



# The mercury flow through a terrestrial songbird food chain in subtropical pine forest: Elucidated by Bayesian isotope mixing model and stable mercury isotopes

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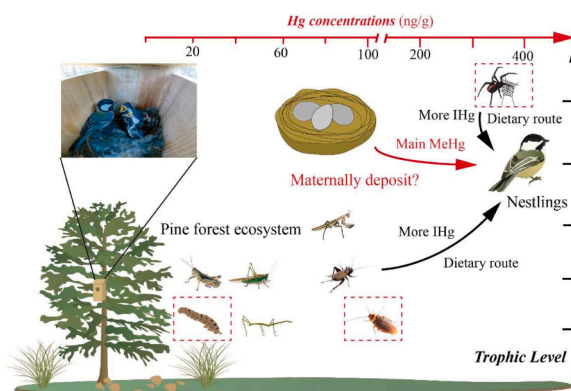
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## HIGHLIGHTS

- The trophic transfers of MeHg and IHg within an avian food chain were investigated.
- Caterpillars, cockroaches, and spiders provide the most Hg to Great Tit nestlings.
- Provisioned invertebrates tended to contribute more IHg to the nestlings.
- Imbalanced Hg integration may influence the Hg isotopes ratios in nestling feathers.

## GRAPHICAL ABSTRACT



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## ABSTRACT

In order to comprehend the transfer of inorganic mercury (IHg) and methylmercury (MeHg) within food chains in terrestrial pine forests, we collected samples of Great Tit nestlings, common invertebrates, plants, and soil in a subtropical pine forest and used Bayesian isotope mixing model analysis, Hg daily intake, and stable Hg isotopes to elucidate the flow of MeHg and IHg in these food chains. Results indicate that caterpillars and cockroaches are the predominant prey items for nestlings, accounting for a combined contribution of 81.5%. Furthermore, caterpillars, cockroaches, and spiders were found to contribute the most (~80%) of both IHg and MeHg that dietary accumulated in nestlings. The provisioned invertebrates tend to supply more IHg and diluting the proportion of MeHg as total Hg (MeHg%). Notably, nestling feathers displayed the highest  $\Delta^{199}\text{Hg}$  values but a relatively lower MeHg%, suggesting an imbalanced incorporation of Hg from maternal transfer and dietary accumulation during

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the nestling stage. This study highlights the efficacy of nestlings as indicators for identifying Hg sources and transfers in avian species and food chains. However, caution must be exercised when using Hg isotope compositions in growing feathers, and the contribution of maternally transferred Hg should not be ignored.

## 1. Introduction

Mercury (Hg) is a globally distributed contaminant known for its toxic nature and long-range transport [11,6]. Methylmercury (MeHg), a highly neurotoxic organic form of Hg, is generated in anaerobic conditions and undergoes biomagnification along food chains, posing risks to wildlife and human health [36,50]. Terrestrial songbirds have emerged as effective indicators of Hg contamination in terrestrial environments [21]. Recent studies have demonstrated that songbirds can acquire Hg or MeHg from aquatic sources through predation on riparian spiders or emergent aquatic insects, thereby contributing to the biomagnification of Hg in food chains [8,66,68].

To understand Hg transfers and biomagnification in avian food chains, it is crucial to determine the dietary composition of songbirds [17]. Traditional methods involving manual observation of feeding behaviors [40,39,48,64] or the identification of prey items from the stomach and fecal samples [54] have limitations, such as laborious fieldwork, low taxonomically precise detection, failure to detect relatively small, rapidly digested, and highly fragmented prey [14,23,53,58], as well as the certain lifestages (e.g., breeding season) when using natural or artificial nest boxes. In contrast, the analysis of stable carbon and nitrogen isotopes ( $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ ) combined with Bayesian isotope mixing models (BIMM) has emerged as a reliable approach for studying animal diets [35,42]. This method has been successfully applied to investigate the diets of wolves [10], raptors [48], and songbirds [2,40,63].

Due to its widespread distribution, large population, small home ranges, insectivorous nature, and ease of monitoring in artificial nest boxes during the breeding season, the Great Tit (*Parus major*) is frequently employed as a model species for studying heavy metal pollution [7,49]. During the breeding season, parent birds feed their nestlings with invertebrates from various trophic levels, including lepidopteran larvae (caterpillars), spiders, and other arthropods [38,64]. Therefore, studying an avian food chain composed of leaves, invertebrates, and nestlings would provide valuable insights into bird dietary compositions and facilitate a deeper understanding of Hg transfers and biomagnification within the local ecosystem. Currently, although the diets of Great Tit nestlings have been extensively documented through video observations [39,64], the application of stable isotopes ( $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ ) in conjunction with BIMM [40] is expected to provide a more detailed understanding of bird diets and the trophic transfers of both IHg and MeHg in songbirds, especially within well-defined avian food chains during the nestling stage.

Stable Hg isotopes have recently emerged as reliable tools for tracing Hg sources and transport processes in the environment [5,59,73]. Mercury isotopes exhibit two types of fractionation: mass-dependent fractionation (MDF, primarily  $\delta^{202}\text{Hg}$ ) and mass-independent fractionation (MIF, primarily  $\Delta^{199}\text{Hg}$  and  $\Delta^{201}\text{Hg}$ ). Previous studies have shown that MIF is absent in the trophic transfers within food chains, such as from fish to their diets [25] or from fish to waterfowl [26], making MIF of Hg isotopes a reliable tool for tracing Hg sources. Currently, Hg isotopes have been widely utilized in raptor feathers and tissues [31], seabird or waterfowl eggs, blood, feathers, and internal tissues [9,26,44,46,45], as well as songbird blood and feathers [61,68]. Although the Hg isotopes in bird blood and feathers can serve as efficient tools to investigate Hg sources or transfers in birds [47,61], the potential and control factors of Hg isotopes in nestling stage of terrestrial songbirds when Hg is rapidly integrated both the dietary intake and maternal deposition, are still poorly understood. Moreover, the difference or comparison of source identification results of multi-isotopes systems

( $\delta^{13}\text{C}$ ,  $\delta^{15}\text{N}$ , and Hg isotopes) combined with BIMM are still unclear and need to be established in food chain studies.

In our previous study, a clear avian food chain of pine needles/grasses–herbivorous invertebrates–carnivorous invertebrates–nestlings was identified in a subtropical pine forest [34], which can serve as ideal targets for elucidating Hg sources in nestlings and the trophic transfers of Hg from diets to nestlings using a combined multi-isotope system. Therefore, in this research, we selected this subtropical pine forest as the study area, collected samples of the inhibited nestling food chains, and employed BIMM and stable Hg isotopes to reconstruct Hg sources and transfers within this pine avian food chain during the breeding season. The primary objectives of this study are as follows: 1) reconstruct the dietary compositions of Great Tit nestlings using BIMM; 2) investigate the stable Hg isotope compositions and fractionations during trophic transfers; and 3) discuss the Hg sources and the trophic transfer processes in the pine avian food chains by integrating the daily intake of Hg and stable Hg isotopes.

## 2. Materials and methods

### 2.1. Study area and sampling

Wenjing Town (24.02–24.44° N, 100.78–101.14° E) is located on Mt. Wuliang and is in close proximity to Mt. Ailao in the central west region of Yunnan Province, Southwest China (Fig. S1). The local climate is characterized as a subtropical monsoon, with an average annual temperature of 20.1 °C and precipitation of 722.9 mm [24]. The nearby average ambient concentration of total gaseous Hg (TGM; 1.6–2.0 ng/m<sup>3</sup> and 1.70 ± 0.41 ng/m<sup>3</sup>; [13,72]) indicates the absence of anthropogenic point source Hg contamination, making it suitable as a background site. In Wenjing, a pure pine forest dominated by *Pinus kesiya* at an altitude of ~1500 m was selected as the study area for installing nest boxes. Around 100 nest boxes were placed at four sampling sites in January 2018 [34].

During the breeding season (April to June) in 2018, we systematically collected surface soil, pine needles, grasses, and various invertebrates (including caterpillars, grasshoppers, katydids, and spiders) in the vicinity of the nest boxes, as well as the feathers of Great Tit nestlings. To ensure sufficient biomass for Hg isotope analysis, an additional collection of feathers from a total of 50 Great Tit nestlings (approximately 14 days old) was conducted in 2019. The collection and treatment procedures for soil, grasses, pine needles, invertebrates, and nestling feathers were detailed in Luo et al. [34]. All sampling activities were conducted with the necessary permissions from the Forestry and Grassland Administration of Jingdong County and the Institute of Geochemistry, Chinese Academy of Sciences.

### 2.2. Analytical methods

#### 2.2.1. Hg determination

The digestion of total Hg (THg) and MeHg in plants, invertebrates (insects and spiders), and soils was conducted following the procedures described by Liang et al. [29,30]. In summary, for THg, approximately 0.2 g of plant and soil samples were digested using 4 mL of HNO<sub>3</sub> at 95 °C in a water bath for three hours. THg was then determined using a cold-vapor atomic absorption spectrometer (CVAFS, Brooks Rand III, USA) according to Method 1631e [56]. For MeHg, about 0.2 g of soil samples were digested with 1.5 mL of CuSO<sub>4</sub> (1 M) and 7.5 mL of HNO<sub>3</sub> (3 M). MeHg in the soil was extracted into 10 mL of CH<sub>2</sub>Cl<sub>2</sub>. About 0.2 g of plant samples and 0.05–0.1 g of invertebrates were digested using a 5

mL solvent of 25% KOH in methanol at 75 °C for three hours. The digests were acidified and then extracted into 10 mL of CH<sub>2</sub>Cl<sub>2</sub>. The MeHg in the solvent digests of both soil and plants was back-extracted into the water phase, purged and trapped, and then measured using gas chromatography coupled with a cold-vapor atomic fluorescence spectrometer (GC-CVAFS, Brooks Rand III, USA) in accordance with USEPA Method 1630 [55].

Due to the limited biomass and relatively lower Hg concentrations in feathers, a modified method based on Hammerschmidt and Fitzgerald [16] and Tsui et al. [61] was used to determine THg and MeHg simultaneously. This modified method has also been successfully applied in studies by Zhang et al. [71] and Xu et al. [68]. In brief, about 0.005 g of feather samples were digested using 3 mL of HNO<sub>3</sub> (4.6 M) at 60 °C for 20 h in 15 mL centrifuge tubes. An aliquot was taken for MeHg determination using GC-CVAFS, following the same procedures used for soil and plant samples. The remaining digests were further digested using HNO<sub>3</sub> at 95 °C for THg measurement, following the procedures applied for soil and plant sample analyses.

Method blanks, duplicates, and certified reference materials (CRMs) were employed for data quality control. The CRMs used for THg in soil, plants, invertebrates, and feathers were yellow-red soil (GSS-5, China), citrus leaves (GBW10020, China), lobster hepatopancreas (Tort-2, Canada), fish muscle (GBW10029, China), and human hair (GBW09101b, China), respectively. Tort-2 and estuarine sediment (CC580, Belgium) were used to validate MeHg in biota samples (invertebrates) and soil samples, while fish muscle was used to validate the data of MeHg and THg in feathers. The recoveries of THg and MeHg in soil, plants, and invertebrates were in the range of 95.7%–103% (THg) and 93.1%–96.1% (MeHg), which were reported in our previous study by Luo et al. [34]. In this study, the recoveries of THg and MeHg using the modified analytical method for feathers were reported as 95.7 ± 9.0% (THg) and 94.1 ± 5.9% (MeHg) in Table S1. The method blanks for plants and invertebrates, soils, and feathers samples were 0.02 ± 0.01 ng/mL, 0.026 ± 0.007 ng/mL, and 0.013 ± 0.001 ng/mL, respectively. The standard deviations of duplicated samples were less than 10%. Hg concentrations in all samples were reported on a dry weight (d.w.) basis, and inorganic Hg (IHg) was calculated as the difference between THg and MeHg.

### 2.2.2. Stable carbon and nitrogen isotopes

The carbon and nitrogen isotope ratios were measured using a gas mass spectrometer (MAT 253, Thermo Scientific, Germany) at the State Key Laboratory of Environmental Geochemistry, Institute of Geochemistry, Chinese Academy of Sciences. The stable isotope ratios are reported in per mill (δ) notation as follows:

$$\delta R_{\text{sample}} = (R_{\text{standard}}/R_{\text{sample}} - 1) \times 1000 \quad (1)$$

where  $R$  represents the abundance ratios of <sup>15</sup>N/<sup>14</sup>N and <sup>13</sup>C/<sup>12</sup>C. Cellulose (IAEA-C3) and ammonium sulfate (IAEA-N1) were used as certificated materials for δ<sup>13</sup>C and δ<sup>15</sup>N. The measured isotope values of δ<sup>13</sup>C and δ<sup>15</sup>N were converted based on Vienna Pee Dee Belemnite (V-PDB) and standard atmospheric nitrogen, respectively.

### 2.2.3. Stable Hg isotopes and MeHg isotope signature estimation

To overcome the low Hg concentrations in the samples, we employed a concentrate method following the procedures outlined by Huang et al. [18] and listed in the supporting information (SI). In summary, the samples underwent thermal combustion in a double-stage furnace (Lindberg/Blue M™ Mini-Mite, Thermo Scientific, Germany), and the resulting Hg was trapped using an 40% reverse aqua regia solution (HNO<sub>3</sub>:HCl = 2:1, v/v). The Hg-trapped solutions were then transferred to brown borosilicate glass bottles, and the THg concentrations were measured using the CVAFS method. Prior to Hg isotope analysis, the trapping solution was adjusted to about 1 ng/mL and 10% acidity.

The Hg isotope compositions were determined using a multi-

collector inductively coupled plasma mass spectrometer (Neptune Plus, Thermo Scientific, Germany) at the State Key Laboratory of Environmental Geochemistry, Institute of Geochemistry, Chinese Academy of Sciences. NIST 3133 Hg solution served as an external standard for sample analysis, and the NIST 997 Tl standard was used to correct the instrumental mass bias. The isotopic values were reported in delta notation, expressed in permil (‰). The mass-dependent fractionation (MDF), denoted as δ<sup>xxx</sup>Hg (with xxx referring to 199, 200, 201, and 202), and the mass-independent fractionation (MIF), represented as Δ<sup>xxx</sup>Hg (with xxx as 199 and 201), were calculated using equations recommended by Bergquist and Blum [4]:

$$\delta^{\text{xxx}}\text{Hg} = 1000 \times [(^{\text{xxx}}\text{Hg}/^{198}\text{Hg})_{\text{sample}} / (^{\text{xxx}}\text{Hg}/^{198}\text{Hg})_{\text{NIST3133}} - 1] \quad (2)$$

$$\Delta^{199}\text{Hg} \approx \delta^{199}\text{Hg} - (0.2520 \times \delta^{202}\text{Hg}) \quad (3)$$

$$\Delta^{201}\text{Hg} \approx \delta^{201}\text{Hg} - (0.7520 \times \delta^{202}\text{Hg}) \quad (4)$$

During the determination process, the Hg isotope compositions of certified reference materials, RM 8610 (UM-Almadén) (0.03 ± 0.06‰ and -0.52 ± 0.08‰; Δ<sup>199</sup>Hg and δ<sup>202</sup>Hg; 2 SD, n = 8), BCR 482 (-0.65 ± 0.08‰ and -1.71 ± 0.11‰; Δ<sup>199</sup>Hg and δ<sup>202</sup>Hg; 2 SD, n = 5), GSS-5 (-0.28 ± 0.08‰ and -1.83 ± 0.10‰; Δ<sup>199</sup>Hg and δ<sup>202</sup>Hg; 2 SD, n = 4), and Tort-2 (0.71‰ and 0.00‰; Δ<sup>199</sup>Hg and δ<sup>202</sup>Hg; n = 1), were found to be consistent with the values reported by Huang et al. [18], Estrade et al. [12], and Kwon et al. [26] (Table S2). The largest 2 SD values of 0.08‰ and 0.11‰ for BCR 482 were used to represent the analytical uncertainty of Δ<sup>199</sup>Hg and δ<sup>202</sup>Hg.

### 2.3. Dietary compositions analysis by the Bayesian isotope mixing model

To estimate the proportional contribution of different food sources to the nestlings' diet, we utilized the "simmr" package of Fits Stable Isotope Mixing Models [41] in the R package (Version 4.2.0; [43]). The model incorporated trophic discrimination factors of Δ<sup>15</sup>N (3.2 ± 0.1‰) and Δ<sup>13</sup>C (2.7 ± 0.1‰) reported by Pagani-Núñez et al. [40] and White and Dawson [63] for songbirds and their prey, respectively (refer to the Supplementary Information (SI) for the R code).

Among the nine invertebrates collected, lacewings were excluded as potential prey items based on previous video recordings [34]. Additionally, since the estimated carbon-to-nitrogen concentration ratios ([C]:[N]) in vertebrates reported by Tsui et al. [60], Girard et al. [15], and White and Dawson [63] were similar, no further corrections were made to the estimated diet contributions, and these values were considered as the final dietary compositions.

### 2.4. Hg daily intake of nestlings

The estimation of Hg daily intake (MDI) for Great Tit nestlings was conducted using the recommended daily food intake rate ( $DIR$ , g/d, dry weight) from Liu et al. [32], Xia et al. [67], and Wu et al. [65]. This method was combined with the body weight ( $BW$ , g) of the nestlings (13.9 ± 0.77 g, here we use 14 g), as reported by Su et al. [52] in a control site. The Hg concentrations ( $C$ , ng/g, dry weight), absorption factors ( $abs$ , MeHg: 95%, IHg: 7%; [57]), the diet contribution proportion ( $\rho$ ), and the Hg daily intake ( $MDI$ , ng/g<sub>BW</sub>/d) of Great Tit nestlings via diets were estimated as follows:

$$DIR = 0.648 BW^{0.651} \quad (5)$$

$$MDI = DIR/BW \times C \times \rho \times abs \quad (6)$$

### 3. Results and discussion

#### 3.1. Hg concentrations

##### 3.1.1. Bird feathers

In Table 1, the concentrations of THg and MeHg in the feathers were measured as  $412 \pm 104$  ng/g and  $79.1 \pm 28.0$  ng/g, respectively. The average THg concentration was similar to the control site value of  $300 \pm 220$  ng/g reported for Great Tit nestlings by Su et al. [52]. It was also comparable to the levels found in nestlings of Russet Sparrow ( $797 \pm 221$  ng/g) and Green-backed Tit ( $521 \pm 156$  ng/g) in a city pine forest, as reported by Zhang et al. [71]. However, the THg concentration in the feathers was significantly lower than the value of  $2960 \pm 1440$  ng/g observed in Hg mining areas [52]. Compared with other studies, the MeHg concentrations in nestling feathers is much lower than that in adult insectivorous songbirds ( $172\text{--}4352$  ng/g) and omnivorous (mean  $156 \pm 41$  ng/g, range  $112\text{--}217$  ng/g) and frugivorous songbirds ( $104\text{--}596$  ng/g) in nearby Mt. Ailao area [28]. Even in another pine forest in Guiyang City, Southwest China, the MeHg concentrations were still lower than those in Green-backed Tit ( $115 \pm 31$  ng/g) and Russet Sparrow nestlings ( $388 \pm 156$  ng/g) reported by Zhang et al. [71].

The proportion of MeHg in relation to THg (hereafter as MeHg%) in the nestling feathers was measured as  $20.0 \pm 7.7\%$ . This value was similar to the MeHg% found in nestlings of Green-backed Tit ( $24 \pm 11\%$ ) but lower than that in Russet Sparrow ( $49 \pm 14\%$ ), as reported by Zhang et al. [71]. However, it was much lower than the results reported for Skuas chicks ( $94.3 \pm 2.9\%$ ) and seabirds ( $84\text{--}97\%$ ) by Renedo et al. [44]. In our opinion, these observed differences might be related to the differences in bird species (terrestrial or aquatic birds), living environments (Hg contaminated or remote areas), the rapid imbalanced incorporation of Hg during feather growth (Ackerman et al., 2013), and the different time scales of Hg integration. For example, the feathers of Great Tit nestling is collected before fledging in  $\sim 14$ -days old, which is much lower than the time before fledging of seabirds chicks, such as Southern and Northern Giant Petrel chick ( $> 100$  days; [19]) and Skuas chicks ( $\sim 40$  days; [22,20]).

**Table 1**

The concentrations of THg, MeHg, and the isotopic compositions of Hg were measured in each taxonomic group within the food chain of the pine forest ecosystem.

Taxon	THg (ng g <sup>-1</sup> )			MeHg (ng g <sup>-1</sup> )		MeHg%	Trophic Level	$\Delta^{199}\text{Hg}$ (2sd)	$\Delta^{201}\text{Hg}$ (2sd)	$\delta^{202}\text{Hg}$ (2sd)
	N	Range	Mean $\pm$ Std	Range	Mean $\pm$ Std	Mean $\pm$ Std		Mean $\pm$ Std	Mean $\pm$ Std	Mean $\pm$ Std
Soil	17	29.0–64.6	43.3 $\pm$ 10.6	0.019–0.272	0.109 $\pm$ 0.073	0.3 $\pm$ 0.2	—	$-0.35 \pm 0.05$	$-0.33 \pm 0.07$	$-1.46 \pm 0.14$
Pine needles	15	2.60–27.0	11.1 $\pm$ 7.7	0.056–0.276	0.127 $\pm$ 0.072	2.2 $\pm$ 2.2	1	$-0.21 \pm 0.08$	$-0.24 \pm 0.14$	$-2.56 \pm 0.36$
Grasses	16	4.17–7.98	6.03 $\pm$ 0.49	0.06–0.265	0.149 $\pm$ 0.072	2.2 $\pm$ 0.5	1	$-0.28 \pm 0.13$	$-0.29 \pm 0.04$	$-1.89 \pm 0.20$
Foliar samples	—	—	—	—	—	—	—	$-0.24 \pm 0.10$	$-0.26 \pm 0.10$	$-2.26 \pm 0.45$
Caterpillars	25 (8) <sup>a</sup>	11.9–53.8	27.2 $\pm$ 13.2	0.65–1.66	1.26 $\pm$ 0.37	3.1 $\pm$ 1.3	1.32	$-0.27 \pm 0.03$	$-0.10 \pm 0.06$	$-2.87 \pm 1.65$
Cockroaches	30 (5)	83.6–139	106 $\pm$ 21.6	10.9–25.8	16.9 $\pm$ 7.87	14.9 $\pm$ 8.1	1.31	$-0.34 \pm 0.06$	$-0.17 \pm 0.04$	$-2.43 \pm 0.07$
Crickets	16 (3)	58.3–103	87.4 $\pm$ 17.0	1.54–3.05	2.53 $\pm$ 0.86	3.4 $\pm$ 1.7	1.45	$-0.23 \pm 0.01$	$-0.22 \pm 0.20$	$-2.28 \pm 0.20$
Grasshoppers	25 (3)	16.8–34.5	24.6 $\pm$ 6.23	0.46–1.63	0.96 $\pm$ 0.41	4.1 $\pm$ 2.2	1.59	$-0.30 \pm 0.03$	$-0.27 \pm 0.06$	$-2.63 \pm 0.04$
Stick insects	5 (2)	36.1–64.2	54.1 $\pm$ 15.6	—	1.62	3.0	1.27	$-0.14$ (0.08)	$-0.08$ (0.08)	$-2.58$ (0.11)
Katydid	20 (3)	30.8–50.1	39.4 $\pm$ 8.0	5.20–10.5	8.40 $\pm$ 2.82	20.9 $\pm$ 9.2	1.61	—	—	—
Mantises	21 (6)	65.6–100	79.5 $\pm$ 15.2	24.4–39.5	31.3 $\pm$ 4.86	37.4 $\pm$ 7.4	3.19	$-0.08 \pm 0.07$	$-0.10 \pm 0.10$	$-1.74 \pm 0.18$
Spiders	30 (3)	303–459	397 $\pm$ 51	116–188	145 $\pm$ 26	39.5 $\pm$ 9.2	4.77	$-0.04 \pm 0.08$	$0.02 \pm 0.07$	$-1.94 \pm 0.10$
Lacewings	6 (2)	97.5–106	102 $\pm$ 5.7	—	36.4	37.3 $\pm$ 1.4	4.82	—	—	—
Great Tit nestlings	50	252–628	412 $\pm$ 104	35.0–157	79.1 $\pm$ 28.0	20.0 $\pm$ 7.7	4.31	$0.47 \pm 0.06$	$0.43 \pm 0.07$	$-2.04 \pm 0.04$

<sup>a</sup> : Number in bracket is the number of homogenized samples.

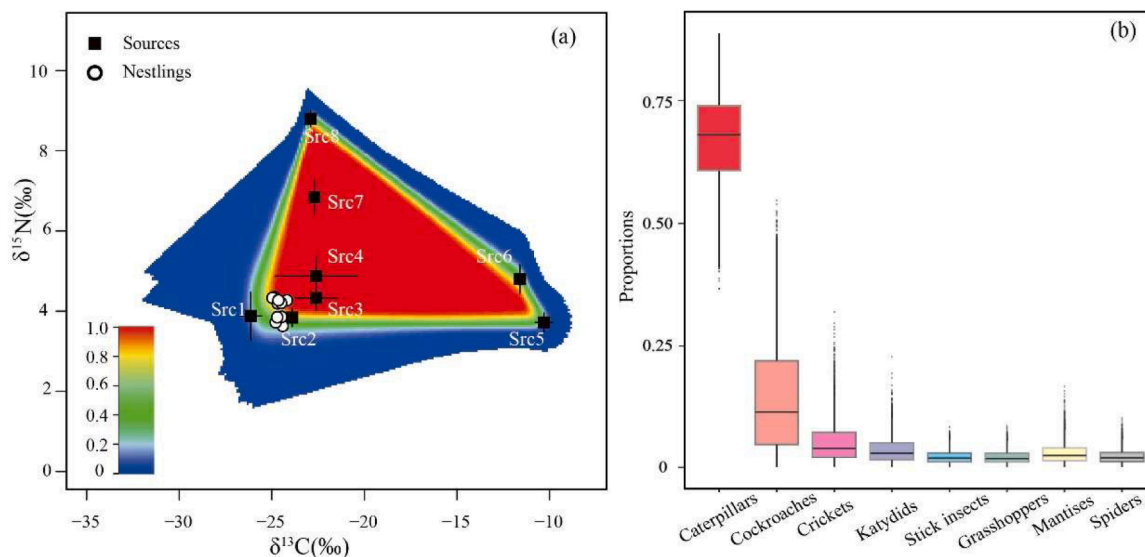
##### 3.1.2. Soils, plants, and invertebrates

Table 1 presents the concentrations of THg and MeHg in soils, pine needles, and invertebrates. For a detailed overview of THg and MeHg concentrations in soil, pine needles, cockroaches, grasshoppers, katydids, stick insects, spiders, lacewings, and mantises, refer to our previous study by Luo et al. [34]. Grasses exhibited similar THg and MeHg concentrations to pine needles, with values of  $6.03 \pm 0.49$  ng/g and  $0.15 \pm 0.07$  ng/g, respectively. In contrast, crickets had lower THg ( $87.4 \pm 17.0$  ng/g) and MeHg ( $2.53 \pm 0.86$  ng/g) concentrations compared to omnivorous cockroaches and carnivorous mantises, spiders, and lacewings. Regarding MeHg%, higher trophic levels such as carnivorous spiders ( $39.5 \pm 9.2\%$ ), lacewings ( $37.3 \pm 1.4\%$ ), and mantises ( $37.4 \pm 7.35\%$ ) exhibited significantly higher MeHg% than herbivorous stick insects ( $3.0\%$ ), caterpillars ( $3.13 \pm 1.33\%$ ), grasshoppers ( $4.07 \pm 2.18\%$ ), crickets ( $3.40 \pm 1.70\%$ ), and omnivorous crickets ( $3.40 \pm 1.70\%$ ) and cockroaches ( $14.9 \pm 8.1\%$ ).

#### 3.2. Dietary compositions of nestlings

Fig. 1(a) confirms the eight potential diet sources of Great Tit nestlings based on  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  measurements, as well as video observations from Luo et al. [34]. The estimated dietary compositions of the nestlings, obtained using BMM, are presented in Fig. 1(b). Among these diets, caterpillars made the highest contribution at  $67.1 \pm 9.1\%$  (72.0%, normalized median, N.M.), followed by cockroaches at  $14.4 \pm 11.4\%$  (12.1%, N.M.). Crickets, katydids, stick insects, grasshoppers, mantises, and spiders accounted for approximately 18.5% (15.9%, N.M.) of the total diet.

The estimated contribution of caterpillars by BMM aligns with the findings from previous video observations in this pine forest ( $67.3\%$ , [34]) as well as video observations ( $75\%$ ) and neck-collar methods ( $44\%$ ) for Great Tit nestlings in Wytham Woods, United Kingdom [64]. Considering the high consistency in the diet composition of tit nestlings over time, even within a one-hour video observation period [37], the dietary compositions of 14-days old nestlings in this study can be regarded as representative of the overall dietary composition from



**Fig. 1.** The mixing space geometry of Great tit nestling prey items for the BMM analysis (a) and the estimated boxplots of each nestling prey item using the simmr package (b). In (a), contour lines and color represent the variation in isotopic values of Great tit nestling feathers (mixture, depicted as white circles), which would influence the probability of a BMM model using prey items (sources, depicted as black rectangles) as reported by Smith et al. [51]. Srcs 1–8 refer to caterpillars, cockroaches, crickets, katydids, stick insects, grasshoppers, mantises, and spiders.

hatching to fledging.

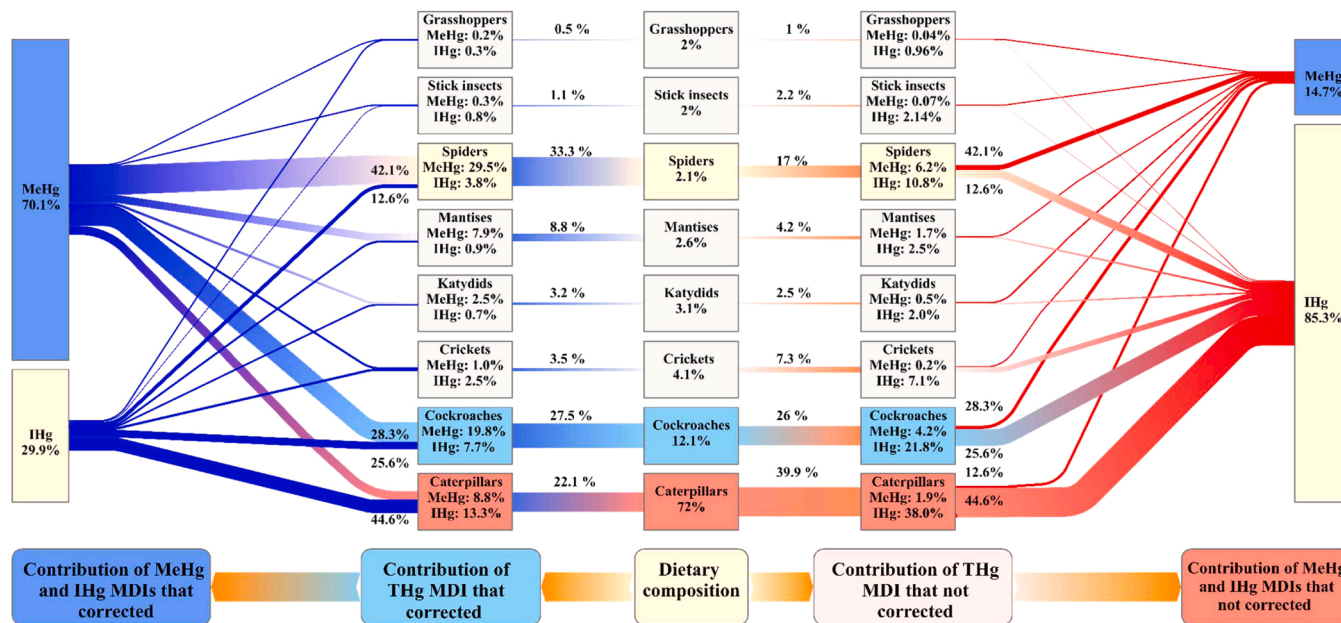
### 3.3. Hg daily intakes of nestlings and Hg flow in the food chain

Based on the estimated total mass (3.61 g/d, dry weight) of nestling diets, the estimated dietary composition, and Hg concentrations, we calculated the MDIs of MeHg and IHg of nestlings at 14 days. We also created a conceptual map of Hg flow in the nestling food chains in terrestrial pine forests using e!Sankey pro (ifu Hamburg, Germany) as depicted in Fig. 2. Since the provisioned items were not fully digested and were often consumed by parents (Fig. S2), and no available absorption factors for Hg in terrestrial songbird nestlings were found, we established two dietary exposure scenarios: one without absorption

factor correction (Scenario 1, Table S3) and one with absorption factor correction (Scenario 2, Table S4).

In Scenario 1, the MDIs of MeHg and IHg for an individual nestling were 1.86 ng/gbw/d and 10.8 ng/gbw/d, respectively, indicating that dietary IHg exposure accounted for the major proportion (85.3%) of Hg exposure in nestlings when absorption factors were not considered. However, in Scenario 2, the MDIs of MeHg and IHg were 1.77 ng/gbw/d and 0.76 ng/gbw/d, respectively, suggesting that dietary MeHg exposure became the major route (70.1%) of Hg exposure in nestlings when absorption factors were taken into account.

Although the Hg daily intake of nestlings was calculated only at 14 days, the contribution rates of provisioned items are likely consistent based on field observations by Pagani-Núñez and Senar [37], suggesting



**Fig. 2.** The flow chart illustrates the MDIs depicting the transfer of IHg and MeHg from invertebrates to nestlings. Panel (left) represents the MDIs after correction, while panel (right) shows the MDIs without correction. The values within the boxes and belts represent the contribution rates of prey items, MDIs of THg, IHg, and MeHg, respectively.

that the relative contribution of Hg daily intake via each prey item also remained stable over the period before fledging. Therefore, from hatching to fledging (about 14 days), caterpillars contributed 44.6% (IHg) and 12.6% (MeHg) of the total MDIs, while spiders, despite being less provisioned, contributed comparable proportions of 12.6% (IHg) and 42.1% (MeHg) of the total MDIs in the two scenarios, respectively. On the other hand, cockroaches contributed similar proportions of 25.6% (MeHg) and 28.3% (IHg) of the total MDIs, respectively. Therefore, these three prey items accounted for a total of 83.0% (MeHg) and 82.8% (IHg) of the total MDIs, indicating that the provision of spiders, cockroaches, and caterpillars were the major dietary exposure pathways for IHg and MeHg in nestlings.

### 3.4. Stable Hg isotope compositions and fractionations

#### 3.4.1. MDF

In Table 1, the highest  $\delta^{202}\text{Hg}$  value ( $-1.46 \pm 0.14\text{‰}$ ) was observed in soil, which was similar to previously reported values in surface forest soil ( $-1.44 \pm 0.24\text{‰}$ ) and soil profiles ( $-1.48 \pm 0.57\text{‰}$ ), but lower than the unweathered bedrock ( $-0.12 \pm 0.31\text{‰}$ ) in the vicinity of Mt. Ailao [62,70]. Pine needles had a  $\delta^{202}\text{Hg}$  value of  $-2.56 \pm 0.36\text{‰}$ , which closely resembled the  $\delta^{202}\text{Hg}$  of their consumer caterpillars ( $-3.50 \pm 1.06\text{‰}$ ). Grasses exhibited a higher  $\delta^{202}\text{Hg}$  value ( $-1.89 \pm 0.20\text{‰}$ ) compared to herbivorous grasshoppers ( $-2.63 \pm 0.04\text{‰}$ ) and stick insects ( $-2.58 \pm 0.11\text{‰}$ , 2 SD). Cockroaches and crickets showed similar values of  $-2.43 \pm 0.07\text{‰}$  and  $-2.28 \pm 0.20\text{‰}$ , respectively, while mantises and spiders exhibited relatively positive values of  $-1.74 \pm 0.18\text{‰}$  and  $-1.94 \pm 0.10\text{‰}$ , respectively. Additionally, the  $\delta^{202}\text{Hg}$  in high trophic nestling feathers was  $-2.04 \pm 0.04\text{‰}$ , which was relatively higher (+0.83‰) than the  $\delta^{202}\text{Hg}$  of their main prey of caterpillars.

Fig. 3 reveals the presence of distinct MDF in the trophic transfers within terrestrial food chains. Notably, foliar samples to herbivorous invertebrates and herbivorous to carnivorous invertebrates exhibited MDF, such as the relatively smaller MDF of  $-0.31\text{‰}$  between pine needles ( $2.2 \pm 2.2\%$ , MeHg%) and caterpillars ( $3.1 \pm 1.3\%$ , MeHg%) and large MDF of  $0.69\text{‰}$  between grasshoppers ( $4.1 \pm 2.2\%$ , MeHg%) to spiders ( $39.5 \pm 9.2\%$ , MeHg%). This large MDF may relate to the various MeHg% between consumers and prey. Moreover, the transfer from caterpillars to nestlings also displayed a relatively higher MDF of  $+0.83\text{‰}$ , which closely resembled the MDF observed in the transfers from fish ( $+0.88\text{‰}$ )/crab ( $+0.96\text{‰}$ )/mussel ( $+0.79\text{‰}$ ) to common eiders [26].

#### 3.4.2. MIF

Soil samples displayed the lowest  $\Delta^{199}\text{Hg}$  value of  $-0.35 \pm 0.05\text{‰}$ , which closely resembled the values observed in forest surface soil ( $-0.36 \pm 0.10\text{‰}$ ) and soil profiles ( $-0.46 \pm 0.24\text{‰}$ ), but were lower than the unweathered bedrock ( $-0.08 \pm 0.06\text{‰}$ ) near Mt. Ailao [62,70]. As primary components of forest food chains, pine needles, and grasses exhibited lower  $\Delta^{199}\text{Hg}$  values of  $-0.21 \pm 0.08\text{‰}$  and  $-0.28 \pm 0.13\text{‰}$ , respectively, which were comparable to the  $\Delta^{199}\text{Hg}$  values in foliar samples ( $-0.29 \pm 0.06\text{‰}$ ) and atmospheric Hg ( $-0.18 \pm 0.03\text{‰}$  and  $-0.08 \pm 0.08\text{‰}$ ; total gaseous Hg, TGM) in the vicinity of Mt. Ailao [13, 33,69]. Invertebrates demonstrated varying  $\Delta^{199}\text{Hg}$  values, including cockroaches ( $-0.34 \pm 0.06\text{‰}$ ), grasshoppers ( $-0.30 \pm 0.03\text{‰}$ ), caterpillars ( $-0.27 \pm 0.03\text{‰}$ ), and stick insects ( $-0.14 \pm 0.08\text{‰}$ , 2 SD). Carnivorous spiders and mantises displayed relatively higher  $\Delta^{199}\text{Hg}$  values of  $-0.04 \pm 0.08\text{‰}$  and  $-0.08 \pm 0.08\text{‰}$ , respectively. As expected, nestling feathers exhibited the highest  $\Delta^{199}\text{Hg}$  values ( $0.47 \pm 0.06\text{‰}$ ), which were similar to those observed in Russet Sparrow nestling feathers ( $0.46 \pm 0.21\text{‰}$ ) and insectivorous adult songbirds ( $0.28\text{--}0.43\text{‰}$ ) in Hg mining areas [68].

In contrast to MDF, minimal MIF was observed in the trophic transfers within avian food chains, such as pine needles to caterpillars ( $-0.07\text{‰}$ ) and grass to grasshoppers ( $-0.02\text{‰}$ )/stick insects ( $-0.14\text{‰}$ ). This finding aligns with the understanding that MIF is absent in trophic

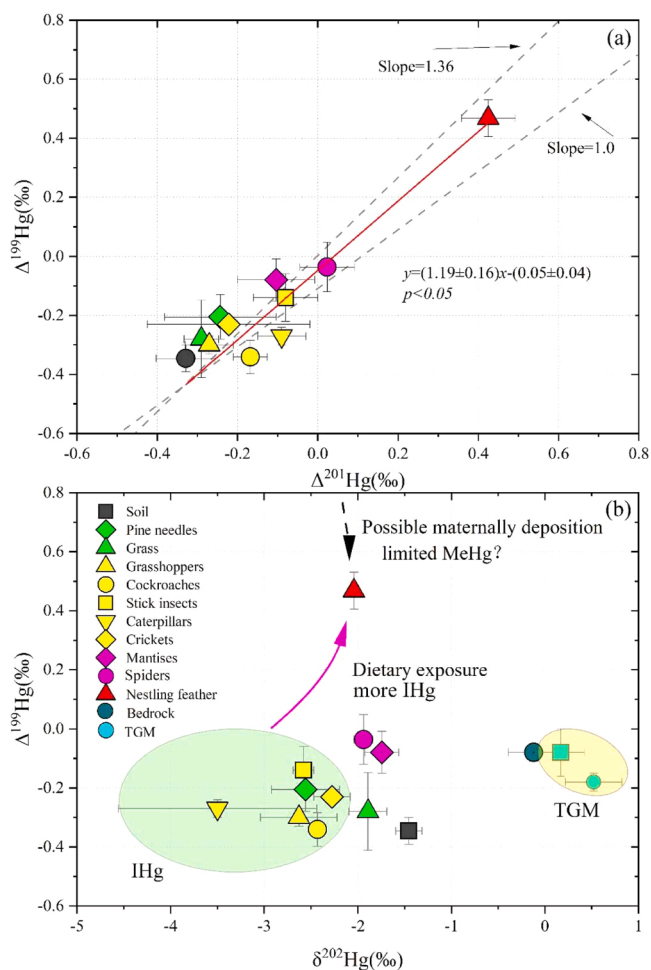


Fig. 3. The correlation between  $\Delta^{199}\text{Hg}$  versus  $\Delta^{201}\text{Hg}$  (a) and the values of  $\Delta^{199}\text{Hg}$  and  $\delta^{202}\text{Hg}$  (b) of soil and food chain samples obtained from the pine forest ecosystem.

transfers within both aquatic and terrestrial food chains [27,68]. However, significant MIFs ( $+0.55\text{‰}$  to  $+0.81\text{‰}$ ) were observed between nestling feathers and their prey items, particularly the primary provisioned caterpillars ( $+0.74\text{‰}$ ). These substantial MIFs differed from the negligible MIF observed in the transfers from fish and crab to Common Eiders [26], but were comparable to the MIF observed between spiders and arachnivorant Chestnut-headed Tesia ( $+0.23\text{‰}$  to  $+0.51\text{‰}$ ), rice grains and granivorous Tree Sparrow ( $+0.50\text{‰}$ ), and raspberries and frugivorous Brown-breasted Bulbul ( $+0.46\text{‰}$ ) in Hg mining areas [68].

#### 3.4.3. Estimating MeHg isotope ratios

Given the limited influence of aquatic food chains on the pine forest food chain, the average Hg isotope compositions in foliar samples ( $-0.24 \pm 0.10\text{‰}$  and  $-2.26 \pm 0.45\text{‰}$ ,  $\Delta^{199}\text{Hg}$  and  $\delta^{202}\text{Hg}$ ) were considered representative of the IHg isotopic compositions in this terrestrial avian food chain. Consequently, the estimated MeHg isotope ratios ( $\Delta^{199}\text{Hg}$  and  $\delta^{202}\text{Hg}$ ) were determined for mantises ( $0.19\text{‰}$  and  $-0.87\text{‰}$ ) and spiders ( $0.27\text{‰}$  and  $-1.45\text{‰}$ ). However, due to the lower proportion of MeHg and the presence of maternally deposited Hg, the MeHg isotope ratios in nestling feathers were not calculated.

### 3.5. Potential reasons for the large MIFs between nestlings and prey items

In local ecosystems, it was seen that lower values of  $\Delta^{199}\text{Hg}$  and  $\delta^{202}\text{Hg}$  were favored in the accumulation of IHg by consumers (e.g., caterpillars and grasshoppers), while higher values of  $\Delta^{199}\text{Hg}$  and

$\delta^{202}\text{Hg}$  were favored in the accumulation of MeHg by high trophic consumers (e.g., spiders and mantises). Thus, one possible explanation for the large MIFs observed between nestlings and prey items is the significant association between MIF and variations in MeHg% (or the mixture of IHg and MeHg) between predators (or consumers) and prey items (or diets), as also noted in our previous study in Hg mining areas [68]. For instance, both conifer pine needles and caterpillars had relatively lower MeHg% (2–3%), resulting in measured Hg isotopic compositions being close to IHg isotope compositions with minimal impact from MeHg isotope compositions. However, in the transfers of caterpillars-nestlings and grasshoppers-spiders, the substantial differences in MeHg% between prey items (3–4%) and predators (20–30%) may lead to the observed large MIFs.

Factors such as Hg mixture from out-habitat sources like aquatic environments [61], bird movement or changes in foraging areas [44, 45], and nutritional stress [25] can attribute to Hg isotope variations in songbirds. However, in a pure terrestrial ecosystem over a relatively short period (~14 days), these factors are unlikely to have a significant effect on the Hg isotope compositions in nestlings. Furthermore, considering the minimal differences in Hg isotope compositions between feathers and blood of Skuas chicks [44], the distinct Hg isotope composition discrepancies observed in adult bird tissues (e.g., blood and feathers; [44,45]) also cannot explain the observed large MIFs.

Feathers provide a reflection of the Hg concentration in the blood during feather growth [3] and are strongly influenced by the dynamic changes in blood Hg concentration, especially in juvenile or chick birds that simultaneously incorporate maternally deposited and diet-derived Hg before fledging [1]. During the short breeding period, the rapid and imbalanced integration of Hg from dietary route and maternal deposition with various values of  $\Delta^{199}\text{Hg}$ , such as the much lower  $\Delta^{199}\text{Hg}$  in prey items in this study but elevated  $\Delta^{199}\text{Hg}$  in murre eggs ( $\sim 0.88\text{‰}$ ) as reported by Day et al. [9] and Green-backed Tit ( $1.37 \pm 0.07\text{‰}$ ) and Russet Sparrow eggs ( $0.88 \pm 0.10\text{‰}$ ) in another pure pine forest (personal unpublished data), could be a possible contributing factor to the observed large MIF between nestling feathers and their prey items.

### 3.6. Hg sources in nestlings and their environmental implications

Maternally deposited Hg and dietary-acquired Hg are two primary sources of Hg for birds during the chick or juvenile stage [1]. As depicted in Fig. 4, since MeHg is predominant in egg contents (Ackerman et al., 2013) as well as the high proportion of MeHg ( $94.2 \pm 12.0\%$ , unpublished data) in the eggs of Great Tits inhabiting both heavily contaminated and control sites in Hg mining areas. Therefore, it is likely that MeHg also prevails in eggs from the studied pine forest. Moreover, with the exclusion of cockroaches and stick insects as outliers for MeHg% and  $\Delta^{199}\text{Hg}$ , the estimated  $\Delta^{199}\text{Hg}$  of MeHg in food chain is  $0.25\text{‰}$  (Fig. S3), suggesting there is another MeHg source with elevated  $\Delta^{199}\text{Hg}$  to Great Tit nestlings. Consequently, due to the significant disparity in MeHg% between eggs ( $\sim 90\%$ ) and feathers ( $\sim 20\%$ ), the dietary pathway appears to be the primary route supplying more IHg to nestlings, leading to a dilution of MeHg levels in growing feathers. Furthermore, despite the pine forest being distant from aquatic or riparian habitats, parent birds with larger foraging ranges may have already acquired Hg from non-pine habitat sources, exhibiting higher  $\Delta^{199}\text{Hg}$  values before the breeding season or egg laying. This factor could explain why nestling feathers exhibited the highest  $\Delta^{199}\text{Hg}$  values but relatively lower MeHg%.

Although Hg isotope compositions in blood have been regarded as a suitable tool for accurately determining Hg sources in birds [44,46,61], our findings suggest that the rapid and imbalanced mixture of dietary-acquired Hg and maternally deposited Hg can influence Hg isotope compositions in nestlings. Therefore, caution should be exercised when utilizing growing nestling feathers to identify Hg sources and discuss the biomagnification of food chains. In future studies, it is crucial and urgent to investigate the differences in Hg isotope composition or fractionation during Hg transfer among internal organs, blood, and feathers in nestlings to quantitatively evaluate the contribution of maternally transmitted Hg to nestlings.

## 4. Conclusions

This investigation revealed that caterpillars, cockroaches, and spiders constituted the primary sources of MeHg and IHg of Great Tit

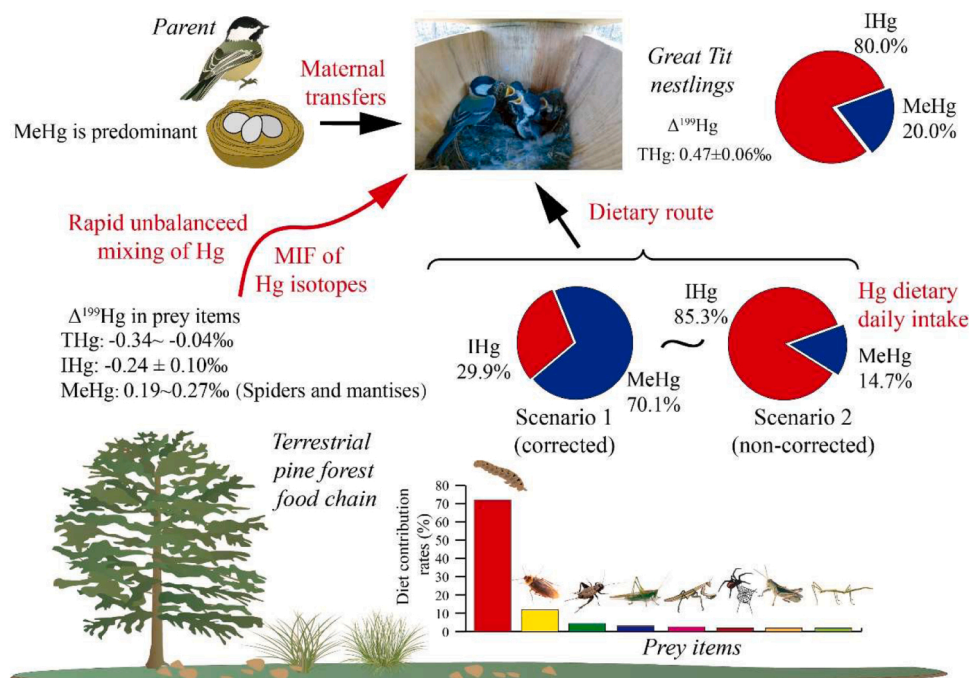


Fig. 4. The conceptual diagram represents the Hg flow from prey items to nestlings within the food chain of the pine forest ecosystem.

nestlings through the dietary pathway. Remarkably high  $\Delta^{199}\text{Hg}$  values in feathers and substantial differences between consumers and their diets, such as caterpillars and cockroaches-nestlings, were observed. As for this large MIF, the inconsistent MeHg% between prey items and nestling feathers as well as the rapid and imbalanced integration of maternally deposited and dietary-acquired Hg during the relatively short breeding stage (from hatching to fledging) are the two most likely explanations. Therefore, caution should be exercised when utilizing the concentration and Hg isotope compositions in growing nestling feathers to reconstruct Hg sources and discuss bioaccumulation in nestlings and juvenile birds. Further studies, such as analyzing Hg isotope compositions in parents and eggs, as well as implementing long-term monitoring of nestlings, would facilitate the quantification of maternally deposited and dietary-acquired Hg in songbird nestlings.

### Environmental implication

In a clear bird food chain identified via artificial nest boxes, Bayesian isotope mixing model and stable mercury isotopes were used to trace the Hg sources and transfers in this food chain. We found that dietary exposure tends to provide more inorganic Hg and dilute the MeHg as total Hg (MeHg%) in nestlings, and the imbalanced Hg incorporation from maternal transfers and dietary intake may affect the MIF of Hg isotopes in nestling growing feathers. Therefore, the Hg isotope compositions in growing feathers should be used with caution, and the maternally transferred Hg in nestlings should also be considered.

### CRedit authorship contribution statement

**Zhidong Xu:** Conceptualization, Methodology, Writing – original draft, Writing – review & editing, Funding acquisition; **Kang Luo:** Investigation, Writing – review & editing, Funding acquisition; **Qinhui Lu:** Investigation, Methodology; **Lihai Shang:** Visualization, Investigation Writing – review & editing; **Jing Tian:** Methodology; **Zhiyun Lu:** Investigation, Supervision; **Qihua Li:** Methodology; **Zhuo Chen:** Reviewing and editing, Supervision; **Guangle Qiu:** Writing – reviewing and editing, 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.

### Data availability

Data will be made available on request.

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### Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.jhazmat.2023.132263](https://doi.org/10.1016/j.jhazmat.2023.132263).

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