



# The effect of aquatic and terrestrial prey availability on metal accumulation in pied flycatcher (*Ficedula hypoleuca*) nestlings

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## ARTICLE INFO

### Keywords:

Dietary exposure  
Metals  
Passerine  
Aquatic subsidies  
Invertebrates

## ABSTRACT

Ingestion of contaminated prey is a major route for metal exposure in terrestrial insectivores. In terrestrial ecosystems adjacent to lakes and streams, emerging aquatic insects can transport metals, accumulated during their larval stage, from aquatic to terrestrial ecosystems. However, contaminant exposure via aquatic insects has often been ignored in terrestrial environments, despite such insects representing a substantial part of the diet for terrestrial insectivores living close to lakes and streams. In this study, we investigated how dietary lead (Pb) and calcium (Ca) exposure from different aquatic and terrestrial prey types affects Pb accumulation in pied flycatcher (*Ficedula hypoleuca*) nestlings living close to a former Pb/zinc (Zn) mine in northern Sweden, which closed in 2001. Stable isotope analysis ( $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ ) of nestling blood and different prey types was used to estimate nestlings' diet. Ants, Lepidoptera larvae and Trichoptera were the most common prey types in the nestlings' diet, in which aquatic prey types (Trichoptera included) accounted for 2.0–96.4%. Ingestion of specific prey groups, such as aquatic insects and ants, were important for Pb accumulation in nestlings, and when access to aquatic prey was low, ants were the predominant source of Pb. The influence of dietary Ca on Pb accumulation was less consistent, but Ca availability was relatively high and often co-occurred with high Pb concentrations in invertebrates. The study shows that both the proportion of different prey and their individual metal concentrations need to be considered when estimating exposure risks for insectivores. Further, it highlights the need to account for metal exposure from emerging aquatic insects for terrestrial insectivores living close to lakes and streams.

## 1. Introduction

Metal contamination is an important threat to the environment (Dudka and Adriano, 1997; Fields, 2003; Mishra et al., 2019), exerting toxic effects on exposed organisms (Janssens et al., 2003a; Eeva and Lehikoinen, 2004; Berglund et al., 2010; Beyer et al., 2013). For instance, birds exposed to lead (Pb) show suppressed immune system functioning (Franson and Pain, 2011), behavioral changes (Janssens et al., 2003b; Burger and Gochfeld, 2005), reduced hemoglobin levels (Beyer et al., 2004; Berglund et al., 2010; Cid et al., 2018) and reduced reproduction success (Berglund et al., 2010).

Dietary exposure has previously been identified as a major route for metal exposure (including Pb), in insectivorous birds but Pb exposure may differ between species depending on the bird's dietary preferences (Eeva et al., 1997; Beck et al., 2013; Belskii and Belskaya, 2013). This is the result of species-specific metal accumulation differences between invertebrates (Hare, 1992; Rainbow, 2002) and may be attributed to the different feeding strategies and behavior of invertebrates (Heikens et al.,

2001; Hare et al., 2003; Gall et al., 2015). For instance, herbivorous invertebrates, such as Lepidoptera larvae, generally contain low metal concentrations, even in contaminated environments, which could be due to them developing an aversion to feeding on metal rich plant material (Gall et al., 2015). Pb accumulation may also depend on the habitat preference of invertebrates, and invertebrates in close contact with contaminated soil may ingest larger amounts of contaminated soil particles compared to aerial- and other invertebrates less exposed to soil (Heikens et al., 2001). However, dietary availability of protective elements may also influence Pb accumulation in a consumer. As an example, high availability of calcium (Ca) reduces Pb uptake in the gut due to competition between Ca and Pb in the gastrovascular tract (Scheuhammer, 1996; Dauwe et al., 2006). A diet rich in Ca could, therefore, reduce Pb accumulation and its toxic effects (Scheuhammer, 1996; Eeva and Lehikoinen, 2004; Espin et al., 2017). As such, available insects and diet choice could have a great influence on the level of Pb exposure that birds experience in contaminated environments.

Previous attempts to identify dietary exposure sources for terrestrial

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<https://doi.org/10.1016/j.envres.2022.112779>

Received 6 January 2021; Received in revised form 24 November 2021; Accepted 18 January 2022

Available online 25 January 2022

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insectivores have generally focused solely on terrestrial prey types (Eeva et al. 1997, 2005; Berglund et al., 2009; Belskii and Belskaya, 2013). However, insectivorous birds may also be exposed to metals acquired through aquatic ecosystems; indeed, birds living near lakes and streams rely heavily on emerging aquatic insects for food (Nakano and Murakami, 2001; Baxter et al., 2005; Uesugi and Murakami, 2007; Treveline et al., 2018). Aquatic insects can transfer metals from contaminated water systems when they emerge as flying adults and become part of terrestrial food webs (Beck et al., 2013; Kraus et al., 2014a; Alberts and Sullivan, 2016; Schindler and Smits, 2017). As such, insectivorous birds living close to lakes and streams are potentially exposed to metals from both aquatic and terrestrial ecosystems, and birds with access to prey of an aquatic origin might, therefore, experience different dietary exposure sources than birds with mainly terrestrial prey. However, the extent to which aquatic insects contribute to dietary metal exposure has seldom been evaluated in comparison to terrestrial prey types.

The aim of the study was to investigate how different prey types affect Pb exposure and uptake in birds, using nestlings of the insectivorous pied flycatcher (*Ficedula hypoleuca*) as the subject species. We assessed the contribution of aquatic and terrestrial prey to nestling diet using  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ . Compared to traditional observational methods, such as prey identification using video recordings and neck collars, stable isotopes can give information about the diet over a longer period of time (Hobson and Clark, 1992; Pagani-Nunez et al., 2017), which better illustrates the continuous dietary metal exposure for predators (Craighead and Bedrosian, 2008). In addition, we evaluated the influence of dietary Ca availability on Pb uptake in flycatchers. Our hypotheses were that i) increased dietary Pb exposure from all prey combined is accompanied by increased Pb accumulation in flycatchers, ii) high concentrations of Ca in the diet decrease Pb accumulation in flycatchers, and iii) aquatic prey types are important contributors to Pb

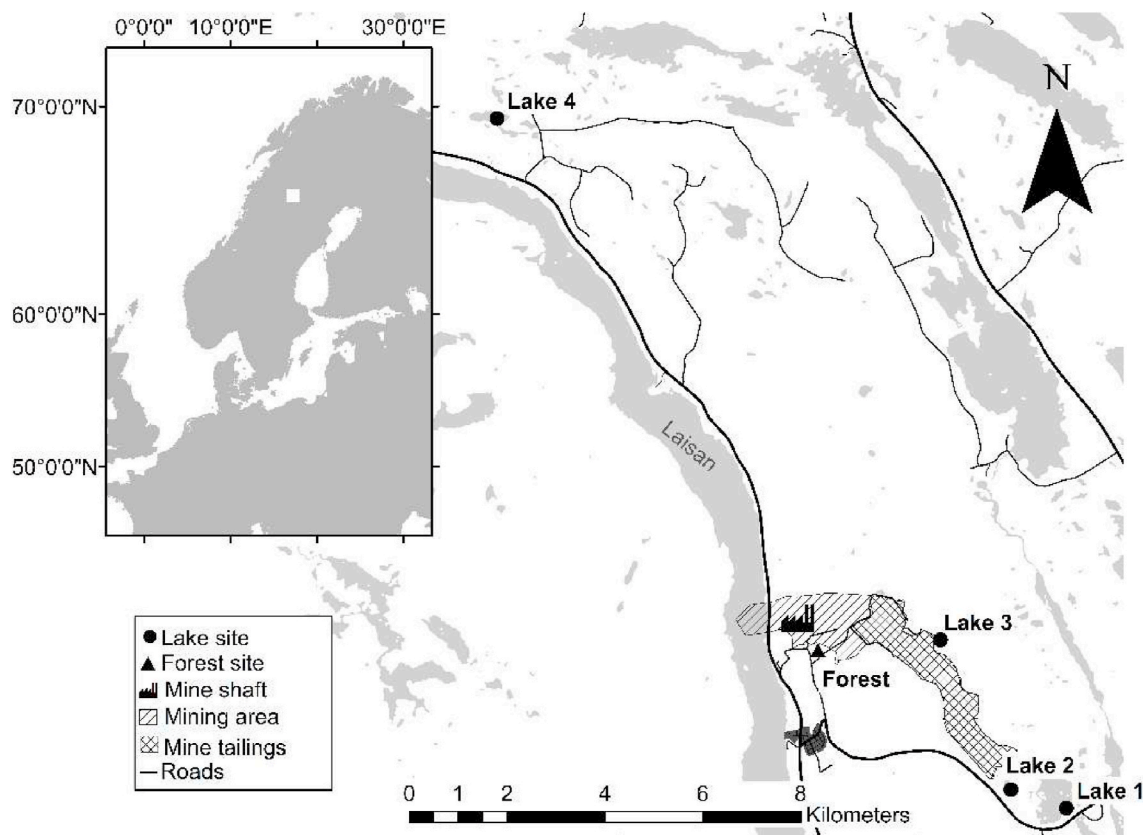
accumulation in flycatchers.

## 2. Material and methods

### 2.1. Study design and site description

The study was conducted during June and July in 2017–2020 at five sites located on the edge of the sub-alpine region near a closed Pb/zinc (Zn) mine in Laisvall, Northern Sweden (0.5–12 km from the mine shaft, Fig. 1). The mine was worked underground, with the excavated ore dominated by galena (PbS) and sphalerite (ZnS) (Rickard et al., 1975). The mine was the largest Pb producer in Europe during production (1943–2001) with a peak production of 95 000 ton/year (Widmark, 1983). Tailing ponds (covering approximately 3 km<sup>2</sup>) and clearing ponds were established east of the mine to prevent particles entering adjacent lakes (Boliden, 1966). Elevated levels of Pb have been observed in the environment surrounding the mine during and after production, which was transported through atmospheric deposition (Berglund and Nyholm, 2011) and by runoff from contaminated soils and tailings to adjacent aquatic environments (Lidman et al., 2020a). Hence, even though metal deposition and transportation has decreased since production stopped, organisms in the area are still exposed to metals (Berglund et al., 2010; Lidman et al., 2020b). The vegetation in the area is dominated by old-growth Norway spruce (*Picea abies*) and Scots pine (*Pinus sylvestris*), with deciduous trees (*Alnus*, *Betula*, *Salix*) along lake shores. Mean annual temperature and precipitation (2017–2018) at the site were 0.36 °C and 625 mm, respectively (Boliden, 2018).

The five sampling sites covered different contamination levels in soils and sediments (Table 1). Four sites were located along lake shores (labelled Lakes 1–4, Fig. 1) and one site was located in a forested area with no lake or large stream within 300 m (labelled Forest, Fig. 1). The



**Fig. 1.** The location of the five study sites near the closed Pb and Zn mine in northern Sweden. Lake sites are indicated by points and the forest site by a triangle. The mining area, illustrated by the grey, dashed area, is partly located under the lake Laisan, and the mine tailings, illustrated by the grey double dashed area, expands both west and south east.

**Table 1**

Lead (Pb) and calcium (Ca) concentration in the organic soil layer (n = 5) and surface sediment (n = 1) at the different sites. Concentrations in soils are provided as means with 95% confidence intervals within brackets, and different letters within the same column indicate significant differences between sites.

Site	Soil Pb (µg/g)	Ca (mg/g)	Sediment Pb (µg/g)	Ca (mg/g)
Lake 1	330 (273–387) <sup>a</sup>	10.13 (7.93–12.33) <sup>ab</sup>	3200	9.12
Lake 2	306 (261–351) <sup>a</sup>	10.36 (7.8–12.92) <sup>ab</sup>	3900	9.08
Lake 3	660 (473–847) <sup>a</sup>	9.44 (8.16–10.72) <sup>ab</sup>	9800	11.49
Lake 4	55.0 (53–58) <sup>b</sup>	9.94 (9.02–10.86) <sup>a</sup>	48	7.79
Forest	8400 (5200–11600) <sup>c</sup>	7.38 (6.07–8.69) <sup>b</sup>	Na	Na

Na: no data available.

forest site and lakes 1–3 are considered polluted (Table 1) and lake 4 serves as reference site. Including a forest site allows us to compare the exposure risk for birds with different access to terrestrial and aquatic insects (Schindler and Smits, 2017) along a contamination gradient. At each site 11–68 nest boxes were placed at a height of 1.5 m with 40 m between boxes, to attract breeding pied flycatchers (Lidman et al., 2020b). At lake sites, the number of nest boxes was limited by the shore length.

## 2.2. Birds and blood sampling

The pied flycatcher is a small passerine bird (12–13 g) that winters in West Africa and migrates to breeding areas in northern Eurasia. They forage between 50 and 135 m away from their nest boxes when using these for breeding (Harvey et al., 1984). During the breeding period (June–July), females lay 5–7 eggs that are incubated for 13–16 days before the nestlings hatch (Lundberg and Alatalo, 1992). Until fledging (14–16 days), adults provide nestlings with a diet consisting of aerial and flightless invertebrates caught on the ground, on vegetation, or in the air (Lundberg and Alatalo, 1992; Veistola et al., 1997; Eeva et al., 2005). The pied flycatcher's ecology has been studied extensively, largely because of its preference for nesting in nest boxes (Lundberg and Alatalo, 1992). As such, the species is well suited for studies in natural environments, due to its prevalence in high latitude environments in Europe (Backström et al., 2013).

We used nestling blood as the tissue for both Ca, Pb and isotope analysis. Blood has previously been used and verified as a good indicator of metal exposure in birds (Craighead and Bedrosian, 2008; Berglund, 2018), and to measure birds' isotopic signal for tracing dietary sources (Hobson and Clark, 1992; Hobson and Bairlein, 2003). Predation by mustelids prevented us from optimal sampling in 2017, and therefore additional sampling of birds was performed in 2018, resulting in 3–5 clutches from each site (Supplementary information Table S1). Nest-boxes were monitored weekly for pied flycatcher occupation and nestling development. Blood samples were retrieved prior to fledging when nestlings were 12–14 days old.

We sampled a maximum of 0.1 ml blood from each nestling, which is the suggested sample volume for birds of this size (Fair et al., 2010). Blood was taken from the jugular vein using a syringe (approved by the Swedish EPA [NV-03323-16] and the Committee for Ethical Review [A4/2016]). The sampling procedure for elemental (Ca and Pb) and isotope analyses differed slightly. Two to four nestlings per clutch were sampled using heparinized syringes to obtain at least 200 µl blood needed for elemental analyses. One nestling per clutch was sampled for isotope analyses using syringes without heparin. Heparin prevents clotting, which simplifies handling of blood samples, but it may interfere with the isotopic signal of blood, and hence we chose these different approaches for the two different analyses. With this design we assume that nestlings from the same clutch are fed a similar composition of prey. Blood samples were stored on ice in the field and at –18 °C in the lab.

Blood samples were freeze dried prior to isotope analysis but elemental analyses were performed on fresh blood.

## 2.3. Invertebrate sampling for isotope and elemental analysis

Invertebrates were collected at the five study sites during the nestling period of the pied flycatchers (15 June – 10 July). We applied two sampling approaches, one to collect invertebrates for elemental analyses (performed in 2017 and 2018) and one to collect invertebrates for isotope analyses (2017–2020). This extended sampling for isotope analyses was needed to obtain enough mass for some invertebrate taxa.

To analyze element concentration in invertebrates we captured aerial invertebrates using five deposition traps/site (18 × 18 cm plastic boxes) placed on the ground (Stenroth et al., 2015) 40 m apart, and we captured ground-living invertebrates with five pitfall traps/site (8.5 cm diameter plastic cup, 500 ml) inserted into the ground level with the soil surface, spaced 20 m apart (Lidman et al., 2020b). The traps were filled with a 50/50 propylene glycol/water mixture to act as a preservative. All traps were emptied every second week during the sampling period. Lepidoptera larvae were collected for isotopic and elemental analysis by shaking vegetation over white fabric. To account for potential leaching of metals during sampling and storing in preservatives, Lepidoptera larvae intended for element analysis were placed into a preservative mixture to mimic the passive sampling. The material collected was stored at 6 °C whilst awaiting further processing.

For the isotope analyses the aerial invertebrates were collected using a sweep net that was passed through the vegetation, ants and spiders were collected from the ground manually, and Lepidoptera larvae were collected as described earlier (but without placing them in preservative mixture). We chose this active sampling method over passive sampling using preservative, as the preservative may interfere with the isotopic signal of the invertebrates. Terrestrial prey types for isotope analysis were selected based on previous estimations of pied flycatcher nestlings' diet, which is known to be dominated by specimens from the invertebrate orders Aranea, Lepidoptera, Diptera and Hymenoptera (Lundberg and Alatalo, 1992; Sanz, 1998; Berglund, 2010). As for aquatic prey types, previous studies have not focused on habitats close to lakes, but we assume that, due to the pied flycatcher's generalist feeding strategy, nestlings are given a combination of available aquatic prey. Captured invertebrates for the isotope analysis were stored at –18 °C.

All collected invertebrates were sorted to order (Nilsson, 1996; Douwes et al., 2004) and separated by ecosystem (aquatic or terrestrial). Members of Diptera were sorted to sub-order (Brachycera or Nematocera) or family (Chironomidae), Hymenoptera to ants (Formicidae) and others, and Odonata to infraorder Anisoptera and suborder Zygoptera. Terrestrial Brachycera and Nematocera are hereafter referred to as 'flies' and 'Nematocera', respectively. Collected spiders were from the family Lycosidae and not sorted further. Sorted material belonging to the same invertebrate group, hereafter referred to as 'prey type', and from the same site was pooled before elemental and isotope analysis. Only prey types that were captured at all sites both in deposition traps and sweep nets (except aquatic insect orders that were generally absent at the forest site), were used in statistical and isotope analyses; this excluded Coleoptera, Plecoptera, Odonata and Hymenoptera other than ants.

## 2.4. Sediment and soil sampling

To estimate the contamination level at each site, sediment samples and samples of the organic soil layer were retrieved for elemental analysis. One sediment core was taken from the deepest part of each lake with a HTH gravity sediment corer (Renberg, 1991) in 2016–2017 (Lidman et al., 2020a). Each core was sliced in the field into 1-cm segments and the top 2 cm were used for further elemental analysis. Samples of the organic soil layer were taken from three locations at each site with a soil borer (Ø 12 cm). The samples were rinsed through a sieve

stack (5/4000-230/63  $\mu\text{m}$ ) to remove plant material. Captured soil particles from the sieves were collected for further analysis. Sediment and soil samples were stored at  $-18^\circ\text{C}$  whilst awaiting further processing.

## 2.5. Isotope analysis and isotopic signal estimation

Invertebrate and blood samples were freeze dried and homogenized with a mortar and pestle before isotope analyses. Three 0.5–1 mg subsamples from each sample (invertebrates and blood) were loaded into  $5 \times 9$  mm tin capsules. Exceptions occurred when only one individual or too little material was available, then only one sample was sent for analysis. The samples were analyzed for stable isotope  $^{12}\text{C}/^{13}\text{C}$  ( $\delta^{13}\text{C}$ ) and  $^{15}\text{N}/^{14}\text{N}$  ( $\delta^{15}\text{N}$ ) ratios at the isotope lab of the Department of Forest Ecology and Management at the Swedish University of Agricultural Sciences in Umeå, where isotopes were determined with an Elemental Analyzer - Isotope Ratio Mass Spectrometer (EA-IRMS) equipped with a Flash EA 2000 (Thermo Fisher Scientific, Bremen, Germany) and a DeltaV (Thermo Fisher Scientific, Bremen, Germany) (Werner et al., 1999). Maize and wheat flour were used as working standards and the instrument was calibrated against reference standards of  $\delta^{13}\text{C}$  (IAEA-600, IAEA-CH-6, and USGS40) and  $\delta^{15}\text{N}$  (IAEA-600, IAEA-N-2, USGS40, and USGS41). The results were corrected for drift and sample size effect (non-linearity).

## 2.6. Elemental analysis

Pb and Ca concentrations in invertebrates and nestling blood were analyzed with ICP-MS (PerkinElmer Elan DRC-e) and ICP-OES (PerkinElmer Optima 200 DV), respectively, after microwave acid digestion (6 mL conc.  $\text{HNO}_3 + 1$  mL  $\text{H}_2\text{O}_2$ ) with a Mars 6 express, following a digestion protocol from CEM (2019). Digested liquid was diluted to 50 mL with MiliQ water and analyzed for element concentrations. Standard reference material (Mussel tissue ERM® - CE278k) was included in each run (75–109% recovery). Prior to elemental analyses, insect samples were freeze dried. Element concentrations in nestling blood are expressed as  $\mu\text{g/g}$  wet-weight, and in insects, Pb and Ca are expressed as  $\mu\text{g/g}$  and  $\text{mg/g}$  dry weight, respectively. Element concentrations below the limit of detection (LOD, 0.01  $\mu\text{g/L}$  and 5  $\mu\text{g/L}$ , respectively, for Pb and Ca) were allocated a value of  $\text{LOD}/\sqrt{2}$  (Serdar et al., 2003).

Freeze-dried soil and sediment samples were homogenized with a pestle and mortar before analyzing element concentrations by X-ray Fluorescence (XRF) using a Bruker S8-Tiger WD-XRF, with LOD of 0.9 ppm and 0.015  $\text{mg/g}$  for Pb and Ca, respectively (Rydberg, 2014). Quality control samples (QC), QC-70314 and QC-73310, were included in the analysis, which contained 23 ppm Pb and 9.08  $\text{mg/g}$  Ca and 285 ppm Pb and 8.29  $\text{mg/g}$  Ca, respectively, with measured values of 25.5 ppm Pb and 9.50  $\text{mg/g}$  Ca, and 281 ppm Pb and 8.55  $\text{mg/g}$  Ca, respectively.

## 2.7. Diet estimation and statistical analyses

Nestling diet was estimated with multiple source mixing models (package: *simmr*, function: *simmr\_out*) in a Bayesian framework with the isotopic signal of the prey as sources and the individual nestling's isotopic signal as the mixture. Thus, one mixing model was constructed for each nestling. Discrimination factors of 2.4 and 1.7 for  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ , respectively, were included in the model to account for isotope discrimination in blood formation (Hobson, 2003). We used four Markov chain Monte Carlo (MCMC) runs until satisfactory convergence (Gelman diagnostics  $<1.2$ ) was reached (Brooks and Gelman, 1998). For one nestling, satisfactory convergence was not achieved and the estimated diet was excluded from further analysis.

Differences in soil contamination patterns were analyzed using a linear model (*lm*) (package: *stats*, function: *lm*) with Pb or Ca concentration in soils as response variables, and site as a fixed factor. Pb

concentrations were log-transformed to meet the assumption of normal distribution. We used 95% confidence intervals (CI) to identify significant effects. No statistical analyses were performed to distinguish differences in sediment contamination, due to the limited number of replicates ( $n = 1$  per site).

In order to analyze differences in nestling diets, we used analysis of variance (ANOVA) (package: *stats*, function: *aov*). First we focused on differences among aquatic dietary sources, and used the dietary contribution (based on the Bayesian mixing model) of aquatic prey as response variable and site and prey type (i.e. black flies, Ephemeroptera, Chironomidae and Trichoptera), as well as the interaction as fixed factors. Second, we focused on differences among terrestrial dietary sources and repeated the same analyses, but for terrestrial prey types (ants, flies, Lepidoptera, Nematocera and spiders). We used Spearman correlation test to assess if the dietary contribution of individual prey types correlated.

We analyzed differences in Pb concentration between prey types using nested ANOVA (package: *nlme*, function: *lme*). First, we analyzed differences in Pb concentrations between aquatic and terrestrial prey types using Pb as response variable, year and ecosystem as fixed factors, and site as a random factor. Second, we analyzed differences in Pb concentrations among aquatic prey types (black flies, Ephemeroptera, Chironomidae and Trichoptera) using Pb as response variable, year and aquatic prey types as fixed factors and site as random factor. Last, we analyzed differences in Pb concentrations among terrestrial prey types (ants, flies, Lepidoptera, Nematocera and spiders) using Pb as response variable, year and terrestrial prey types as fixed factors and site as random factor. All models included year as fixed factor to examine possible differences between sampling years. All models also included site as random factor to remove the effect of different contamination level among sites. Pb concentrations in prey types were log-transformed to meet the assumption of normal distribution and the ANOVA was followed by a TukeyHSD-test (package: *stats*, function: *TukeyHSD*) for pair-wise comparison of prey types. A similar procedure was performed to analyze differences in Ca concentrations, by running the same models, but with Ca as response variable. As we found no differences in element concentrations among aquatic prey types ( $p \leq 0.42$ ), aquatic prey was combined in the following analyses.

To assess whether Pb and Ca accumulation in birds differed among sites and between sampling years, two-way ANOVA (package: *stats*, function: *aov*) was used with Pb or Ca concentration in nestling blood as the response variable and site and year as fixed factors. Element concentrations in bird blood were log-transformed to meet the assumption of normal distribution and the ANOVA was followed by a TukeyHSD-test for pair-wise comparisons among sites. The p-values in the TukeyHSD-tests were adjusted for multiple comparisons with Tukey HSD correction method.

The dietary element exposure from different prey types for each sampled nestling was estimated by multiplying the proportion of each prey type, obtained from the Bayesian mixing model, by the corresponding element concentration for that prey type and site. Differences in dietary Pb and Ca exposure from individual prey types (aquatic, ants, flies, Lepidoptera, Nematocera and spiders) were analyzed with three-way ANOVA using either Ca or Pb dietary exposure as the response variable and prey type, year and site as fixed factors, without interaction effects. The dietary Pb and Ca exposure were log transformed prior to analysis to meet the assumption of normal distribution. The ANOVA was followed by a TukeyHSD-test (adjusted for multiple comparisons) for pair-wise comparison of dietary Pb and Ca exposure among prey types and sites. To assess whether the dietary Pb and Ca contribution from the same prey types was correlated, we calculated a Spearman correlation.

We used general linear models (package: *stat*, function: *glm*) with Gaussian distribution and a log-link function to estimate how dietary Pb and Ca exposure affected Pb accumulation in birds. We used four model sets to disentangle the effects of prey types. All models included Pb concentration in nestling blood as the response variable. To test the



influence of the total dietary element exposure and potential interaction between Ca and Pb exposure, on Pb accumulation in nestlings, the first model set included year, total dietary Pb exposure, total dietary Ca exposure, and the interaction between Pb and Ca as fixed effects. The total dietary exposure for each element was calculated by summing up the dietary element exposure from all prey type for each nestling, and these are expressed as the total amount Pb or Ca per gram diet, respectively. The second model set aimed at exploring the influence of aquatic vs terrestrial dietary element exposure and Pb accumulation in nestlings, and included year, the dietary Pb exposure from all terrestrial prey types, the dietary Ca exposure from all terrestrial prey types, the dietary Pb from all aquatic prey types, the dietary Ca exposure from all aquatic prey types and the interaction between Ca and Pb from terrestrial and aquatic prey types, respectively, as fixed effects. The third model set aimed at exploring the dietary exposure from terrestrial prey types in more detail, and the impact on Pb accumulation in nestlings, and included year, dietary Pb exposure from individual terrestrial prey types, dietary Ca exposure from individual terrestrial prey types, and the interactions between Ca and Pb for each prey type as fixed effects. For model set 1, 2 and 3, model selection was performed based on Akaike's Information Criterion corrected for small sample sizes (AICc, package: MuMin, function: dredge), where models were retained if  $\Delta AICc \leq 2$  (Burnham et al., 2011). As we found no significant differences in element concentrations among aquatic prey types ( $p \leq 0.42$ ) and birds in forest sites had access to other aquatic prey than birds in lake sites (see result section), no corresponding model for aquatic prey types was performed. To test potential interaction effects between the most common prey items, and their impact on Pb accumulation in nestlings, the fourth model set included the three-way and two-way interactions between dietary contribution of Pb from aquatic prey, ants and Lepidoptera.

Dietary Pb and Ca exposure and diet proportions of different prey types are expressed as mean  $\pm$  1 SD. The significance level was set to  $p \leq 0.05$ . All statistical tests and plots were performed using R Version 4.0.3 (R Core Team, 2020) in R studio Version 1.1.456 (RStudio Team, 2020). All plots, except the 3D plots (package: lattice), were plotted using the ggplot2 package.

### 3. Results

#### 3.1. Pb and Ca in soil and sediment

Concentrations of Pb showed large spatial variation in sediments and soil (Table 1) and were in the range 55–8400  $\mu\text{g/g}$  and 48–9800  $\mu\text{g/g}$ , respectively, for soil and sediment. Among lake sites, Pb concentrations were generally higher in sediment than soil, except for the reference lake (lake 4), and the highest sediment Pb concentration was found in lake 3. However, because we only collected one sediment core per lake, no statistical analyses could be performed. The highest Pb concentrations in soils were found at the forest site ( $\beta = 9.03$ ,  $p < 0.0001$ ), and the lowest Pb concentrations in soils were found near the reference lake ( $\beta = 4.07$ ,  $p < 0.0001$ ). There was little variation in Ca concentration in sediment and soil (7.39–10.36 mg/g), but the Ca concentrations in soils from the forest site were significantly lower than those at lake 4 ( $\beta = 0.74$ ,  $p < 0.0001$ , Table 1).

#### 3.2. Isotope signals

Sampled prey types had  $\delta^{13}\text{C}$  values that ranged from  $-37.43$  to  $-25.32$  (Supplementary information Fig. S1 and Table S2) and allowed us, in part, to separate aquatic from terrestrial prey types, with aquatic insects, except Ephemeroptera from lake 4 and Trichoptera, being generally more depleted in  $\delta^{13}\text{C}$ .  $\delta^{15}\text{N}$  ranged between  $-5.26$  and  $11.03$  and was more depleted in the herbivorous Lepidoptera larvae ( $-5.26$  to  $2.16$ ) than in other terrestrial prey types. The variation in isotopic signal for nestling blood was narrow ( $-28.29$  to  $-25.69$  and  $3.84$  to  $5.46$  for

$\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ , respectively) and was in the range of their prey resources (Supplementary information Table S3 and Fig. S1).

#### 3.3. Diet

In total, aquatic prey types accounted for between 2.00 and 96.4% of nestling diet (Supplementary information Table S1), with higher contribution of aquatic prey types at lake sites (Fig. 2A). Among aquatic prey, there were significant differences in the dietary contribution of different prey types ( $F_{3,72} = 5.3$ ,  $p < 0.01$ ), and this also varied among sites (significant interaction:  $F_{12,72} = 2.1$ ,  $p = 0.03$ ). For example, at lake sites, one of the most common aquatic prey item was Trichoptera ( $0.136 \pm 0.205$  g/g), with significantly higher dietary contribution than black flies ( $p < 0.01$ , Fig. 2B). However, at forest sites, blackflies were the only identified aquatic prey, among the aquatic groups included in the study. The most common terrestrial prey items in nestling diet were ants ( $0.24 \pm 0.186$  g/g) and Lepidoptera larvae ( $0.23 \pm 0.147$  g/g, Fig. 2C) with significantly higher dietary contribution than spiders, flies and Nematocera ( $p < 0.01$  for all). However, different terrestrial prey types dominated the diet at the different sites (significant interaction:  $F_{16,90} = 2.5$ ,  $p < 0.01$ , Fig. 2, Supplementary information Table S1). Higher dietary contribution by aquatic prey were accompanied by lower contribution by ants ( $r = -0.93$ ,  $p < 0.0001$ ) and Lepidoptera ( $r = -0.67$ ,  $p <$

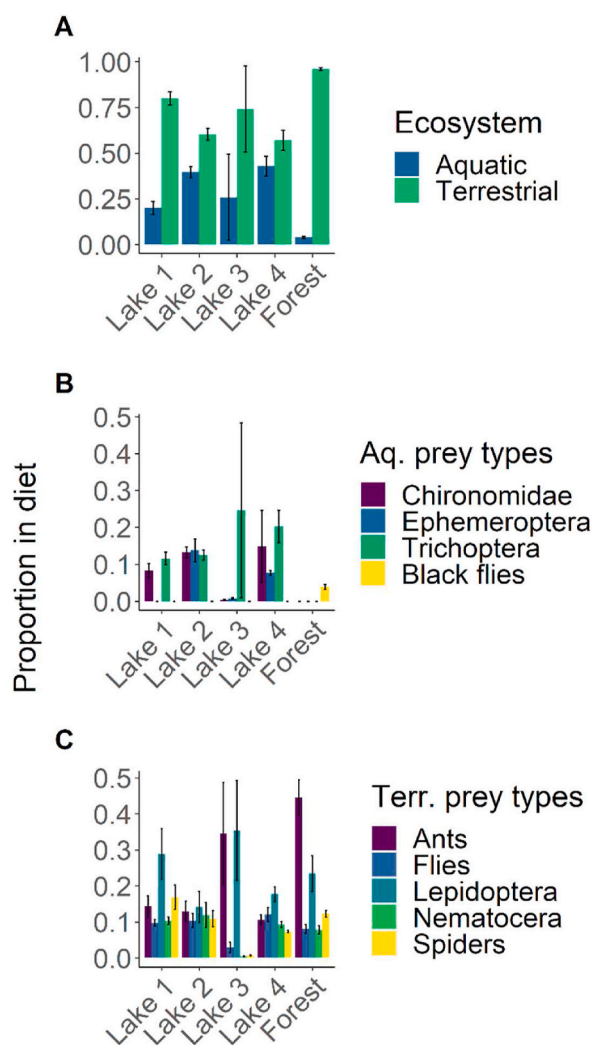


Fig. 2. Proportion ( $\pm 1$  SD) of different prey types in the nestlings' diet at the different sites; A) proportion of prey from different ecosystems, B) proportion of different aquatic prey types, and C) proportion of different terrestrial prey types in nestling diet. For sample size see Supplementary Table S1.

0.001) and in turn the dietary contribution of ants and Lepidoptera was positively correlated ( $r = 0.44$ ,  $p = 0.04$ ). In addition, the dietary contribution of Nematocera and flies ( $r = 0.75$ ,  $p < 0.0001$ ), Nematocera and spiders ( $r = 0.55$ ,  $p < 0.01$ ) as well as spiders and flies were positively correlated ( $r = 0.52$ ,  $p = 0.01$ ).

### 3.4. Element concentration in invertebrates and nestling blood

Element concentrations in invertebrates varied among sites and prey types and ranged from 0.064 to 162  $\mu\text{g Pb/g}$  and 0.466–4.33  $\text{mg Ca/g}$  (Table 2), with the highest Pb concentration in ants from the forest site and highest Ca concentration in Chironomids from lake 1. Pb concentrations at lake 4 (the reference lake) were generally lower than the concentrations at the polluted sites (Table 2). There was no statistical difference in Pb concentrations between aquatic and terrestrial prey types ( $F_{1,32} = 0.20$ ,  $p = 0.66$ ), but aquatic prey had, on average, higher Ca concentrations ( $2.41 \pm 1.07 \text{ mg/g}$  vs.  $1.59 \pm 0.92 \text{ mg/g}$ ) than terrestrial prey types ( $F_{1,32} = 7.50$ ,  $p = 0.01$ ). There was no difference within aquatic prey types for either Pb ( $F_{3,5} = 1.02$ ,  $p = 0.46$ ) or Ca concentrations ( $F_{3,5} = 1.13$ ,  $p = 0.42$ ) and aquatic sources were, therefore, combined in further analyses. Among the terrestrial prey types, there were significant differences with respect to both Pb ( $F_{4,16} = 33.2$ ,  $p < 0.0001$ ) and Ca concentrations ( $F_{4,16} = 13.2$ ,  $p < 0.001$ ). Pb concentrations were significantly lower in Lepidoptera larvae ( $0.23 \pm 0.25 \mu\text{g/g}$ ) than all other terrestrial prey types ( $p < 0.0014$  for all). Ca concentrations were significantly higher ( $p = 0.049$ ) in Nematocera ( $2.84 \pm 0.70 \text{ mg/g}$ ) compared to spiders ( $1.24 \pm 0.18 \text{ mg/g}$ ,  $p = 0.002$ ), ants ( $1.18 \pm 0.46 \text{ mg/g}$ ,  $p = 0.001$ ) and Lepidoptera larvae ( $0.64 \pm 0.12 \text{ mg/g}$ ,  $p < 0.001$ ), and they were also significantly higher in flies ( $2.05 \pm 0.81 \text{ mg/g}$ ) compared to Lepidoptera larvae ( $p = 0.005$ ).

Pb concentrations in nestling blood ranged from 0.027 to 0.59  $\mu\text{g Pb/g}$  blood (Fig. 3). Pb concentration was significantly different among sites ( $F_{4,15} = 5.02$ ,  $p = 0.009$ ) and were higher in nestlings from lake 3 and the forest site than from lake 4 (reference lake) ( $p = 0.02$  for both). Ca concentrations in nestling blood ranged from and 51.9–66.8  $\text{mg Ca/g}$  blood, with no differences ( $F_{4,15} = 0.80$ ,  $p = 0.54$ ) among sites (Fig. 3). No difference was observed between years for neither Pb ( $F_{1,15} = 0.26$ ,  $p = 0.62$ ) nor Ca ( $F_{1,15} = 3.72$ ,  $p = 0.07$ ) blood concentrations.

### 3.5. Dietary exposure and metal accumulation

The calculated total dietary Pb exposure for nestlings (based on the dietary contribution from each prey type and their element concentration) ranged from 0.79 to 108  $\mu\text{g Pb/g}$  and the dietary Pb exposure from individual prey types ranged from 0.01 to 72.3  $\mu\text{g Pb/g}$ , of which aquatic prey accounted for 35.3% ( $\pm 31.4$ ) of the dietary Pb exposure, with a lower contribution at the forest site (Table 3). The contribution of Pb differed depending on prey types ( $F_{5,15} = 40.0$ ,  $p < 0.0001$ ), but not year ( $F_{1,15} = 0.35$ ,  $p = 0.56$ ). Ants and aquatic prey accounted for significantly higher Pb exposure than all other prey types ( $p < 0.05$  for all), whereas Lepidoptera accounted for significantly less Pb than all other prey types ( $p < 0.0001$  for all), resulting in the following exposure

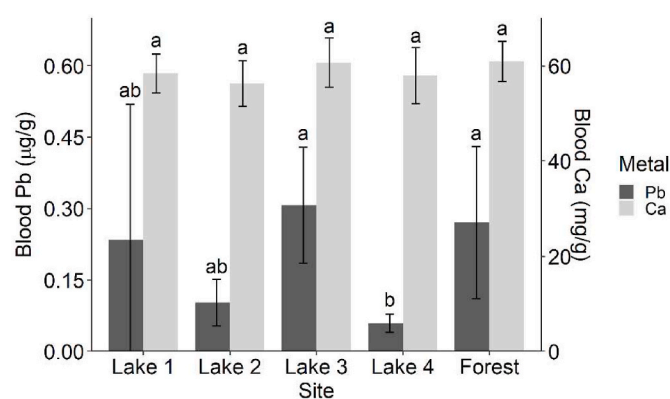


Fig. 3. Average Pb and Ca concentration ( $\pm 1$  SD) in nestling blood at the different sites. Different letters (a or b) above error bars indicate significant differences in Pb and Ca concentration among sites.

pattern: ants = aquatic > flies = spiders = Nematocera > Lepidoptera. The dietary Pb exposure also differed among sites ( $F_{4,15} = 40.4$ ,  $p < 0.0001$ ), according to the following pattern: forest > lake 1 = lake 2 > lake 3 > lake 4.

The total dietary Ca exposure ranged from 1.04 to 2.49  $\text{mg Ca/g}$  and the dietary Ca exposure from individual prey types ranged from 0.04 to 5.62  $\text{mg/g}$ , of which aquatic prey accounted for 25.2% ( $\pm 21.0$ ) of the dietary Ca exposure, with lower proportions at the forest site (Table 3). There were significant differences among prey types ( $F_{5,15} = 5.0$ ,  $p < 0.0001$ ), but not between years ( $F_{1,15} = 0.31$ ,  $p = 0.58$ ). Spiders accounted for significantly less dietary Ca exposure than ants, aquatic prey and Lepidoptera ( $p < 0.04$  for all), and Nematocera also accounted for less Ca than aquatic prey ( $p = 0.02$ ). The dietary Ca contribution differed among sites ( $F_{4,15} = 13.5$ ,  $p < 0.0001$ ), with prey types from lake 3 accounting for significantly less Ca than prey types from all other sites ( $p < 0.001$ ), resulting in the following pattern: lake 4 = lake 1 = lake 2 = forest > lake 3. The dietary Ca and Pb exposure were positively correlated in ants ( $p < 0.001$ ,  $r = 0.77$ ), aquatic insects ( $p = 0.013$ ,  $r = 0.54$ ) and spiders ( $p < 0.001$ ,  $r = 0.71$ ), but they were not correlated for the other prey types ( $p > 0.27$  for all).

The importance of dietary Pb and Ca for Pb accumulation in nestlings was assessed based on four model sets. In model set one we analyzed the total contribution of Ca and Pb, and the best supported model (Supplementary Table S4) indicated that an interaction effect of increased total dietary Ca and Pb exposure resulted in significantly higher Pb accumulation in birds, but there was also a slight increase in Pb accumulation at low dietary Pb and Ca exposure ( $\beta = 0.09$ ,  $p = 0.03$ , Fig. 4). Individually, total dietary Pb or Ca exposure from all prey types combined showed a significant negative relationship with Pb accumulation in nestlings ( $\beta = -0.14$ ,  $p = 0.04$  and  $\beta = -2.00$ ,  $p = 0.01$ , respectively). In model set two, where the impact of aquatic and terrestrial prey types was separated, similar patterns were observed. The best supported model (Supplementary Table S4) indicated that there were positive

Table 2

Lead (Pb) and calcium (Ca) concentration in bulk samples of prey types from different sites. Pb and Ca concentrations are expressed as  $\mu\text{g/g}$  and  $\text{mg/g}$  dry weight respectively. Significant differences in Pb concentration among terrestrial prey are indicated by different letters, and significant differences in Ca concentration among terrestrial prey are indicated by different Greek letters. No significant differences were found among aquatic prey.

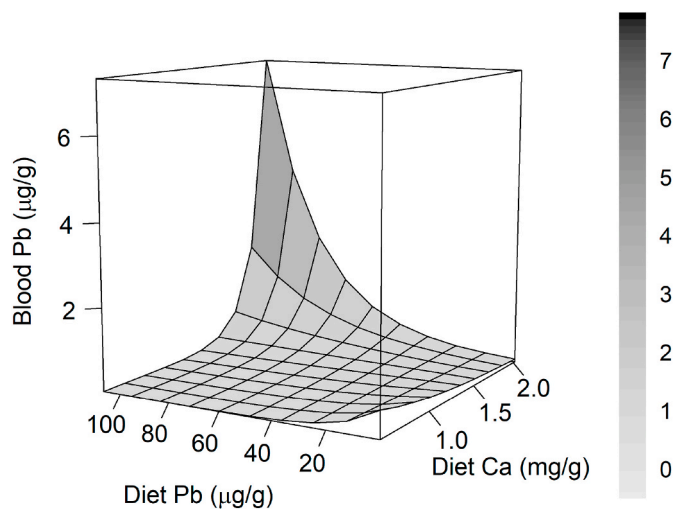
Site	Terrestrial										Aquatic									
	Ants		Flies		Lepidoptera		Nematocera		Spider		Black flies		Ephemeroptera		Chironomidae		Trichoptera			
	Pb <sup>a</sup>	Ca <sup>ap</sup>	Pb <sup>a</sup>	Ca <sup>βγ</sup>	Pb <sup>b</sup>	Ca <sup>α</sup>	Pb <sup>a</sup>	Ca <sup>γ</sup>	Pb <sup>a</sup>	Ca <sup>ap</sup>	Pb	Ca	Pb	Ca	Pb	Ca	Pb	Ca		
Lake 1	9.79	1.57	8.93	1.46	0.10	0.62	8.53	2.55	6.51	1.25	Na	Na	5.35	2.15	147	4.33	11.5	1.91		
Lake 2	25.3	0.82	12.0	1.56	0.17	0.47	13.9	2.72	4.84	0.95	Na	Na	7.65	1.62	6.90	1.58	31.4	3.08		
Lake 3	18.8	1.35	23.2	1.61	0.14	0.81	22.0	3.51	6.10	1.39	Na	Na	22.0	4.01	34.5	1.62	10.8	1.40		
Lake 4	1.56	0.59	0.26	3.39	0.06	0.63	0.57	3.53	4.08	1.41	Na	Na	<0.001	3.81	4.65	2.29	0.09	1.12		
Forest	162	1.60	57.6	2.25	0.66	0.68	33.4	1.87	33.9	1.22	29.9	3.97	Na	Na	Na	Na	Na	Na		

Na: no data available.

**Table 3**

Estimated dietary lead (Pb, µg/g dry weight) and calcium (Ca, mg/g dry weight) exposure from each prey type to pied flycatcher nestlings. Dietary exposure are average concentrations for each site and  $\pm$  1SD are provided in brackets. Significant differences in Pb concentration among prey and sites are indicated by different letters, and significant differences in Ca concentration among prey and sites are indicated by different Greek letters.

Site	n	Ants Pb <sup>a</sup>	Ca <sup>α,β</sup>	Aquatic Pb <sup>a</sup>	Ca <sup>α</sup>	Flies Pb <sup>b</sup>	Ca <sup>α,β,γ</sup>	Lepidoptera Pb <sup>c</sup>	Ca <sup>α,β</sup>	Nematocera Pb <sup>b</sup>	Ca <sup>β,γ</sup>	Spiders Pb <sup>b</sup>	Ca <sup>γ</sup>	Total exposure Pb	Ca
Lake 1 <sub>a,α</sub>	3	1.28 (0.46)	0.21 (0.07)	13.6 (5.20)	0.58 (0.20)	0.87 (0.14)	0.14 (0.02)	0.03 (0.01)	0.18 (0.08)	0.88 (0.17)	0.26 (0.05)	1.10 (0.38)	0.21 (0.07)	17.8 (5.09)	1.60 (0.17)
Lake 2 <sub>a,α</sub>	4	3.27 (1.43)	0.11 (0.05)	5.91 (0.48)	1.40 (0.12)	1.24 (0.46)	0.16 (0.06)	0.02 (0.01)	0.07 (0.04)	1.66 (0.98)	0.33 (0.19)	0.53 (0.22)	0.10 (0.04)	12.6 (2.29)	1.58 (0.12)
Lake 3 <sub>b,α</sub>	4	6.51 (5.34)	0.46 (0.38)	2.96 (5.08)	0.38 (0.65)	0.67 (0.67)	0.05 (0.05)	0.05 (0.04)	0.29 (0.22)	0.10 (0.03)	0.02 (0.01)	0.05 (0.01)	0.01 (0.003)	10.2 (2.97)	1.03 (0.39)
Lake 4 <sub>c,β</sub>	5	0.16 (0.05)	0.06 (0.02)	0.71 (1.01)	0.86 (0.35)	0.03 (0.01)	0.41 (0.15)	0.01 (0.002)	0.11 (0.03)	0.05 (0.01)	0.33 (0.06)	0.30 (0.03)	0.10 (0.01)	1.27 (0.92)	1.86 (0.11)
Forest <sub>d,α</sub>	5	72.3 (17.9)	0.71 (0.18)	1.16 (0.45)	0.15 (0.06)	4.65 (1.62)	0.18 (0.06)	0.16 (0.07)	0.16 (0.07)	2.61 (0.88)	0.15 (0.05)	4.16 (0.76)	0.15 (0.03)	85.1 (17.0)	1.50 (0.11)
Average		19.3 (31.6)	0.35 (0.30)	4.07 (5.12)	0.43 (0.36)	1.60 (1.96)	0.21 (0.17)	0.06 (0.07)	0.34 (0.24)	1.10 (1.19)	0.17 (0.12)	1.33 (1.69)	0.15 (0.12)	27.5 (33.5)	1.65 (0.37)



**Fig. 4.** The effect of increased total dietary Pb and Ca exposure and their interaction on Pb concentration in nestling blood. Darker color indicates higher Pb concentrations in nestling blood.

interactions between terrestrial derived dietary Ca and Pb exposure and Pb accumulation in birds ( $\beta = 0.04$ ,  $p < 0.01$ , Supplementary Fig. S1A), as well as between aquatic derived dietary Ca and Pb exposure and Pb accumulation in birds ( $\beta = 0.55$ ,  $p < 0.001$ , Supplementary Fig. S2B). Again, the contribution of solely Pb from terrestrial ( $\beta = -0.06$ ,  $p < 0.01$ ) or aquatic sources ( $\beta = -0.20$ ,  $p = 0.01$ , Fig. 5A), as well as the dietary contribution of Ca from aquatic sources ( $\beta = -5.03$ ,  $p < 0.0001$ ) showed negative relationships with Pb in nestlings, and apart from one bird, nestlings from lake 3 and the forest site accumulated high concentrations of Pb but had limited contribution from aquatic prey types (Fig. 5A). No relationship was found for Ca from terrestrial prey types ( $\beta = -0.17$ ,  $p = 0.70$ ). In model set three, the dietary contribution from terrestrial sources were explored in more detail, evaluating the contribution by ants, flies, Lepidoptera, Nematocera and spiders. The best supported model (Supplementary Table S4) indicated that Pb from ants ( $\beta = 0.22$ ,  $p < 0.0001$ , Fig. 5B) and Ca from Lepidoptera ( $\beta = 1.28$ ,  $p < 0.01$ ) as well as Nematocera ( $\beta = 7.3$ ,  $p < 0.0001$ ) showed a positive relationship with Pb in birds, but increased Pb from spiders ( $\beta = -3.8$ ,  $p < 0.0001$ ), and increased Ca from flies ( $\beta = -7.0$ ,  $p < 0.0001$ ) and spiders ( $\beta = -9.4$ ,  $p < 0.0001$ ), had a negative relationship with Pb in nestling blood. In addition, there were significant interactions between dietary exposure of Pb and Ca from spiders ( $\beta = 18.8$ ,  $p < 0.0001$ ) and ants ( $\beta = -0.19$ ,  $p < 0.0001$ ), where the combination of high dietary Ca and Pb resulted in high Pb accumulation. In model set four, interaction

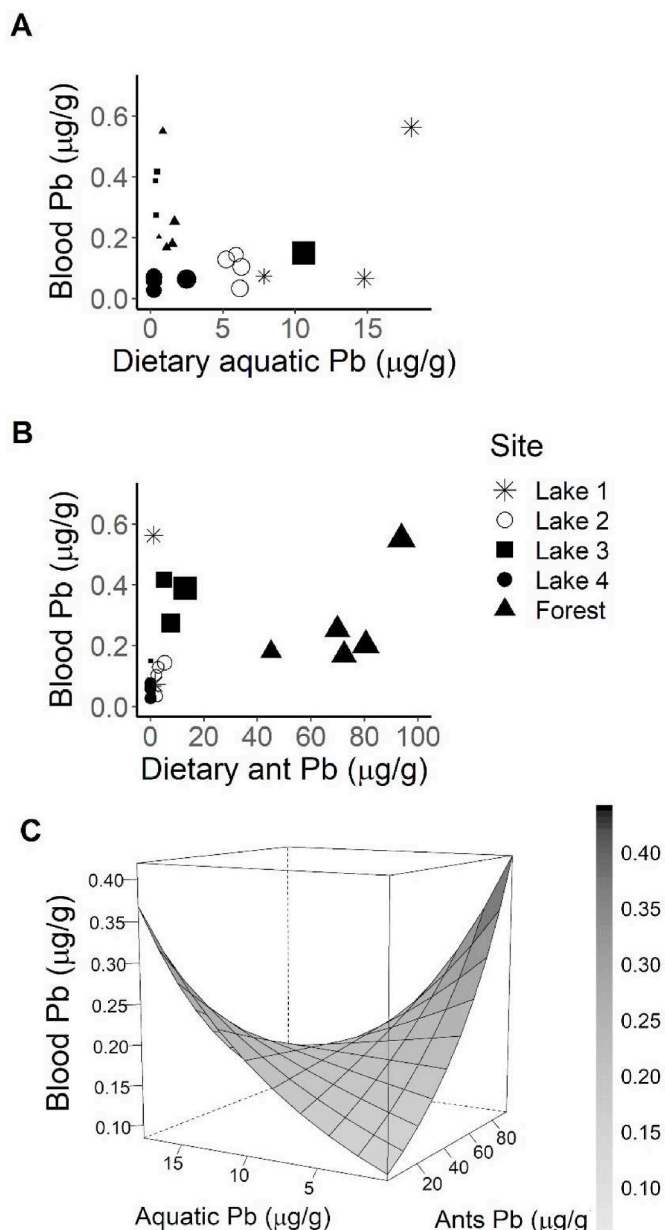
effects of dietary Pb contribution from the most common prey items (ants, aquatic prey and Lepidoptera) were explored, indicating a significant interaction between Pb exposure from ants and aquatic prey, and accumulation of Pb in nestlings ( $\beta = 0.02$ ,  $p < 0.01$ ). Either increased contribution of Pb from ants or aquatic prey, but not both, resulted in high Pb accumulation in nestlings (Fig. 5C). No interaction was found for the other prey items (ants:Lepidoptera,  $p = 0.49$ , aquatic:Lepidoptera,  $p = 0.96$ ) nor was there a three-way interaction ( $p = 0.12$ ).

#### 4. Discussion

Contrary to our expectations, increased total dietary Pb exposure alone did not result in increased Pb accumulation in nestlings. Instead, the contribution from specific prey items, originating from both aquatic and terrestrial ecosystems, could better predict Pb accumulation. The metal concentration of specific prey types is crucial for determining the exposure risk for predators (Belskii and Belskaya, 2013), although the risk will be reduced if the prey type is uncommon. As such, it is important to consider both the proportion of a specific prey item and the respective metal concentration.

Ants, Lepidoptera and aquatic prey were the most common prey types and, in addition, aquatic prey items and ants also contributed to the highest dietary Pb and Ca exposure. Previous studies have shown that tree swallows (*Tachycineta bicolor*) breeding close to aquatic ecosystems accumulate metals via emerging aquatic insects (Beck et al., 2013, 2014), but surprisingly, increased contribution of aquatic derived Pb was accompanied by decreased Pb accumulation in nestlings in our study. However, when taking potential interaction effects between the most common prey items (ants, aquatic prey, and Lepidoptera) into account, it was apparent that birds accumulated high Pb concentrations either when aquatic prey or ants contributed with large amounts of Pb, and a diet rich in aquatic prey was generally accompanied by a diet low in ants. Thus, when availability of aquatic insects is low, replacing a diet of aquatic prey with a diet of ants may result in equal accumulation of Pb in contaminated environments. Whether this pattern is due to prey availability, prey preference or some other factor, needs to be explored further. However, high availability of aquatic prey may improve nestling health (Lidman et al., 2020b) and hence be a preferred prey. Although pied flycatchers are considered generalists (Eeva et al., 2005), nestlings are provided more easily digested food, i.e. more soft prey such as aquatic insects or Lepidoptera larvae (Lundberg and Alatalo, 1992), which was also identified as common prey in the present study. Aquatic prey comprised 13–96% of nestling diets at the lake sites (Supplementary information Table S1), which is within the range of previous estimates of annual contribution from aquatic insects to adult insectivorous passerines' diet (Nakano and Murakami, 2001; Iwata et al., 2003; Uesugi and Murakami, 2007; Beck et al., 2013, 2014). In the present study,





**Fig. 5.** The effect on Pb concentration in nestling blood by A) dietary Pb exposure from aquatic insects, B) dietary Pb exposure from ants, and C) the interaction between Pb exposure from ants and aquatic insects on Pb concentration in nestling blood. Size of symbol reflects the relative proportion of ants (A) or aquatic insects (B) in nestling diet. Darker color on in the 3D plot indicates higher Pb concentrations in nestling blood.

aquatic prey types were less common (lower proportions) at the two sites with the highest Pb accumulation in nestlings (lake 3 and the forest site), which may explain the negative relationship between aquatic-derived Pb exposure and Pb accumulation in birds.

Although aquatic insects have been identified as a potential route for dietary metal exposure for terrestrial insectivores (Beck et al., 2013; Kraus, 2019), dietary metal exposure from emerging aquatic insects may be less important than their terrestrial counterparts, due to low retention of metals during metamorphosis from aquatic larvae to flying adults and limited feeding as adults for some species (Kraus et al., 2014b; Wesner et al., 2017; Kraus, 2019). A high exposure to aquatic derived metals are also limited to areas close to lakes and streams, as the influence of aquatic sources appears to diminish within a few hundred meters of the shoreline (Schindler and Smits, 2017). In addition, high exposure to

metals during larval stages may reduce insect emergence due to toxic effects during metamorphosis (Schmidt et al., 2013; Kraus et al., 2014a). This would result in reduced availability of emerging aquatic prey and could explain the relatively low proportion of aquatic prey (and contribution of aquatic-derived Pb) at all but one clutch at lake 3, which also had the highest sediment Pb concentrations. This is further in accordance with our previous findings, indicating reduced insect emergence in the most contaminated lakes within the study system (Lidman et al., 2020a) and could suggest that the Pb transfer between aquatic and terrestrial ecosystems is limited in highly contaminated lakes (Schmidt et al., 2013; Kraus et al., 2014a).

Ants contributed substantially to Pb accumulation in contaminated environments and nestlings from the forest site were exposed to more than 10 times higher dietary Pb from ants than birds from lake sites, but nestlings from the forest site accumulated similar Pb concentrations as nestlings from the contaminated lake sites. This indicates that there could be limitations to absorption and accumulation of Pb from ants, which may be attributed to a high proportion of Pb stored in the less readily digested exoskeleton (Hall et al., 1998; Sarica et al., 2005; Tollett et al., 2009). There was a strong positive correlation between Ca and Pb concentration in ants and it is also plausible that absorption of Pb from ants was reduced due to availability of Ca-rich food (Scheuhammer, 1996). Another factor that could complicate the relationships between metal contribution from a specific prey type and accumulation in nestlings may be that not all of the metals found in invertebrates are available for uptake (Dauwe et al., 2004). The metal concentration in prey items corresponds to both absorbed and accumulated internal metal, as well as any unabsorbed metal content in their gut, including metals that have formed stable complexes with other ligands (for example soil particles) and, therefore, may be less bioavailable. Ingestion or external contamination of soil particles is more likely occurring in soil and ground dwelling organisms such as ants and a substantial part of metals in ants may therefore be unavailable for uptake compared to metals accumulated in invertebrates with less soil contact, e.g. Lepidoptera larvae. The birds with a diet rich in ants ingested more Lepidoptera, which, in opposite to ants, had low Pb content. The low accumulation of Pb in Lepidoptera is supported by previous studies (Belskii and Belskaya, 2013; Gall et al., 2015) and Lepidoptera larvae in particular have been identified as important prey for pied flycatchers and other insectivorous passerines (Lundberg and Alatalo, 1992; Belskii et al., 2005; Eeva et al., 2005). An increased proportion of Lepidoptera in the diet has further been suggested to reduce the accumulation of Pb in nestlings (Belskii et al., 2005; Eeva et al., 2005), but this was not found in the present study. As such, co-occurrence of a diet rich in Lepidoptera, which contains relatively little Pb, and ants, which contain more Pb, may confound the interpretation of those dietary components.

The negative relationship between total terrestrial dietary Pb and Pb accumulation in nestlings, but also between total dietary Pb and Pb accumulation is unexpected. This might be an indication that our mixing model lack a common prey type of high Pb content. Hymenoptera (including ants), spiders, Lepidoptera, Coleoptera, Diptera, and to some extent Homoptera and Hemiptera have been identified as the most common prey categories for flycatchers (Lundberg and Alatalo, 1992; Eeva et al., 2005; Berglund, 2010; Belskii and Belskaya, 2013). Of these, only Coleoptera, Homoptera and Hemiptera was unaccounted for in the present study, but all three prey groups accumulate low to moderate concentrations of Pb, in relation to other arthropods (Heikens et al., 2001; Belskii et al., 2005). Thus, including those prey groups in the model should not explain the unexpected patterns. Based on previous element analyses of invertebrates, Isopods and Collembola are suggested to be rich in Pb in contaminated environments (Heikens et al., 2001), but as far as we know there is no report on their frequency in pied flycatcher nestling diets. However, the small size of Collembola would suggest that they aren't among the flycatchers preferred prey.

Although there were indications that increased Ca availability may protect nestlings from accumulating Pb, there were also indications of



the opposite pattern. Increased total Ca exposure, exposure to Ca from aquatic prey types and from specific terrestrial prey types (flies and spiders) decreased the Pb accumulation in nestlings, but increased Ca exposure from both Nematocera and Lepidoptera was accompanied by increased Pb accumulation in nestlings. In addition, increased total dietary Ca exposure in combination with increased total dietary Pb exposure increased the Pb accumulation in nestling blood. The latter is probably a result of cocorrelation of Ca and Pb in many prey items (e.g. ants, aquatic insects and spiders). Increased availability of Ca is known to reduce the Pb accumulation in birds as Pb follow the same absorption route as Ca in the gut (Scheuhammer, 1996; Dauwe et al., 2006; Berglund et al., 2011). When Ca availability is limited, organisms produce more Ca-binding proteins to increase the dietary absorption of Ca, but those proteins also allow Pb absorption, especially when Ca is scarce. Thus, in theory, as aquatic prey was generally rich in Ca, birds feeding on aquatic prey may experience some protection against Pb absorption. However, it is also plausible that the Ca availability is adequate at the mining site and that there is little need for a high absorption efficiency in the gut to balance the Ca homeostasis. This is indicated by higher Ca availability in nestling diets from contaminated sites compared to the reference lake. In addition, the mining sites have been subjected to liming and are not considered Ca poor.

The mixing model suggested that ants, aquatic prey, and Lepidoptera were common prey types for pied flycatchers (Eeva et al., 1997; Belskii and Belskaya, 2013). However, in contrast to previous studies, spiders were not among the identified common prey. Stable isotopes and mixing models have proved to be a reliable method to estimate the major diet composition pattern in birds (Ramos et al., 2009; Pagani-Nunez et al., 2017), but the accuracy is reduced for organisms with more complex diets (Pagani-Nunez et al., 2017), such as for generalists like the pied flycatcher. The model becomes even more complex when aquatic prey types have a terrestrial signal, which was the case for Trichoptera, which presumably feed on allochthonous carbon from the terrestrial environment (Graça, 2001). Further, the diet model relies on the inclusion of all potential prey types. Even though we, as far as possible, selected invertebrate groups that have previously been identified as important prey items for pied flycatcher nestlings, there is a risk that we may have unintentionally included non-important or excluded important prey from the model, which could explain the slightly increased Pb accumulation at low dietary Ca and Pb exposure or some of the unexpected patterns between Pb exposure and Pb accumulation. One potential example is the exclusion of Coleoptera, which are known to be an important prey for pied flycatcher nestlings (Lundberg and Alatalo, 1992; Berglund et al., 2010; Nicolaus et al., 2019). Coleoptera was found at too low a frequency during our sampling campaign to be included in the mixing model, and Coleoptera are known to accumulate lower Pb concentrations than many other arthropods (Heikens et al., 2001). Thus a high proportion of Coleoptera in the diet is expected to decrease the Pb accumulation in nestlings. Apart from limitations with the mixing model *per se*, the available prey will also influence the model predictions between prey exposure and Pb accumulation. As an example, the model prediction for elevated dietary Pb contribution from ants and aquatic insects combined (Fig. 5C) is counter intuitive, predicting that this scenario would result in lower blood lead levels than a diet rich in either ants or aquatic insects alone. However, as there were no nestlings sampled that had a diet rich in both of these prey categories, the model cannot predict such a scenario. If birds from contaminated sites would have a diet rich in both ants and aquatic prey, we would expect a high accumulation of Pb. As such, care should be taken to include all relevant prey.

## 5. Conclusion

Nestlings accumulated Pb at contaminated sites, but Pb accumulation did not correspond to the total calculated dietary exposure. Instead, estimated dietary elemental exposure from specific prey types, such as

ants and aquatic insects, influenced Pb accumulation despite there being little difference in element concentration between all prey groups. Thus, it is important to consider the proportion of prey in the diet as well as individual metal concentrations, to better identify the prey that contributes to metal accumulation in the consumer. On lake shores, the contribution of metals from adjacent aquatic ecosystems was apparent if the access to aquatic prey was high, but if the availability of aquatic prey was low, ants was the predominant source to Pb. This suggests that both terrestrial and aquatic prey need to be included in risk assessments to estimate the dietary metal exposure and transfer of metal within the terrestrial food web.

## Author statement

**Johan Lidman:** Conceptualization, Writing – original draft, Methodology, Investigation, Visualization, Project administrator; **Åsa M.M. Berglund:** Conceptualization, Writing – review & editing, Investigation, Validation, Resources, Supervision, Funding acquisition

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Acknowledgement

We thank Tove Westberg, Andreas Wallmark and Lisa Malm for their help during fieldwork, David Hall for statistical support and Richard Bindler for proofreading the manuscript. The research project was performed within the industrial doctoral school at Umeå University in collaboration with Boliden AB. Financial support was received by Helge Ax:son Foundation (to Johan Lidman), The Swedish Research Council [2018-05470] (to Åsa Berglund), J. Gust. Richert Foundation [2017-00373] (to Åsa Berglund), and Göran Gustafssons Foundation [1803] (to Åsa Berglund).

## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.envres.2022.112779>.

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