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.

Boonyarittichaikij R., Pomian B., Dekeukeleire D., Lens L., Bonte D., Verheyen K., Pasmans F., Martel A. & Verbrugghe E. (2020). Season as a discriminating factor for faecal metabolomic composition of great tits (Parus major). Belgian Journal of Zoology 150: 169–184. https://doi.org/10.26496/bjz.2020.79
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; Marcoal 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 (Beauchercq 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 (Teysnier 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; Teysnier et al. 2018a) and environmental factors such as alterations in food sources (Grond et al. 2018; Teysnier 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 (Teysnier 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 17000 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-ExacteTM 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 140000 full width at half maximum at 1 Hz. Other instrumental parameters are presented in Table 1.
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 (Van Den 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: \( R^2 < > 0.5, R^2 Y > 0.5, Q^2 Y > 0.5, CV \) 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) ≥ 0.5 and p ≥ -0.025 and p ≥ 0.02 for the positive ions and p(corr) ≥ 0.5 and p ≥ -0.03 and p ≥ 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 \( R^2 (Y) \),

### 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 (Van Den 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: \( R^2 < > 0.5, R^2 Y > 0.5, Q^2 Y > 0.5, CV \) 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) ≥ 0.5 and p ≥ -0.025 and p ≥ 0.02 for the positive ions and p(corr) ≥ 0.5 and p ≥ -0.03 and p ≥ 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 \( R^2 (Y) \),

172
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 ≤ 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.
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 (Köhler 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) (Rytkönén 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’ky 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 microbiologically-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 microbiologically-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.

Acknowledgements

We thank Luc Willems for technical support and Robbe De Beelde, Pieter Vantieghem, Lieze Rouffaer and Isabelle Clairhout for help with the fieldwork. We would like to acknowledge Prof. Lynn Vanhaecke for her guidance and expertise in unravelling the great tit metabolome. This work was supported by the UGent GOA project Scaling up Functional Biodiversity Research: from Individuals to Landscapes and Back (TREEWEB). E.V. was supported by the Research Foundation Flanders [FWO grants 12E6616N and 1507119N].

Declaration of Conflicting Interests

The authors declare that there is no conflict of interest.
References

BEAULERCQ S., LEFÈVRE A., MONTIGNY F., COLLIN A., TESSERAUD S., LETERIER C., EMOND P. & GUILLOTEAU L.A. (2019). A multiplatform metabolomic approach to characterize fecal signatures of negative postnatal events in chicks: a pilot study. Journal of Animal Science and Biotechnology 10: 21. https://doi.org/10.1186/s40104-019-0335-8

CIRULLI E.T., GUO L., SWISHER C.L., SHAH N., HUANG L., NAPIER L.A., KIRKNESS E.F., SPECTOR T.D., CASKEY C.T., THORENS B., VENTER J.C., TELENTI A. (2019). Profound perturbation of the metabolome in obesity is associated with health risk. Cell Metabolism 29: 488–500. https://doi.org/10.1016/j.cmet.2018.09.022

DAWSON W.R. (1985). Relation of oxygen consumption and evaporative water loss to temperature in the cardinal. Physiological Zoology 31: 37–48. https://doi.org/10.1086/physzool.31.1.30155377

DORR B.S., HANSON-DORR K. C., ASSADI-PORTER F.M., SELEN E.S., HEALY K.A. & HORAK K.E. (2019). Effects of repeated sublethal external exposure to deep water horizon oil on the avian metabolome. Scientific Reports 9: 1–12. https://doi.org/10.1038/s41598-018-36688-3

GOLET G.H. & IRONS D.B. (1999). Raising young reduces body condition and fat stores in Black-legged Kittiwakes. Oecologia 120: 530–538. https://doi.org/10.1007/s004420050887

GREGORY K.E., BIRD S.S., GROSS V.S., MARUR V.R., LAZAREV A.V., WALKER W.A. & KRISTAL B.S. (2013). Method development for fecal lipidomics profiling. Analytical Chemistry 85: 1114–112. https://doi.org/10.1021/ac303011k

GROND K., SANDERCOCK B.K., JUMPPONEN A. & ZEGLIN L.H. (2018). The avian gut microbiota: community, physiology and function in wild birds. Journal of Avian Biology 49: e01788. https://doi.org/10.1111/jav.01788

HART J.S. (1962). Seasonal acclimatization in four species of small wild birds. Physiological Zoology 35: 224–236.

KOHL K.D., AMAYA J., PASSEMENT C.A., DEARING M.D. & MCCUE M.D. (2014). Unique and shared responses of the gut microbiota to prolonged fasting: a comparative study across five classes of vertebrate hosts. FEMS Microbiology Ecology 90: 883–894. https://doi.org/10.1111/1574-6941.12442

LAMICHHANE S., SEN P., DICKENS A.M., OREŠIČ M. & BERTRAM H.C. (2018). Gut metabolome meets microbiome: A methodological perspective to understand the relationship between host and microbe. Methods 149: 3–12. https://doi.org/10.1016/j.ymeth.2018.04.029

LE GALL G., NOOR S.O., RIDGWAY K., SCOVELL L., JAMIESON C., JOHNSON I.T., COLQUHOUN I.J., KEMSLEY E.K. & NARBAD A. (2011). Metabolomics of fecal extracts detects altered metabolic activity of gut microbiota in ulcerative colitis and irritable bowel syndrome. Journal of Proteome Research 10: 4208–4218. https://doi.org/10.1021/pr2003598

LEY R.E., TURNBAUGH P.J., KLEIN S. & GORDON J.I. (2006). Microbial ecology: human gut microbes associated with obesity. Nature 444: 1022–1023. https://doi.org/10.1038/4441022a

LI D., GAO C., ZHANG F., YANG R., LAN C., MA Y. & WANG J. (2020). Seven facts and five initiatives for gut microbiome research. Protein & Cell 11: 391–400. https://doi.org/10.1007/s13238-020-00697-8

LI Z., QUAN G., JIANG X., YANG Y., DING X., ZHANG D., WANG X., HARDWIDGE P.R., REN W. & ZHU G. (2018). Effects of metabolites derived from gut microbiota and hosts on pathogens. Frontiers in Cellular and Infection Microbiology 8: 314. https://doi.org/10.3389/fcimb.2018.00314

LINNAEUS D. (1758). Systema Naturae per regna tria naturae, secundum classes, ordines, genera, species, cum characteribus, differentiis, synonymis, locis. Editio decima, reformata [10th revised edition], vol. 1. Laurentius Salvius, Holmiae.
BOONYARITTICHAIKIJ R. et al., Seasonal variation in great tit faecal metabolome

MARCObAL A., KASHyAP P.C., NErLSON T.A., ARONov P.A., DONIA M.S., SPORMANN A., FISCHBACH M.A. & SONnenBURG J.L. (2013). A metabolomic view of how the human gut microbiota impacts the host metabolome using humanized and gnotobiotic mice. The ISME Journal 7: 1933–1943. https://doi.org/10.1038/ismej.2013.89

MILLER D.S. (1939). A study of the physiology of the sparrow thyroid. Journal of Experimental Zoology 80: 259–281. https://doi.org/10.1002/jez.1400800207

PEDERSEN H.K., GUDMUNDSDOTTIR V., NIelsen H.B., HYOTYLAINEN T., NIelsen T., JENSEN B.A.H., FORSLUND K., HILDEBRAND F., PRIFTI E., FALONY G., LE CHATELIER E., LEVENZ F., DORÉ J., MATTILA I., PLICHTA D.R., PÖHÖ P., HELLGREN L.I., ARUMUGAM M., SUNAGAWA S., VIEIRa-SILVA S., JORGENSEN T., HOLM J.B., TRÖST K., CONSORTIUM M., KRISTIANSEN K., BRIX S., RAES J., WANG J., HANSEN T., BORK P., BRUNAK S., ORESIC M., EHRLICH SD. & PEDErSEN O. (2016). Human gut microbes impact host serum metabolome and insulin sensitivity. Nature 535: 376–381. https://doi.org/10.1038/nature18646

PEIG J. & GREEN A.J. (2009). New perspectives for estimating body condition from mass/length data: the scaled mass index as an alternative method. Oikos 118: 1883–1891. https://doi.org/10.1111/j.1600-0706.2009.17643.x

ROUFFAER L.O., STRUBBE D., TEYSSIER A., SALLEH HUDIN N., VAN DEN AEBEELE A.-M., COX I., HAESENDONCK R., DELMEE M., HAEBEBROUCK F., PASMANS F., LENs L. & MARTEL A. (2017). Effects of urbanization on host-pathogen interactions, using Yersinia in house sparrows as a model. PLoS ONE 12: e0189509. https://doi.org/10.1371/journal.pone.0189509

RYTKÖNEN S., VESTERINEN E.J., WESTERDUIN C., LEVIÄKANGAS T., VATKA E., MUTANEN M., VÄLMÄKI P., HUKKANEN M., SUOKAS M. & ORELL M. (2018). From feces to data: A metaborcoding method for analyzing consumed and available prey in a bird-insect food web. Ecology and Evolution 9: 631–639. https://doi.org/10.1002/ece3.4787

SCHRIMPE-RUTLEDGE A.C., CodreANU S.G., SherroD S.D. & MCLEAN J.A. (2016). Untargeted metabolomics strategies – challenges and emerging directions. Journal of the American Society for Mass Spectrometry 27: 1897–1905. https://doi.org/10.1007/s13361-016-1469-y

SVESSON L. (1992). Identification Guide to European Passerines. British Trust for Ornithology, Thetford, UK.

SWANSOn D.L. (2010). Seasonal metabolic variation in birds: functional and mechanistic correlates. In: Thompson C.F. (ed.) Current Ornithology. Vol. 17: 75–129. Springer, New York.

TANG Z.Z., CHEN G., HONG Q., HUANG S., SMITH H.M., SHAH R.D., SCHOLZ M. & FERGUSOn J.F. (2019). Multi-omic analysis of the microbiome and metabolome in healthy subjects reveals microbiome-dependent relationships between diet and metabolites. Frontiers in Genetics 10: 454. https://doi.org/10.3389/fgene.2019.00454

TEYSSIER A., LENS L., MATThYSEn E. & White J. (2018a). Dynamics of gut microbiota diversity during the early development of an avian host: evidence from a cross-foster experiment. Frontiers in Microbiology 9: 1524. https://doi.org/10.3389/fmicb.2018.01524

TEYSSIER A., ROUFFAER L. O., SALEH HUDIN N., STRUBBE D., MATThYSEn E., LENS L. & White J. (2018b). Inside the guts of the city: Urban-induced alterations of the gut microbiota in a wild passerine. The Science of the Total Environment 612: 1276–1286. https://doi.org/10.1016/j.scitotenv.2017.09.035

TREUREN W.V. & DODD D (2020). Microbial contribution to the human metabolome: implications for health and disease. The Annual Review of Pathology: Mechanisms of Disease 15: 345–69. https://doi.org/10.1146/annurev-pathol-020117-043559
TURNBAUGH P.J., HAMADY M., YATSUNENKO T., CANTAREL B.L., DUNCAN A., LEY R.E., SOGIN M.L., JONES W.J., ROE B.A., AFFOURIT T.P., EGHO M., HENRISSAT B., HEATH A.C., KNIGHT R. & GORDON J.I. (2009). A core gut microbiome in obese and lean twins. *Nature* 457: 480–484. https://doi.org/10.1038/nature07540

VAN DEN BERG R.A., HOFESLOOT H.C., WESTERHUIS J.A., SMILDE A.K. & VAN DER WERF M.J. (2006). Centering, scaling, and transformations: improving the biological information content of metabolomics data. *BMC Genomics* 7: 142. https://doi.org/10.1186/1471-2164-7-142

VANDEN BUSSCHE J., MARZORATI M., LAUKENS D. & VANHAECKE L. (2015). Validated high resolution mass spectrometry-based approach for metabolomic fingerprinting of the human gut phenotype. *Analytical Chemistry* 87: 10927–10934. https://doi.org/10.1021/acs.analchem.5b02688

VAN DONGEN W.F., WHITE J., BRANDT H.B., MOODLEY Y., MERKLING T., LECLAIRE S., BLANCHARD P., DANCHIN E., HATCH S.A. & WAGNER R.H. (2013). Age-related differences in the cloacal microbiota of a wild bird species. *BMC Ecology* 13: 11. https://doi.org/10.1186/1472-6785-13-11

VAN MEULEBROEK L., BUSSCHE J.V., DE CLERCQ N., STEPPE K. & VANHAECKE L. (2015). A metabolomics approach to unravel the regulating role of phytohormones towards carotenoid metabolism in tomato fruit. *Metabolomics* 11: 667–683. https://doi.org/10.1007/s11306-014-0728-9

VAN MEULEBROEK L., DE PAEPE E., VERCRUYSSE V., POMIAN B., BOS S., LAPAUW B. & VANHAECKE L. (2017). Holistic lipidomics of the human gut phenotype using validated ultra-high-performance liquid chromatography coupled to hybrid orbitrap mass spectrometry. *Analytical Chemistry* 89: 12502–1251. https://doi.org/10.1021/acs.analchem.7b03606

VEĽKÝ M., KAŇUCH P. & KRIŠTÍN A. (2011). Food composition of wintering great tits (*Parus major*): habitat and seasonal aspects. *Folia Zoologica* 60: 228–236. https://doi.org/10.25225/fozo.v60.i3.a7.2011

VERNOCCI P., DEL CHIERICO F. & PUTIGNANI L. (2016). Gut microbiota profiling: metabolomics based approach to unravel compounds affecting human health. *Frontiers in Microbiology* 7: 1144. https://doi.org/10.3389/fmicb.2016.01144

VIDEVAL E., STRANH M., ENGELBRECHT A., CLOETE S. & CORNWALLIS C.K. (2017). Measuring the gut microbiome in birds: Comparison of faecal and cloacal sampling. *Molecular Ecology Resources* 18: 424–434. https://doi.org/10.1111/1755-0998.12744

VISCONTI A., LE ROY C.I., ROSA F., ROSSI N., MARTIN T.C., MOHNEY R.P., LI W., DE RINALDIS E., BELL J.T., VENTER J.C., NELSON K.E., SPECTOR T.D. & FALCHI M. (2019). Interplay between the human gut microbiome and host metabolism. *Nature Communications* 10: 4505. https://doi.org/10.1038/s41467-019-12476-z

ZIERER J., JACKSON M.A., KASTENMÜLLER G., MANGINO M., LONG T., TELENTI A., MOHNEY R.P., SMALL K.S., BELL J.T., STEVES C.J., VALDES A.M., SPECTOR T.D. & MENNl C. (2018). The fecal metabolome as a functional readout of the gut microbiome. *Nature Genetics* 50: 790–795. https://doi.org/10.1038/s41588-018-0135-7

Manuscript received: 20 April 2020
Manuscript accepted: 24 September 2020
Published on: 6 October 2020
Branch editor: Frederik Hendrickx
Sample characteristics: per bird ID the sex (♂ / 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        |
| 59V39009 | ♂   | Adult      | 17.15           |                 | autumn 2016        |
| 59V39011 | ♀   | Adult      | 18.43           |                 | autumn 2016        |
| 59V39056 | ♀   | First-year | 18.57           | 18.12           | autumn 2016        |
| 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        |
| 59V39156 | ♀   | Adult      | 15.84           | 15.45           | autumn 2016        |
| 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        |
| 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        |
| 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        |
| 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        |
| 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       |
| 59V39527 | ♂   | First-year| 16.44           | 16.29           | autumn 2016       |
| 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       |
| 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       |
| 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       |
| 59V39764 | ♂   | First-year| 16.99           | 16.18           | autumn 2016       |
| 59V39765 | ♀   | First-year| 17.62           |                 | autumn 2016       |
| 59V39766 | ♀   | First-year| 19.93           | 19.33           | autumn 2016       |
| 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       |
| 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       |
| 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       |
| 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       |
| 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       |
| 59V2482  | ♂   | 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   | $R^2(X)$ | $R^2(Y)$ | $Q^2$ | 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  |                     |
| 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   | RX  | RY  | Q²  | 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 |         |      |                  |                     |
| 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).