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Season as a discriminating factor for faecal metabolomic composition of great tits (*Parus major*)

Roschong Boonyarittichaikij [1,2], Beata Pomian [3], Daan Dekeukeleire [4], Luc Lens [4], Dries Bonte [4], Kris Verheyen [5], Frank Pasmans [1], An Martel [1,¶] & Elin Verbrugghe [1,¶,*]

[1] Wildlife Health Ghent, Department of Pathology, Bacteriology and Avian Diseases, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, 9820 Merelbeke, Belgium.
[2] Department of Clinical Sciences and Public Health, Faculty of Veterinary Science, Mahidol University, Phuttamonthon, Nakhon Pathom, 73170, Thailand.
[3] Laboratory of Chemical Analysis, Department of Veterinary Public Health and Food Safety, Faculty of Veterinary Medicine, Ghent University, Merelbeke, Belgium.
[4] Terrestrial Ecology Unit, Department of Biology, Ghent University, K.L. Ledeganckstraat 35, 9000 Ghent, Belgium.
[5] Forest & Nature Lab, Department of Environment, Ghent University, Geraardsbergsesteenweg 267, 9090 Gontrode, Belgium.

[¶] Shared senior authorship

[*] Corresponding author: Elin.Verbrugghe@ugent.be

Abstract. The microbiome of wild birds has been associated with health status and risk of disease development, but underlying metabolomic mechanisms are still unknown. Metabolites produced by microbial organisms may affect host metabolic processes and by doing so influence host health. Here we provide for the first time data on the faecal metabolome of wild great tits (*Parus major*) by analyzing metabolites associations with age, sex, season and body condition. Using untargeted metabolomics, we analyzed faecal samples from 112 great tits that were caught in a deciduous forest fragment in Flanders (Belgium) during late autumn and 19 animals that were re-captured during early spring. In this study, no significant associations between the faecal metabolites and age, sex and body condition were observed. However, season was shown to be a discriminating factor for the metabolomic composition of great tits, suggesting an impact of environmental factors.

Keywords. Metabolomics, great tit, faeces, season, scaled mass index.

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Introduction

Over the last 15 years, the microbiome has become an important topic in research (LI *et al*. 2020). Especially in mammalian species the microbiome has received increasing research attention due to its possible role in health status and risk of disease development (LEY *et al*. 2006; TURNBAUGH *et al*. 2009; PEDERSEN *et al*. 2016). However, the mechanisms underlying microbiome-mediated health effects are often unclear as usually a functional readout is missing and metabolite profiles related to the gut microbiota could bring researchers to unknown insights (LE GALL *et al*. 2011; MARCOBAL *et al*. 2013; VERNOCCHI *et al*. 2016; TANG *et al*. 2019). By the production of small molecules (metabolites) that accumulate in the gut and circulate throughout the body, the microbiome impacts numerous aspects of a host's biology (TREUREN & DODD 2020). Human studies show that the faecal metabolome largely reflects gut microbial composition, explaining around 70% of its variance (ZIERER *et al*. 2018; VISCONTI *et al*. 2019).

In contrast to mammalian and especially human research, studies examining the gut microbiome in birds are rather limited (GROND *et al*. 2018). However, there are indications that the microbiome plays a pivotal role in the health of wild birds. So far, studies investigating the avian metabolome are heavily biased towards poultry (BEAUCLERCQ *et al*. 2019; DORR *et al*. 2019). In great tit nestlings (*Parus major*; Phylum Chordata, Class Aves; LINNAEUS 1758), a widely distributed bird species throughout Europe, higher diversity and stability in microbiota composition are linked to a higher relative body mass (TEYSSIER *et al*. 2018a). With metabolomics and the gut microbiome being so strongly related, it has thereby been hypothesized that beneficial effects of the microbiome on host condition and fitness are mediated by gut microbiome metabolites (LAMICHHANE *et al*. 2018).

The microbiome only codes microbial possibilities rather than their actual activity (ZIERER *et al*. 2018), whereas the metabolome provides essential information regarding the microbial functionality (ZIERER *et al*. 2018; VISCONTI *et al*. 2019). We here examine whether the faecal metabolome of great tits, as a proxy for the intestinal microbiome function, correlates with host fitness, by exploring the relationship between the faecal metabolomic composition and body condition (scaled mass index, SMI) (PEIG & GREEN 2009). As the gut microbiome is linked to various life-history traits including age (VAN DONGEN *et al*. 2013; TEYSSIER *et al*. 2018a) and environmental factors such as alterations in food sources (GROND *et al*. 2018; TEYSSIER *et al*. 2018b), we also analyzed the associations between the faecal metabolome of great tits and environmental factors (season) or life-history traits (age and sex). We expected to identify the major drivers that influence the faecal metabolome of great tits. More specifically, we expected that the metabolome would be linked to the body condition of the great tits, which could lead to the identification of body health markers (TEYSSIER *et al*. 2018a).

Material and methods

Field study and sample collection

Great tits were captured in a 39.5 ha mixed deciduous forest fragment in Gontrode, Belgium (coordinates: 50.975° N, 3.799° E). During the autumn of 2016 (November till early December 2016) and spring 2017 (early March 2017), mist netting sessions and weekly night checks in nest boxes were carried out. The mist nets were set up twice to four times a week for approximately 4–5 hours and they were checked every 20 min. No animals died during the sampling protocol. All individuals were ringed and measured (tarsus length to the nearest 0.01 mm; wing length (to the tip of the longest primary feathers) to the nearest 0.5 mm and body mass to the nearest 0.1 g), aged (first-year bird or adult) and sexed (based on plumage characteristics; SVENSSON 1992). In addition, a primary physical examination was performed before any protocol was started. Only clinically healthy birds (e.g., no ruffled feathers, no diarrhea, no altered behavior or bad body condition) were sampled. In total, 112 great tits were caught during late autumn 2016, and 19 of these were re-captured during the early spring of 2017. Upon capture, faecal

samples (n = 131) were collected by placing the animals in a clean and sterilized cotton bag and faeces was collected from the bag surface. All samples were kept in sterile Eppendorf tubes at -70 °C until analysis.

The body condition was calculated using the scaled mass index (SMI) (PEIG & GREEN 2009). This index adjusts the mass of all individuals to that which they would have obtained if they all had the same body size, using the equation of the linear regression of ln-body mass on ln-tarsus length estimated by type-II (standardized major axis; SMA) regression (Supplementary Table 1). The faecal metabolome of the great tits was linked to the SMI using Simca™ 14 (Umetrics, Malmo, Sweden), to analyze categorical variables. Therefore, based on the SMI median (17.76 g), the birds were divided in two groups (n = 65 per group). Alternatively, we also grouped them based on the highest 10% (≥ 19.12 g) and lowest 10% (≤ 16.33 g) of the SMI (n = 13 per group).

Bird ringing and handling were carried out under license and guidelines of the Belgian Ringing Scheme and the Flemish authorities (Agentschap voor Natuur en Bos; ANB/BL-FF/V15-00034). All trapping and sampling protocols were approved and permitted by the Ethical Committee VIB (the Flanders Institute for Biotechnology) Ghent site (EC2015-023).

Chemicals and reagents

Ultra-high performance liquid chromatography-mass spectrometry (UHPLC-MS) grade solvents, including methanol (MeOH) and acetonitrile, from Biosolve (Valkenswaard, the Netherlands) were used. Water was purified by a Milli-Q system (Millipore, Brussels, Belgium) and formic acid (FA) was obtained from Sigma-Aldrich (Saint Louis, MO, USA).

Liquid chromatography

Faecal samples were homogenized and lyophilized. Afterwards, 2 ml ice cold 80% MeOH was added to 33.33 mg lyophilized sample. The volume of the solvent was adjusted to the available amount of sample. Then, 25 µl of 100 ng/µl internal standards (D-valine-D8, L-alanine-D3 and cortisol-D4) were added and each sample was vortexed and subsequently centrifuged at 17 000 g for 10 min. The supernatant was transferred to a liquid chromatography-mass spectrometry (LC-MS) vial. A quality control (QC) was prepared by pooling 100 µl of all individual samples. This pool was divided into 2 vials, which were used for column conditioning (external QC samples, EQC) and data normalization (internal QC samples, IQC). EQC samples were analyzed in duplicate preceding the batch run and IQC samples were analyzed in duplicate after each set of 10 samples, which were analyzed in a randomized order. UHPLC-Quadrupole-Orbitrap HRMS analysis was achieved on a Dionex UltiMate 3000 XRS UHPLC system (Thermo Fisher Scientific, San Jose, CA, USA). The compounds were separated on an Acquity® UPLC HSS T3 column (150 × 2.1 mm, 1.8 µm) (Waters, Manchester, UK) at 45°C, with a mobile phase flow rate of 400 µl/min. The phase consisted of (A) 0.1% FA in water and (B) 0.1% FA in acetonitrile. A gradient elution program was applied as follows: 0–1.5 min 98% A and 2% B, 1.5–7 min 98% A and 2% B, 7–8 min 75% A and 25% B, 8–12 min 40% A and 60% B, 12–14 min 5% A and 95% B, 14–14.1 min 5% A and 95% B, 14.1–18 min 98% A and 2% B. The injection volume was 10 µl.

Mass spectrometry

A Q-ExactiveTM Orbitrap mass spectrometer (Thermo Fisher Scientific, San Jose, USA) equipped with heated electrospray ionization (HESI-II source), was used in polarity switching mode. Accurate mass spectra were acquired with an *m/z* scan range of 53.4–800 Da, at a mass resolution of 140 000 full width at half maximum at 1 Hz. Other instrumental parameters are presented in Table 1.

TABLE 1

Instrumental parameters for Orbitrap mass spectrometry.

Parameter	Value	Parameter	Value
Sheath gas flow (AU)	50	HESI-II positions	0/B/1
Auxiliary gas flow (AU)	25	Max infection time (ms)	80
Sweep gas flow (AU)	5	S-lens RF level	50
Capillary temperature (°C)	250	Fragmentation	Off
Heater temperature (°C)	350	AGC target	500000
Spray Voltage	3		

Data analysis and statistics

In untargeted metabolomics, different steps are required for data acquisition and analysis, as described by (Van Meulebroek *et al*. 2015). The first step involves data preprocessing with Sieve™ 2.1 (Thermo Fisher Scientific, San Jose, USA). In this study, the data for each ionization mode (+ or -) were processed separately during peak list generation to achieve better model characteristics in Simca™ 14. First, appropriate parameter settings were applied as follows: *m*/*z*: 53.4–800 dalton, retention time: 0.5–16 min; *m*/*z* width: 6 ppm; maximum peak width: 0.75 min, peak intensity threshold: 1 000 000 arbitrary units (AU), and maximum number of frames: 20 000. Afterwards, peak alignment was applied. In the final step, a number of discriminative parameters, used to retain only the most relevant ions, were set as follows: ratio (the average ion abundances between samples of different groups): < 0.66 or > 1.5 and P-value < 0.05. Data normalization was performed by dividing the peak intensity of a particular metabolite in a sample by the mean peak intensity of that metabolite in the following two internal QC samples (Vanden Bussche *et al*. 2015).

The second phase in the general workflow involves predictive modelling of the retained ions to discover discriminating metabolites taking season (autumn 2016 vs spring 2017), age (first-year bird vs adult), sex (male vs female) and body condition (SMI) into account. For this, the normalized ion abundances were imported in Simca™ 14, the data were log-transformed to induce normality and scaled by the Pareto method (dividing each variable by the square root of the standard deviation), which reduces the relative importance of larger values and partially preserves data structure (Van Den Berg *et al*. 2006). An unsupervised principal component analysis (PCA-X) model was created to look for potential outliers. In addition, an orthogonal partial least-square discriminant analysis (OPLS-DA) was used to evaluate potential discriminating metabolites between different sample groups (season, age, sex, and body condition (SMI)). The validation criteria were as follows: $R^2X > 0.5$, $R^2Y > 0.5$, $Q^2Y > 0.5$, CV Anova $p < 0.05$ and a good permutation test ($n = 100$). An S-plot was created in the OPLS model to select the discriminating metabolites in significant models, using the following criteria: S-plot with cut-off values of $p(corr) \geq 0.5$ and $p \geq -0.025$ and $p \geq 0.02$ for the positive ions and $p(corr) \geq 0.5$ and $p \geq -0.03$ and $p \geq 0.03$ for the negative ions, Jack-knifed confidence intervals (not across 0), and a variable importance in projection (VIP) scores >1.

Results

For both the positive and negative ionization a patterning was uncovered between the 19 samples from autumn 2016 and the samples from the same birds recaptured in spring 2017 (Fig. 1). In order to evaluate season as a discriminating factor for faecal metabolomic composition in great tits, OPLS-DA models were constructed (Fig. 2). The validity of the supervised OPLS-DA model was evaluated through $R^2(Y)$,

$Q^2(Y)$, CV-ANOVA testing (Supplementary Table 2) and permutation tests (Supplementary Fig. 1). Taking season as a discriminating factor, values obtained for $R^2(X)$, $R^2(Y)$ and $Q^2(Y)$ were respectively 0.569, 0.996 and 0.699 in the positive ionization mode, and respectively 0.543, 0.990, 0.800 in the negative ionization mode. Moreover, CV-ANOVA analysis ($p \leq 0.001$) demonstrated that the obtained OPLS-DA models were highly significant. None of the other factors (age, sex and body condition) had an impact on great tit faecal metabolomic composition with $R^2(X) < 0.5$, $R^2(Y) < 0.5$, $Q^2(Y) < 0.5$ or $p > 0.05$ (Supplementary Table 2).

After model building, S-plots were constructed that retain those metabolites that were specifically associated with great tit faecal samples in autumn 2016 or spring 2017 (Fig. 3). After removing cluster ions, 5 positively charged metabolites and 14 ions negatively charged were retained (Supplementary Table 3). These were screened against an in-house database comprising 300 metabolites, but no match was found.

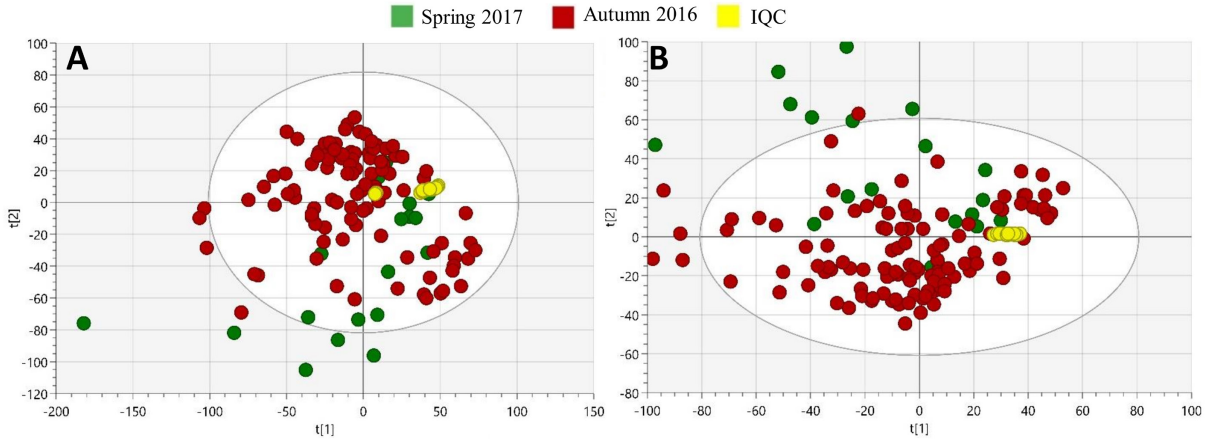

Fig. 1 – **Plots from multivariate statistical analysis.** Score plots of the PCA-X model for the great tit faecal samples in **(A)** positive and **(B)** negative ionization mode. The green, red and yellow symbols represent the faecal samples in spring 2017, in autumn 2016 and internal quality control (IQC) samples, respectively.

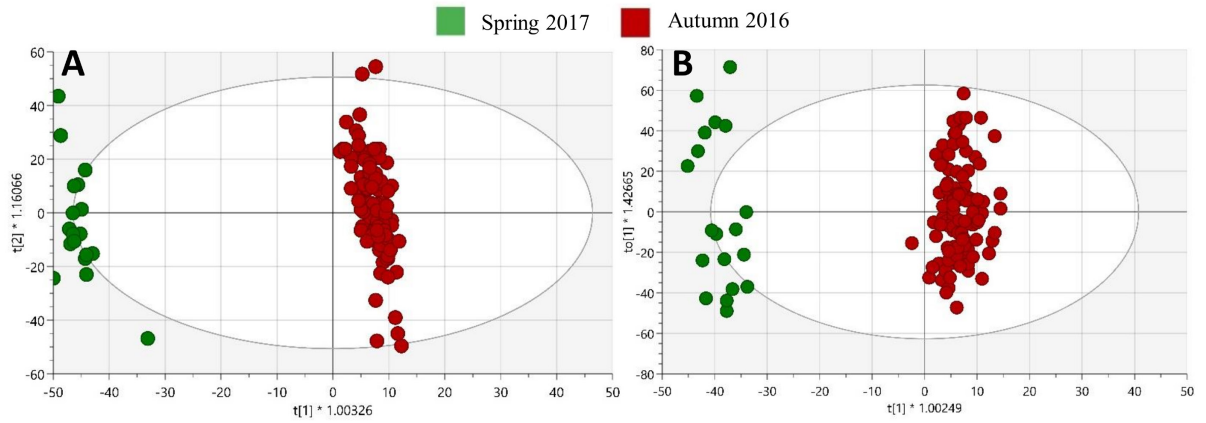

Fig. 2 – **OPLS-DA analysis.** Score plots of a partial least-squares discriminant analysis model for a dataset containing great tit faecal samples collected in autumn 2016 (red) and in spring 2017 (green), in **(A)** positive and **(B)** negative ionization mode.

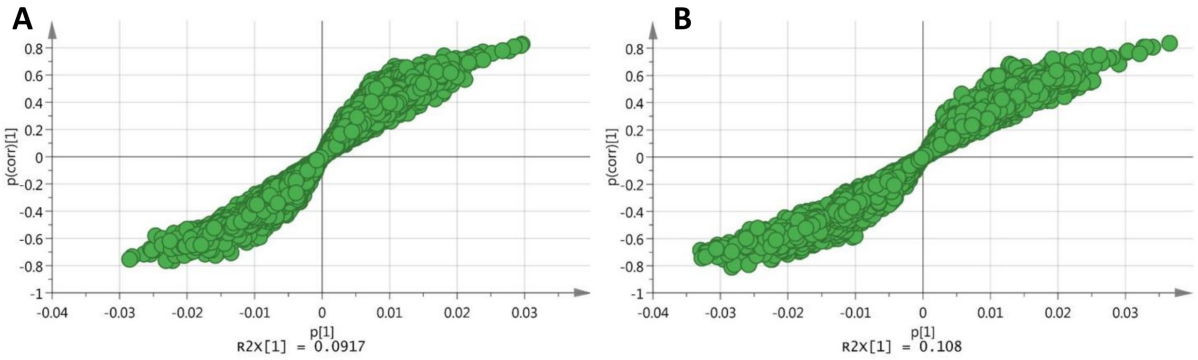

Fig. 3 – **S-plots.** S-plots for faecal samples of great tits in **(A)** positive and **(B)** negative ionization mode, wherein each dot represents a metabolite.

Discussion

In humans it has been shown that the body mass index (BMI) is associated with the metabolome, making the metabolome profile a strong indicator of body health (Cirulli *et al*. 2019). When analyzing the faecal metabolome of great tits, no such association was observed as the SMI was not correlated with metabolomic composition. Thus, our data indicates that the metabolome of great tits is not linked to the overall body condition of these birds. However, when analyzing the interactions between the body condition and the metabolomic profile using Simca™ 14, the SMI was evaluated as a categorical variable, possibly hiding an effect. Also, in this study we only analyzed great tits originating from the same forest plot, whereas environmental factors such as alterations in habitat and food sources have been shown to influence the avian microbiota composition (Teyssier *et al*. 2018b) and SMI (Rouffaer *et al*. 2017). In addition, in this study, we only sampled healthy birds that didn't show any clinical signs. Possibly the lack of unhealthy individuals or death animals could have masked a link between the SMI and faecal metabolomic composition. As such it would be interesting to take a habitat effect into account by analyzing birds from different capture locations and/or including unhealthy or deceased animals.

Unlike SMI, seasonal changes were shown to affect the intestinal metabolomic composition of great tits. The influence of seasonal changes on metabolic rates in birds has been a topic of interest for decades (Miller 1939; Dawson 1958; Hart 1962), with temperature being one of the major modifiers of metabolic level in endothermic animals (Swanson 2010). The sampled great tits face changes in cold exposure and thermostatic costs and they are experiencing interacting effects of shorter days for foraging, longer nights of forced fasting and relatively low availability of food during autumn/winter periods (Swanson 2010). Besides, during the breeding season, changes in metabolomic composition related to the energetics of reproduction might also be expected (Golet & Irons 1999). All the seasonal changes could influence host physiology and, as a consequence, the faecal metabolomic profile. For example, the absence of free nutrients or physiological responses of a host to fasting may result in the selective development of resident microorganisms and cause complementary shifts in diversity and abundances of taxa, which could lead to changes in the faecal metabolomics profiles (Kohl *et al*. 2014). Another possibility for seasonal changes in faecal metabolites could be the altered diet of the birds. During their breeding and post-breeding, great tits preferably forage on invertebrates in all developmental phases (including Lepidoptera, Araneidae, Hemiptera, Diptera, Hymenoptera, Coleoptera) (Rytkönen *et al.* 2018). In times when the invertebrate food supply is limited (autumn/winter), the major component of the diet includes plant material such as buds and seeds of beech, hazel and oak, but also seeds provided at bird tables (Vel'ký *et al*. 2011). As food source and food quality have been shown to pose a differential

selection pressure on the gut microbiome of wild birds (GROND *et al*. 2018), it is also possible that the changes in metabolomic composition are linked to seasonal alterations in diet composition.

In this study, we analyzed faecal samples instead of cloacal swabs. This was done for two reasons, namely (1) due to the noninvasive nature of the sample collection and (2) because faeces is a more representative matrix in comparison to cloacal swabs. Faeces typifies the unique link with the gastrointestinal functionality, encompassing gut integrity and digestive and absorptive processes (GREGORY *et al*. 2013). It strongly reflects dietary intake and shows the interactions between a host and the gut microbiome and has been put forward as the essential biological matrix for in-depth metabolomic and microbiome studies (VANDEN BUSSCHE *et al*. 2015; VAN MEULEBROEK *et al*. 2017; VIDEVALL *et al*. 2017).

Screening of the metabolites specifically linked to season against an in-house database did not result in identification of these metabolites and because of the limited faecal mass per sample we were restricted in further identification attempts using MS/MS analysis (SCHRIMPE-RUTLEDGE *et al*. 2016). Intestinal metabolites mainly originate from gut microbiota and the host itself. Host metabolites include for example free fatty acids, amino acids and vitamins, but metabolites derived from gut microbiota are also essential for intestinal homeostasis including for example bacteriocins, short-chain fatty acids and quorum-sensing autoinducers (LI *et al*. 2018). With the host microbiome playing an important role in maintaining host physiology and the metabolome largely reflecting the gut microbial composition (70%) (ZIERER *et al*. 2018; VISCONTI *et al*. 2019), these processes are inextricably linked. At this point, without identification of the discriminating metabolites it is however not possible to state whether the metabolites are host- or microbially-derived. Yet, with the gained knowledge, this study could for example serve as a reference study for future research using a multi-omics approach investigating the relationship between environment, the microbiome and the circulating host- and microbially-derived metabolome. Our results for example might open new perspectives to identify global relationships between specific dietary compounds, the circulating metabolites, how this is shaped by changes in the gut microbiome and what the consequences are on health status and future health risk.

Summarized, we provided a study that for the first time analyzes the relation between environmental factors, life-history traits and body condition of wild great tits with the faecal metabolome. We hypothesized that the avian faecal metabolomic composition could be used as a proxy for host health, but no such interactions were found. However, as all birds originated from the same location, a correlation may be masked. Furthermore, all birds were clinically healthy and therefore, might show no impact on body condition yet. Instead, season was identified as a discriminating factor for the great tit metabolome. As such, our data highlight the influence of environmental factors on the host metabolome.

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Declaration of Conflicting Interests

The authors declare that there is no conflict of interest.

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SUPPLEMENTARY TABLE 1

Sample characteristics: per bird ID the sex (male (♂) / female (♀)), age (first-year bird/adult), SMI and season of faecal sampling (autumn 2016/spring 2017) are given.

Bird ID	Sex	Age	SMI autumn 2016	SMI spring 2017	Season of sampling
58V92657	♂	Adult	17.37		autumn 2016
58V92955	♂	Adult	17.31		autumn 2016
59V39008	♀	Adult	17.13	16.99	autumn 2016
					spring 2017
59V39009	♂	Adult	17.15		autumn 2016
59V39011	♀	Adult	18.43		autumn 2016
59V39056	♀	First-year	18.57	18.12	autumn 2016
					spring 2017
59V39115	♂	Adult	18.97		autumn 2016
59V39118	♂	Adult	18.38		autumn 2016
59V39121	♂	Adult	17.29		autumn 2016
59V39143	♀	Adult	18.31		autumn 2016
59V39151	♂	Adult	16.65	15.97	autumn 2016
					spring 2017
59V39156	♀	Adult	15.84	15.45	autumn 2016
					spring 2017
59V39177	♀	Adult	20.14		autumn 2016
59V39184	♀	Adult	18.83		autumn 2016
59V39185	♀	Adult	18.26		autumn 2016
59V39187	♂	Adult	17.17	15.79	autumn 2016
					spring 2017
59V39198	♂	Adult	17.89		autumn 2016
59V39201	♂	Adult	18.02		autumn 2016
59V39202	♀	Adult	17.15		autumn 2016
59V39203	♀	Adult	18.06	17.58	autumn 2016
					spring 2017
59V39204	♂	Adult	17.12		autumn 2016
59V39211	♂	Adult	16.08		autumn 2016
59V39227	♂	First-year	18.35		autumn 2016
59V39232	♂	First-year	18.35	16.98	autumn 2016
					spring 2017
59V39241	♀	First-year	19.34		autumn 2016
59V39243	♀	First-year	17.70		autumn 2016
59V39260	♂	First-year	16.87		autumn 2016
59V39298	♂	First-year	16.45		autumn 2016
59V39408	♀	First-year	18.75		autumn 2016
59V39409	♀	First-year	18.80		autumn 2016
59V39439	♀	First-year	19.58		autumn 2016
59V39446	♀	First-year	16.77		autumn 2016
59V39480	♂	Adult	17.82		autumn 2016
59V39483	♂	First-year	18.21	17.55	autumn 2016
					spring 2017
59V39484	♂	First-year	16.96		autumn 2016

Bird ID	Sex	Age	SMI autumn 2016	SMI spring 2017	Season of sampling
59V39485	♂	Adult	18.20	18.05	autumn 2016
					spring 2017
59V39527	♂	First-year	16.44	16.29	autumn 2016
					spring 2017
59V39529	♀	First-year	17.50		autumn 2016
59V39535	♀	First-year	17.34		autumn 2016
59V39575	♂	First-year	16.46		autumn 2016
59V39580	♂	First-year	17.27		autumn 2016
59V39586	♀	First-year	20.12	18.01	autumn 2016
					spring 2017
59V39587	♂	First-year	18.85		autumn 2016
59V39596	♂	First-year	16.81		autumn 2016
59V39745	♀	First-year	17.03		autumn 2016
59V39746	♂	Adult	21.44		autumn 2016
59V39747	♂	Adult	18.90		autumn 2016
59V39748	♂	First-year	17.51		autumn 2016
59V39749	♀	First-year	16.22		autumn 2016
59V39750	♀	First-year	17.81		autumn 2016
59V39751	♀	First-year	17.85		autumn 2016
59V39752	♀	First-year	19.35		autumn 2016
59V39756	♀	First-year	ND		autumn 2016
59V39757	♀	First-year	18.03		autumn 2016
59V39758	♀	First-year	17.63	17.58	autumn 2016
					spring 2017
59V39759	♂	First-year	17.36		autumn 2016
59V39760	♂	First-year	17.65		autumn 2016
59V39761	♀	First-year	17.13		autumn 2016
59V39762	♀	First-year	18.18		autumn 2016
59V39763	♀	Adult	16.58	17.59	autumn 2016
					spring 2017
59V39764	♂	First-year	16.99	16.18	autumn 2016
					spring 2017
59V39765	♀	First-year	17.62		autumn 2016
59V39766	♀	First-year	19.93	19.33	autumn 2016
					spring 2017
59V39767	♂	First-year	18.67		autumn 2016
59V39768	♀	First-year	19.66		autumn 2016
59V39771	♂	First-year	18.38		autumn 2016
59V39773	♀	First-year	17.46		autumn 2016
59V39774	♀	First-year	17.66		autumn 2016
59V39776	♂	First-year	19.20	18.20	autumn 2016
					spring 2017
59V39777	♀	First-year	18.67		autumn 2016
59V39778	♀	First-year	18.42		autumn 2016
59V39779	♂	First-year	18.97		autumn 2016
59V39781	♂	First-year	17.91		autumn 2016
59V39782	♂	First-year	18.16		autumn 2016
59V39831	♂	First-year	19.12		autumn 2016

Bird ID	Sex	Age	SMI autumn 2016	SMI spring 2017	Season of sampling
59V39833	♀	First-year	17.75		autumn 2016
59V39834	♀	First-year	17.98		autumn 2016
59V39835	♂	First-year	18.09		autumn 2016
59V39836	♀	First-year	15.70		autumn 2016
59V39837	♀	First-year	16.25		autumn 2016
59V39838	♀	First-year	17.18		autumn 2016
59V39839	♀	First-year	17.49		autumn 2016
59V39841	♀	First-year	18.02		autumn 2016
59V39842	♀	First-year	18.63		autumn 2016
59V39844	♂	Adult	17.30		autumn 2016
59V39845	♂	First-year	17.99	16.45	autumn 2016
					spring 2017
59V39847	♂	First-year	16.49		autumn 2016
59V39849	♂	Adult	20.84		autumn 2016
59V39850	♂	First-year	18.03		autumn 2016
59V39851	♂	First-year	18.25		autumn 2016
59V39852	♂	Adult	16.33		autumn 2016
59V39853	♀	First-year	18.15		autumn 2016
59V39854	♀	Adult	16.72		autumn 2016
59V39855	♀	First-year	17.77		autumn 2016
59V39856	♂	Adult	17.50		autumn 2016
59V39857	♂	Adult	17.33		autumn 2016
59V39858	♂	First-year	18.02		autumn 2016
59V39860	♂	First-year	17.87	17.86	autumn 2016
					spring 2017
59V39861	♀	First-year	18.46		autumn 2016
59V39862	♂	First-year	17.67		autumn 2016
59V39863	♂	First-year	18.66		autumn 2016
59V39864	♀	First-year	16.64		autumn 2016
59V39865	♀	First-year	17.04		autumn 2016
59V39867	♂	First-year	16.49		autumn 2016
59V39868	♀	First-year	18.99	18.25	autumn 2016
					spring 2017
59V39871	♀	First-year	16.32		autumn 2016
59V39947	♂	First-year	19.74		autumn 2016
59V39951	♂	First-year	18.90		autumn 2016
59V39997	♀	First-year	17.80		autumn 2016
59V39998	♂	Adult	17.30		autumn 2016
59V39999	♂	First-year	15.25		autumn 2016
59V92482	♂	Adult	17.55		autumn 2016

SUPPLEMENTARY TABLE 2

Evaluation of possible discriminating factors for faecal metabolomic composition in great tits. The models were evaluated through $R^2(X)$, $R^2(Y)$, $Q^2(Y)$ and CV-ANOVA testing.

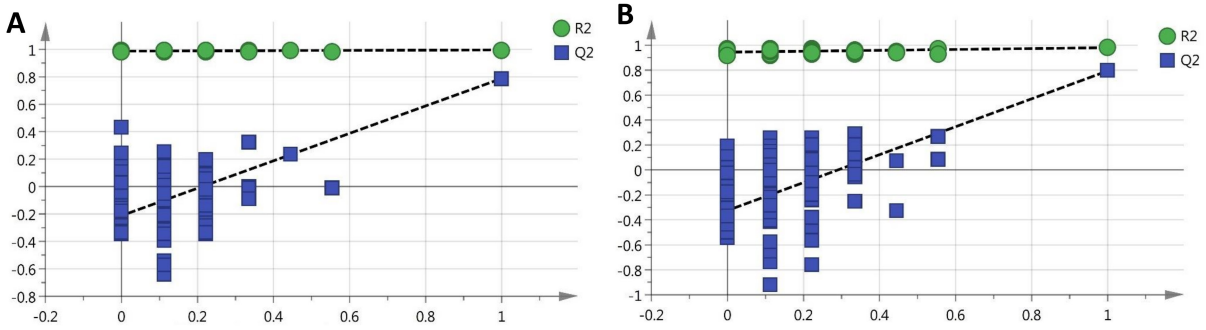
	Model: discriminating factor	**N**	**R^2X**	**R^2Y**	**Q^2**	**cv anova p-value**	**principal component**
	Season (autumn vs spring)	38	0.569	0.996	0.699	0.001*	1+5+0
	Season (autumn vs spring): only Female	18	0.588	0.987	0.230	0.913	1+3+0
	Season (autumn vs spring): only Male	20	0.625	1.000	0.755	0.035*	1+5+0
	Season (autumn vs spring): only Adult	14	0.707	0.999	0.818	0.085	1+3+0
	Season (autumn vs spring): only First-year birds	24	0.539	0.996	0.611	0.065	1+4+0
	Sex (male vs female)	112	0.143	0.527	0.165	1.000	1+8+0
	Sex (male vs female): only adult	34	Can't construct				
	Sex (male vs female): only First-Year bird	78	Can't construct				
+ ions	Age (Adult vs First-year bird)	112	0.171	0.164	0.041	0.099	1+0+0
	Age (Adult vs First-year bird): only Female	54	0.155	0.720	0.023	0.891	1+1+0
	Age (Adult vs First-year bird): only Male	58	0.234	0.172	0.020	0.576	1+0+0
	Body condition (2 groups: median)	111	0.166	0.145	-0.059	1.000	1+0+0
	Body condition (2 groups: median): only Female	53	0.110	0.342	-0.077	1.000	1+0+0
	Body condition (2 groups: median): only Male	58	Can't construct				
	Body condition (2 groups: median): only Adult	34	Can't construct				
	Body condition (2 groups: median): only First-year bird	77	0.110	0.232	-0.169	1.000	1+0+0
	Body condition (10% highest SMI vs 10% lowest SMI)	22	0.720	0,06	0.010	1.000	1+0+0

	Model: discriminating factor	N	R^2X	R^2Y	Q^2	cv anova p-value	principal component
	Season (autumn vs spring)	38	0.543	0.990	0.800	$p < 0.001$*	1+4+0
	Season (autumn vs spring): only Female	18	0.743	1.000	0.698	0.055	1+6+0
	Season (autumn vs spring): only Male	20	0.618	0.998	0.819	0.003	1+4+0
	Season (autumn vs spring): only Adult	14	0.699	1.000	0.848	0.139	1+4+0
	Season (autumn vs spring): only First-year bird	24	0.502	0.978	0.848	$p < 0.001$*	1+2+0
	Sex (male vs female)	112	Can't construct				
	Sex (male vs female): only adult	34	Can't construct				
	Sex (male vs female): only First-year bird	78	Can't construct				
- ions	Age (Adult vs First-year bird)	112	0.163	0.193	0.044	0.088	1+0+0
	Age (Adult vs First-year bird): only Female	54	0.182	0.709	-0.097	1.000	1+1+0
	Age (Adult vs First-year bird): only Male	58	0.237	0.174	0.020	0.756	1+0+0
	Body condition (2 groups)	111	0.363	0.494	-0.012	1.000	1+2+0
	Body condition (2 groups): only Female	53	0.091	0.350	-0.151	1.000	1+0+0
	Body condition (2 groups): only Male	58	0.218	0.229	-0.024	1.000	1+0+0
	Body condition (2 groups): only Adult	34	Can't construct				
	Body condition (2 groups): only First-year bird	77	93	0.200	-0.018	1.000	1+0+0
	Body condition (10% highest SMI vs 10% lowest SMI)	22	0,61	0.970	0,513	0.187	1+3+0

SUPPLEMENTARY TABLE 3

Selected metabolites with *m/z* and retention time.

	Metabolite ID	*m/z*	Time(min)	Mostly present
+ ions	787	116.0966	5.54	Spring 2017
	7686	266.1384	6.40	Spring 2017
	8399	277.2005	7.97	Spring 2017
	10281	305.2317	9.24	Autumn 2016
	11226	321.2267	8.17	Autumn 2016
- ions	1431	289.0329	1.03	Spring 2017
	1886	211.0274	6.34	Spring 2017
	1977	216.0182	6.00	Spring 2017
	2187	223.0259	6.39	Spring 2017
	3159	515.1187	8.90	Spring 2017
	6854	368.1065	7.68	Spring 2017
	8085	409.2076	6.93	Spring 2017
	8627	434.0474	6.15	Spring 2017
	8761	437.2388	7.98	Autumn 2016
	8922	447.0598	6.36	Spring 2017
	9279	465.2701	9.25	Autumn 2016
	9477	473.2156	8.42	Autumn 2016
	9666	481.2651	7.62	Autumn 2016
	11938	785.2292	9.21	Spring 2017



Supplementary Fig. 1 – **Permutation plots.** Permutation plots as a validation criterion for an orthogonal partial least-squares discriminant analysis model for a dataset containing the faecal samples in **(A)** positive and **(B)** negative ionization mode (n = 100).