tit and pied flycatcher respectively. Our results suggest that populations under exposure to metal pollution may experience increased vitamin A deficiency, and that the two passerine species, while obtaining similar micronutrients in food, respond differently to environmental disturbance of nutrients.

1. Introduction

Calcium (Ca) represents one of the most important micronutrients for breeding insectivorous birds (Reynolds et al., 2004). Calcium constitutes the main component of eggshell and it is limiting for skeleton mineralization and nestling growth (Reynolds et al., 2004; Tilgar et al., 2004, 2005). The availability of dietary calcium is crucial for small insectivores, which cannot store adequate amounts of calcium in their skeleton (Dutta et al., 1998; Graveland and van Gijzen, 1994). To make things more complex, natural calcium availability varies depending on soil characteristics (Patten, 2007). For example, metal deposition from anthropogenic activities including mining and smelting, alter the composition and pH of soil, consequently limiting calcium availability (Maynard et al., 2014; Pabian and Brittingham, 2012).

The requirements of calcium vary among bird species and are influenced by life-history traits such as clutch size, migratory status and feeding strategies (Bar, 2008; Graveland, 1996; Pahl et al., 1997; Patten, 2007; Reynolds et al., 2004; Tilgar et al., 2004). Lack or surplus of dietary calcium may also have consequences for the homeostasis of other micronutrients such as vitamins (Schwalfenberg and Genuis, 2015). In fact, interactions between calcium and vitamins have been evidenced in other vertebrates. For example, vitamin A deficient diets have resulted in less deposited calcium in bones of birds and mammals (Navia and Harris, 1980), while supplementing calcium and vitamin D_3 in diet have decreased circulating vitamin E (alpha-tocopherol) in humans (Chai et al., 2012).

Similarly to calcium, vitamins (and carotenoids) may also become limiting nutrients for growth and development in wild animals (Cortes et al., 2006; de Ayala et al., 2006; Marri and Richner, 2014; Matrkova and Remes, 2014; Parolini et al., 2015). It is known that the main cause for vitamin deficiency is its limited consumption in food (Balk et al., 2009; Dierenfeld, 1989; Honour et al., 1995). However, deficiency can

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also develop as a consequence of internal disturbance of homeostasis, intensified when external stress in the form of diseases or ambient contaminant exposure follows (Hõrak et al., 2004).

In free-ranging vertebrates, the disruption of vitamin metabolism by environmental contamination has been documented in populations exposed to metals and organic contaminants (Martinez-Haro et al., 2011; Rodríguez-Estival et al., 2011; Routti et al., 2008). To date, only a few studies have reported vitamin profiles in free-living birds inhabiting environments disturbed by man (Hõrak et al., 2004; Martinez-Haro et al., 2011; Ruiz et al., 2016), and to our best knowledge, the effect of dietary calcium with respect to vitamins has not been investigated in developing young wild birds.

Here, we studied two insectivorous birds, the great tit (*Parus major*) and the pied flycatcher (*Ficedula hypoleuca*), two passerines with different calcium requirements. Resident tits lay bigger clutches and present a highly mineralized skeleton (Gosler, 1993; Tilgar et al., 2005) while the flycatchers lay fewer eggs and present lower body mass adapted for long-distance migration (Lundberg and Alatalo, 1992).

In this study, we aimed to 1) Assess baseline levels and differences in circulatory fat-soluble vitamins and carotenoids between species and their effect on growth and survival probability; 2) Evaluate vitamin status in metal polluted and reference sites; 3) Investigate the effect of calcium supplementation on vitamin profiles; 4) Compare variation of vitamin levels between developmental stages (nestlings vs. egg) and 5) Assess natural nutrient availability by measuring soil properties and caterpillar abundance.

Based on previous comparative studies in the same area (Eeva et al., 2000, 2012), we expect vitamins and carotenoids to differ between species due to the species specific diets, susceptibility to metal-related pollution, calcium turnover and the timing of breeding with food abundance. At the same time, we expect the variation in circulating nutrients to be strongly affected by the diet availability. Regarding the metal-related effects on circulating nutrients, we expect that circulating vitamins will be lower in the polluted zone. However, we recognize that calcium supplementation may alter these predictions, due to interactions between A, D_3 and calcium.

2. Materials and methods

2.1. Study species

The great tit (*Parus major*) is a ubiquitous European resident passerine species. Adults weigh 18–19 g and their mean clutch size is 9 eggs (Eeva and Lehikoinen, 1995). The pied flycatcher (*Ficedula hypoleuca*) is a migratory passerine weighing on average 12–13 g, with an average clutch size of 6 eggs (Eeva and Lehikoinen, 1995). The pied flycatcher winters in West Africa, beginning its migration in March towards the north and Eastern Europe where it arrives in early May (Scandinavian populations)(Lundberg and Alatalo, 1992). Both species are insectivores, the diet of nestlings consisting primarily on lepidopterans (caterpillars), beetles, spiders and hymenopterans (Cramp and Perrins, 1993). They are cavity-nesters which readily accept man-constructed nest boxes. Thus, monitoring them on a regular basis can be easily accomplished during their breeding season. For these reasons, they are considered good bioindicator species of pollutant exposure (Belskii et al., 1995; Berglund et al., 2015; Dauwe et al., 2005; Eeva et al., 2000; Janssens et al., 2001; Sánchez-Virosta et al., 2015).

2.2. Experimental design and sampling

From April-July 2014, we monitored breeding great tits and pied flycatchers in the vicinity of the Copper-Nickel smelter (61°20′ N, 22°10′ E) in Harjavalta, southwestern Finland. The smelter, established in 1940, emits a combination of metal elements into the surrounding area including arsenic (As), cadmium (Cd), copper (Cu), lead (Pb), mercury (Hg), nickel (Ni), sulphur (S) and zinc (Zn). Although emissions have decreased since 1990, as a result of improved technology, disturbances caused by metal deposition have been described in vegetation and wild animals (Kiikkilä 2003). We examined ca. 400 nest boxes (Lambrechts et al., 2010) from the beginning of nest building until fledging. Egg laying, hatching and nestling development were also recorded. A nest was randomly assigned to either a Ca-supplemented or Control treatment when nest building was at an advanced stage. For the treatment, round plastic feeders (3 cm diameter) were placed inside the nest box containing 5 g of commercial (Versele-Laga) crushed mussel shells (Ca-supplemented). The feeders were left empty for the Control-treatment. Ca-consumption was quantified in 3 stages: egg laying, incubation and nestling period by weighing the remaining supplement at the end of each period and replacing that with 5 g of fresh supplement after egg laying and incubation. At the end of the nestling period, the remaining calcium was weighed and the feeder was removed from the nest box. In addition, nests were grouped according to the distance from the smelter as follows: Polluted ($<$ 2 km) and Unpolluted ($>$ 5 km).

Egg laying was recorded from the laying of first egg. One egg (3rd − 5th egg in the sequence) was collected for egg yolk vitamin measurements (µg/g, dry mass; reported in Espín et al., 2016a). At hatching date nestlings were assigned the age d0, and subsequently in numerical order until the age of fledging. At d3, nestlings were counted and the wing length was recorded. At d7 the nestlings were banded individually and combined fecal sacs from 2 to 3 nestlings per brood were collected directly into a 1.5 mL Eppendorf tube and stored at −20 °C until metal analysis. Morphometric measurements: length (mm) of wing, tarsus and head and weight (g) of every nestling in a brood were recorded at d7 for both species. These measurements were recorded again at d12 (pied flycatcher) and at d14 (great tit), and used to calculate growth rates. The two-day difference in measurements is due to the earlier fledging date of pied flycatchers.

Blood samples were collected at two time points: on d9 and d14 for the great tit (except one early brood which was sampled at d7) and d7 and d12 for the pied flycatcher. Blood was collected from the brachial vein using a heparinized capillary tube up to a volume of 75 µL per nestling. Capillaries were centrifuged to separate the red blood cell fraction from the blood plasma using a LW Scientific ZIPocrit Hematocrit Centrifuge at 4400*g* for 5 min at room temperature. At both time points, red blood cells were separated for each individual and on d7/d9 plasma was pooled by brood to obtain a volume of 90 µL for vitamin analysis. After spinning, both samples were immediately flash frozen in liquid nitrogen and stored at −80 °C until analysis. The plasma samples were protected from light at all times to avoid the photolytic degradation of the vitamins. Blood plasma collected at the second time point was used for biochemistry analysis reported in Espín et al. (2016b) and the red blood cell fraction is the scope of upcoming studies.

Natural food availability was assessed by the frass-fall method (Southwood, 1978), which indirectly quantifies the biomass of caterpillars in tree canopies by measuring their falling fecal material (Eeva et al., 1997, 1998). The sampling took place between 28 April and 29 July 2014. We placed round plastic funnels (diameter 34 cm) at 1 m height around the trunk of birch (*Betula* spp.) and pine (*Pinus sylvestris*) trees in ten study sites. Plastic collectors at the bottom of the funnels were emptied once every two weeks. Thus, a total of seven periods of an average of 13.3 days per period, were recorded. The contents were stored in paper bags, separated from litter and weighted. Frass mass per day (ng/day) is used as a carotenoid-rich natural food proxy and hereafter denoted as caterpillar index in a study site.

2.3. Plasma vitamin analysis

The vitamin analysis protocol was based on Priego Capote et al. (2007) and also described in Ruiz et al. (2016). First, the protein fraction was precipitated with 1 mL methanol, vortex-mixed for 15 min