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Do fecal bile acid analyses allow taxonomic discrimination between neotropical mustelids?

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ABSTRACT
The use of fecal samples analysis to differentiate species is becoming more frequent in recent years. We performed a semi-quantitative fecal bile acid analysis of five neotropical mustelids to access the diversity of bile acids, evaluating their taxonomic discriminatory potential. Fecal samples were collected from captive specimens and analyzed through liquid chromatography high-resolution mass spectrometry (LC-HRMS). We found significant differences between the analyzed species in four bile acids, supporting our method as a promising tool for taxonomic discrimination among mammals. Bile acids are endogenous compounds fundamental in biochemical pathways, so detecting interspecific differences may be relevant to understanding the physiology, health status and metabolic responses of mustelids to environmental changes.

Keywords: chemical profiles, Mustelidae, neotropics, taxonomy

RESUMO - Perfis ácidos biliares fecais permitem a discriminação taxonômica entre espécies de mustelídeos neotropicais? A diferenciação taxonômica de espécies tem sido cada vez mais suportada por análises de amostras fecais e abordagens moleculares. A partir da análise semiquantitativa de ácidos biliares fecais de cinco mustelídeos neotropicais acessamos a diversidade destes compostos, avaliando seu potencial na discriminação taxonômica de espécies. Amostras fecais coletadas de espécimes cativos foram analisadas por cromatografia líquida acoplada a espectrômetro de massas de alta performance. Diferenças significativas em quatro ácidos biliares foram detectadas entre as espécies analisadas. Os ácidos biliares são compostos endógenos fundamentais em vias bioquímicas, assim detectar diferenças interespecíficas pode ser relevante para o conhecimento da fisiologia, estado de saúde e respostas metabólicas dos mustelídeos às mudanças ambientais.

Palavras-chave: Mustelidae, neotrópicos, perfis químicos, taxonomia

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Chemical compounds have orchestrated a wide range of intra and interspecific interactions of organisms, from group cohesion interactions, social and reproductive status to resource delimitation, territorial dynamics, and perception of environmental conditions (Campbell-Palmer & Rosell 2011). Bile acids are chemical compounds endogenously made –i.e., produced inside the organism by its own metabolism and intestinal microbiota– that also act in crucial biochemical pathways, such as the regulation of innumerable metabolic processes, including glucose biosynthesis, homeostasis, and hormonal expression (Molinaro et al. 2017).

These compounds present low volatility and high molecular stability. Thus, they can persist for a long time in the feces of mammals deposited in the environment (Nasini et al. 2013). Bile acid profiles obtained from fecal samples have shown to be useful for species discrimination in the wild, including canids, felids, and procyonids (Salame-Méndez et al. 2012; Mastella et al. 2021), contributing to non-invasive monitoring and distribution information of large and medium-sized mammal species in the Neotropical region.

Although the concentration of each bile acid may suffer changes associated with the diet and throughout the life of a given organism (Matysik et al. 2016), qualitative fecal bile acids approaches –chemical profiles (presence/absence)– have shown to be useful in discriminating closely related species of mammals (Mastella et al. 2021). Thus, qualitative analyses may be limited in detecting compounds in low concentration, masking the diversity of bile acids in chemical profiles.

For mustelids, generalist mesopredators that occur in a wide variety of environments and are under some threat level around the world (Macdonald et al. 2017), chemical compounds supply indirect information about them, such as their occurrence, reproductive and health status, especially as chemical cues are often used in their communication (intra and/or interspecific) (Macdonald et al. 2017). Even so, the chemical ecology of mustelids is still unknown (Almeida et al. 2022).

Here, we assessed neotropical mustelids fecal bile acids semi-quantitatively in order to evaluate their interspecific variation to potential taxonomic discrimination among them. Fecal samples from individuals of giant otter *Pteronura brasiliensis* (Zimmerman, 1780), Neotropical otter *Lontra longicaudis* (Olfers, 1818), tayra *Eira barbara* (Linnaeus, 1758), and two grisons species -the lesser grison *Galictis cuja* (Molina, 1782) and the greater grison *Galictis vittata* (Schreber, 1776) were collected in 10 Brazilian Zoological Centers. Sampling was carried out in: (i) Parque Ecológico de Americana –two females and one male of lesser grison, and one male of tayra; (ii) Parque Ecológico de São Carlos –one female and one male of lesser grison, one female of Neotropical otter, two males of tayra; (iii) Parque Zoobotânico Vale –one female of Neotropical otter and two males of tayra; (iv) Parque Zoológico de Sapucaia do Sul –one male of lesser grison, one female and one male of Neotropical otter, one male of tayra; (v) Zoológico de Brasília –one female of giant otter, one female and one male of greater grison; (vi) Zoológico de Pomerode –two females and five males of lesser grison, two females of Neotropical otter, one female and one male of tayra; (vii) Zoológico Municipal de Canoas –one male of lesser grison and one of Neotropical otter; (viii) Zoológico Municipal de Curitiba –one male of lesser grison, two females and two males of Neotropical otter, one male of tayra; (ix) Zoológico Municipal de Uberaba –one female of Neotropical otter; (x) Zoológico da Universidade de Caxias do Sul –one male of lesser grison.

All fecal samples were collected in the respective individual's enclosure as soon as possible after the individual's evacuation. All samples were from healthy (non-diarrheal) adult individuals following a known diet: carnivorous diet (including fish) in Neotropical and giant otters; mostly omnivorous diet in grisons (with meat in larger proportion than fruits); and omnivorous diet in tayras (meat and fruits in the same proportion). The sampling was performed over three to five days, with 2-3 samples being collected from different days, and all samples were stored in a freezer. The semi-quantitative fecal bile acids analysis was performed by liquid-liquid extraction followed by an untargeted liquid chromatography-high-resolution mass spectrometry analysis (LC-HRMS), as described in Alves et al. (2021), aiming to evaluate the taxonomic discriminatory potential.

To allow the identification of bile acids, a standard mix sample containing the following bile acids was analyzed with the samples: lithocholic acid (LCA), glycocholic acid (GCA), taurocholic acid (TCA), cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA) and dehydrocholic acid (DHCA). Raw LC-HRMS data pre-processing, including peak selection, deconvolution, alignment, and MS/MS compound annotation using the NIST 2020 MSMS database was performed in MS-Dial software (RIKEN, version 3.98) (Tsugawa et al. 2015). All data were manually inspected to verify spectral matches with the database and the standard mix sample to avoid and discard ambiguous peak identification. All data were normalized by the TIC (total ion current), mean-centered, and range scaled to reduce the differences between large and small peak values (Van den Berg et al. 2006; Almeida et al. 2022).

For statistical analyses, assuming that the sample peak areas of each compound represent a proxy for compound abundance in each sample, we have used a least-squares analysis to index the samples to the corresponding individual considering possible non-standard variations. Followed by the repeated-measures ANOVA and the paired contrast test adjusted to the Tukey test to determine which bile acids significantly contributed to species discrimination. These analyses were performed in RStudio (Version 1.2.5019, RStudio Team 2020).

Beyond the seven standard compounds that were identified in samples, five other bile acids were detected and putatively annotated by LC-HRMS: 12-ketodeoxycholic acid (12-keto-DCA); 3-oxocholic acid (3-oxoCA); glycoursodeoxycholic acid (GUDCA); sulfocholic acid (sulfo-CA). We found significant signal intensity differences in four bile acids: cholic acid (CA; $F=4.40$; $P<0.005$); glycocholic acid (GCA; $F=2.365$;

$P<0.05$); sulfocholic acid (sulfo-CA; $F=6.508$; $P<0.001$) and taurocholic acid (TCA; $F=4.067$; $P<0.005$).

Tukey HSD tests showed that TCA signal intensity was significantly different between the giant otter and each one of the remaining species ($P<0.05$) in all comparisons (Fig. 1). Neotropical otter and the lesser grison showed significant differences in signal intensity of GCA ($P<0.05$) and sulfo-CA ($P<0.005$). Finally, lesser grison and tayra showed different signal intensities in CA ($P<0.005$) and sulfo-CA profiles ($P<0.005$).

All bile acids were detected in Neotropical mustelid species, being the first report for the greater grison and the giant otter. On the other hand, the presence of dehydrocholic acid is known in the fecal chemical composition of lesser grison (Guerrero et al. 2006), as well as the presence of cholic acid and chenodeoxycholic acid in the fecal chemical composition of the Neotropical otter and tayra (Hagey et al. 2010).

The semi-quantitative analysis successfully revealed differences between bile acid proportions and, thus, among the evaluated neotropical mustelid species. This method seems to be a robust alternative compared to taxonomic discrimination based only on qualitative analyses (presence/absence) (Mastella et al. 2021), often adopted from thin-layer chromatography (Guerrero et al. 2006; Araujo et al. 2010).

In addition, bile acids composition seems to be stable within orders and even families of mammals (Hagey et al. 2010). It is unclear how the concentrations of bile acids vary throughout the animals' life, which could compromise the potential for taxonomic discrimination, particularly in closely related taxa (Matysik et al. 2016).

The adoption of more sensitive and comprehensive chemical approaches seems to be a powerful and promising tool to explore biomarkers for taxonomic discrimination in mustelids and other mammals. LC-HRMS seems to be efficient for revealing bile acids from feces exposed to some environmental degradation, such as those from captive animals, suggesting that the stability of bile acids and the high resolving power of this method may be useful for future chemical research of fecal samples found in nature, whose environmental exposure and storage conditions may be adverse (Almeida et al. 2022).

Monitoring fecal bile acids, in particular, may also allow detecting clues regarding chemical communications, physiological and metabolic adjustments of mammals in response to environmental changes and, eventually, diseases (Molinaro et al. 2017). Bile acids are also responsible for antiapoptotic, anti-inflammatory, and antioxidant effects (Daruich et al. 2019). After all, bile acids act in fundamental biochemical pathways to the maintenance of life (Molinaro et al. 2017; Daruich et al. 2019). So, monitoring bile acids seems promising for detecting the metabolic response of mustelids to environmental changes, reinforcing its potential applicability in future monitoring and conservation studies.

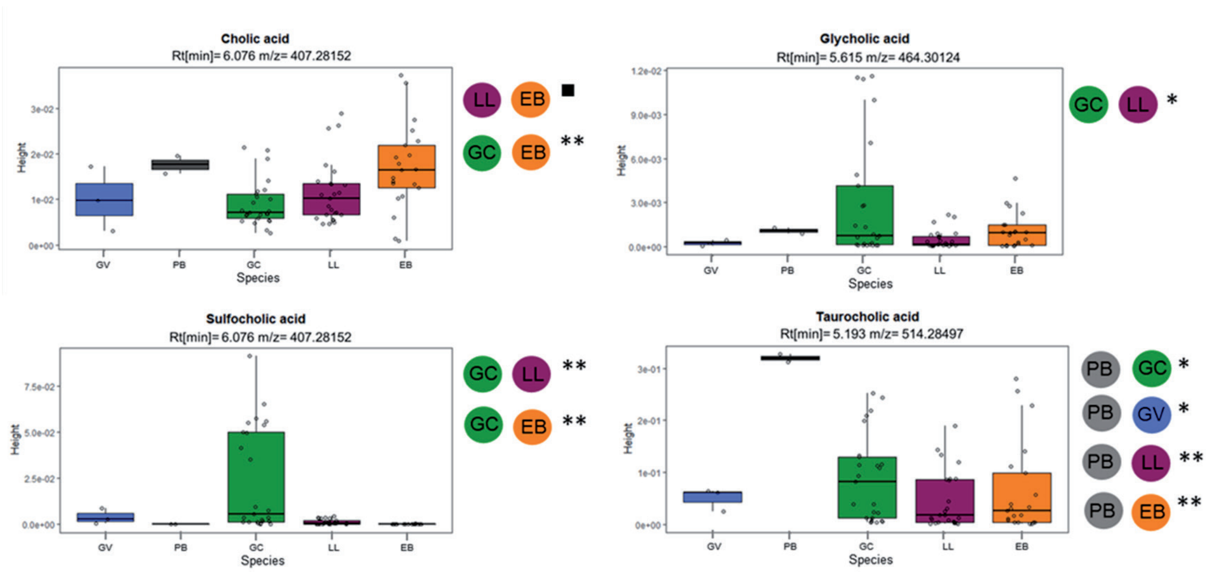

Figure 1. Boxplots showing the targeted bile acids intensity variation in each of the five analyzed mustelids: ***<0.001; **0.001-0.01; *0.01-0.05; n>0.05. PB: *Pteronura brasiliensis* (gray); GV: *Galictis vittata* (blue); GC: *Galictis cuja* (green); LL: *Lontra longicaudis* (purple); EB: *Eira barbara* (orange).

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