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ORIGINAL PAPER

DART mass spectrometry as a potential tool for the differentiation of captive-bred and wild lion bones

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Abstract

In recent years lion bones have been legally traded internationally to Asian markets from captive-bred sources in South Africa. There are also indications of increasing instances of illegal international trade in wild lion bones. The existence of parallel captive and wild supplies of lion bone are a cause of law enforcement concern regarding the potential for the laundering of illegally sourced bones through legal trade, and present a problem for the assessment of the conservation impact of wild lion bone trade due to the difficulty of determining what market-share wild and captive-bred lion bones account for. Captive-bred and wild lion bone are visually indistinguishable and no reliable method currently exists for distinguishing them. We present a preliminary study that explores the use of DART mass spectrometry as a method to differentiate between captive-bred and wild lion bones. We find that DART is able to differentiate between a batch of captive-bred South African lion bone and a batch of wild lion bone and suggest that DART mass spectrometry shows strong potential as a tool for the regulation and investigation of lion bone trade. Further testing is needed to prove the suitability of this technique. Therefore, we suggest that further research focuses on testing the capability of DART to differentiate between contemporary wild and captive-bred lion bone originating from South Africa, and attempts to identify chemical markers in bone that can be used as indicators of captive-bred origin.

Keywords Conservation · Wildlife trade · IWT · Regulation · Law enforcement · Forensics · Panthera leo

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Introduction

Wildlife trade is a multi-billion dollar industry that encompasses a great variety of organisms across a broad range of uses, and occurs in both legal and illegal guises (Broad et al. 2014). The overexploitation of wildlife for trade purposes can have severe impacts on species conservation (Hemley 1994) and the unregulated, illegal trade in wildlife is believed to be amongst the largest illegal industries in the world with significant links to corruption, violence, subversion of development and social stability, and the spread of zoonotic diseases of global health concern (Zimmerman 2003; Warchol 2004; Macdonald and Laurenson 2006; Rosen and Smith 2010; UN 2018; Gore et al. 2019; Volpato et al. 2020).

Sustainable wildlife trade, typically from farmed or ranched sources, has been suggested as a means to reduce the prevalence and negative impacts of illegal wildlife trade—through the reduction of financial incentives for illegal trade, whilst maintaining positive livelihood contributions of legal trade (Bulte and Damania 2005; Damania and Bulte 2007). However, the simultaneous existence of both legal and illegal supplies of wildlife products has led to concern regarding the potential for laundering of products from illegal sources into legal markets and thus negatively impacting wild populations (Fischer 2004; Lyons and Natusch 2011; Jimenez-Bustamante and Rentería 2018). In addition to conservation-based concerns there are also wider societal concerns regarding the facilitation of transnational organised crime by wildlife markets (Warchol 2004; van Uhm 2018). Supply-side approaches to reduction of wildlife exploitation thus require means to verify authenticity and thereby reduce possibilities for criminal activity (i.e. smuggling and laundering) in order to be successful (Damania and Bulte 2007). The trade in lion (*Panthera leo*) bones and body parts exemplifies such concerns.

In the wild, lions are classified as vulnerable, having disappeared from ~ 92% of their historic range and declined by ~ 43% during the two decades between 1993 and 2014, leaving an estimated 23,000–39,000 individuals living in the wild (Bauer et al. 2016). In addition to established threats (see Bauer et al. 2015) the illegal trade in wild lion parts is believed to be increasing and concerns have been raised that associated poaching may become a threat to wild lion populations (Williams et al. 2017a; Everatt et al. 2019). Increasingly, instances of seizures of illegal lion products linked to intercontinental trade are reported, especially to East and Southeast Asia (e.g. EAGLE 2017, 2018; Everatt et al. 2019).

In South Africa there are more than 300 registered facilities for breeding and rearing at least 7800 lions, as indicated on Threatened or Protected Species (ToPS) permits for South Africa, a number which excludes keeping-only facilities—the inclusion of which is likely to increase the estimated number of facilities to over 400, however absolute numbers are not known (Williams et al. 2015; Williams and 't Sas-Rolfes 2019). The international commercial trade in the skeletons (bones, teeth, and claws) of these captive-bred lions is permitted from South Africa under CITES Appendix II. Although in August 2019 export was placed on-hold following a domestic law judgement whereby export quotas for lion skeletons set in 2017 and 2018 were deemed to have been unlawful and unconstitutional due to an insufficient consideration of animal welfare (Republic of South Africa 2019). Nevertheless, at present there still remains the potential for future legal trade in lion skeletons. Commercial international trade in the bones and skeletal parts of wild lions is not permitted by CITES (CITES 2016). In total, over 6000 skeletons have been legally permitted for export from South Africa to Southeast Asia since

2008, where they are believed to be used in traditional Asian medicine, health tonics, and ornaments (Williams et al. 2015).

A number of conservation, political, and socio-economic concerns have been raised surrounding the trade in lion bones and skeletons, including the influence of transnational crime (Coals et al. 2019). Organised crime groups have been linked to the legal trade in lion bones from South Africa (Williams et al. 2015) and illegal sales of lion bone and attempts to smuggle lion bones and bone products out of South Africa have been reported (e.g. Outhwaite 2018; De Telegraaf 2019). Such reports feed concern regarding the potential for laundering wild-origin lion bone through captive-bred lion bone trade both in South Africa and further down the supply chain in Asia (EIA 2017; EMS 2018).

Captive-bred and wild lion bones are indistinguishable to the naked-eye and thus present a challenge to enforcement authorities regarding the detection and prevention of laundering. In recent years South African exports of lion bone have been subject to DNA-profiling tests and physical inspections at various stages of the export process from farm to airport as required under a quota implementation protocol (DEA 2017). The purpose of these tests are primarily to detect laundering of the bones of other felid species, specifically tiger (*Panthera tigris*) (Dalton et al. 2018), and to ensure that multiple lion skeletons are not declared as one for export (Williams et al. 2021) but not whether skeletons initially sampled on farms came from captive-bred or wild sources. Genetic techniques can prove useful and effective for the detection of laundering of wild products into captive-bred trade (Ogden et al. 2009; Hogg et al. 2018). However, no genetic test can specifically distinguish captive-bred from wild lions (Miller et al. 2014).

Chemical elemental techniques, predominantly stable isotope and X-ray fluorescence analyses, have been used to differentiate between captive-bred or wild specimens of wildlife (Hinsley et al. 2016; Sugiyama et al. 2018; Brandis et al. 2018; He et al. 2018) but recent attempts to differentiate captive-bred and wild lion hair through carbon and nitrogen stable isotope analyses have not been conclusive (Hutchinson and Roberts 2020). However, advances in technology have significantly increased the speed and convenience of multi-compound mass spectrometry analyses through use of Direct Analysis in Real Time (DART) mass spectrometry (Cody et al. 2005a, b). DART is an ambient atmospheric ion source which produces analyte ions by reaction of ionised components of the air with the sample which may be solid, liquid, or gas. Analyte ions then pass into a mass spectrometer; the spectra produced are suitable for determination of multiple analytes and assigning tentative chemical formulas to mass peaks (Kim et al. 2010; Smoluch et al. 2016). DART mass spectrometry has proven effective for the identification of illegal substances, notably explosives and drugs (e.g. Nilles et al. 2010; Grange and Sovocool 2011), and is particularly useful in forensic and security applications where the generation of results are time-sensitive (Pavlovich et al. 2018). DART is increasingly employed in law enforcement, however, usage is not yet prevalent in wildlife forensics and wildlife crime detection. Nevertheless, it has been used to identify and differentiate between timber species in trade (Cody et al. 2012; Lancaster et al. 2012; Espinoza et al. 2014) and to identify rhinoceros horn (Price et al. 2018).

Bone incorporates metabolised chemical inputs accumulated throughout an animal's life (Meier-Augenstein 2017). Therefore, the chemical composition of bones can be used to determine animals' captive or wild provenance based on differences in chemical input (e.g. Kays and Feranec 2011). It would be expected that observable differences between captive and wild lion bones might be due primarily to diet, but could also include other inputs such as veterinary drugs and environmental chemicals such as pesticides. Wild lions have a varying diet, predominantly comprised of medium to large sized ungulates, with specific prey

species eaten depending on the regional ungulate composition (Hayward and Kerley 2005; Davidson et al. 2013). Commercially captive-bred lions in South Africa are typically fed livestock; often mortalities from cattle farms, or other domestic animals such as chickens and donkeys, often supplemented with 'predator powder' vitamin and mineral additives (www.vtech.co.za) (pers. obs.). Commercial lion breeding and keeping facilities are often associated with agricultural and livestock-producing land, particularly in the Free State Province where the majority of commercial lion captive-breeding facilities are located (pers. obs.; Williams and 't Sas-Rolfes 2019). Although the majority of captive-bred lions' food is livestock they may also be fed commercially raised wild ungulate species—which form a large part of South Africa's commercial game meat and game hunting industries (pers. obs.). Nevertheless, in general it is assumed that predators raised in captivity tend to have more restricted diets than their wild counterparts (e.g. Kays and Feranec 2011).

We conducted a preliminary study to test whether DART mass spectrometry could potentially be used to differentiate between a batch of captive-bred and a batch of wild sourced lion bones. We expected differences in the chemical composition of lion bone batches to be identifiable with the multiple-compound spectra approach of DART mass spectrometry. We also intended to tentatively identify diagnostic compounds for any observable difference between captive-bred and wild lion bone.

Method

Material

One phalangeal bone was taken from each of 29 verified captive-bred lion skeletons from commercial breeding facilities in South Africa. Captive-bred bone samples were sourced from a commercial trader in captive-bred lion skeletons and were accessioned with the National Zoological Gardens of South Africa (Table 3 in Appendix 1). The trader sources material from a range of legal lion breeding and keeping facilities in South Africa. We understand that the material used in this study came from at least 8 different facilities. We therefore believe the captive-bred samples used in this study are representative of the type of material present in commercial lion skeleton exports. For this proof of concept study we used bone samples from the skulls of six historical museum specimens recorded to have been collected from wild lions in Anglo-Egyptian Sudan in the early twentieth century (Table 4 in Appendix 2). The choice of markedly different geographical regions, ages, and bone types between the captive and wild samples was thought to be appropriate for this test of concept because if differences were not evident from such seemingly different sample types then they were less likely to be distinguishable in contemporary wild lions from Southern Africa. A randomly selected captive-bred sample was withheld from analysis and was used as an 'unknown' sample to test validity of the classification.

Sample preparation

In order to present material of sufficient thinness and homogenous structure to the DART ion source each bone was drilled to a depth of 10 mm at low-medium speed (to avoid friction burning the bone) with a 3 mm drill-bit to create fine bone powder. The drill-bit was cleaned in between taking each sample through immersion in 99% ethanol and wiping with paper towel. Powders from each bone sample were individually suspended in ~ 10 ml of

99% ethanol which was pipette-dropped (two drops per sample) onto DART Quickstrip fine mesh grids balanced over free space so that the ethanol did not come into contact with any surface apart from the DART Quickstrip mesh. The ethanol was then allowed to evaporate from the DART Quickstrip mesh before the resulting powder, which adhered to the Quickstrip mesh, was introduced to the DART ion source using an automated rail. The 29 captive-bred samples were tested singly whilst the six wild samples were analysed in duplicate (total bones = 6; total spectra generated = 12). All samples (captive-bred and wild) were analysed at the Proteomics & Metabolomics Facility, The Roslin Institute, University of Edinburgh, Scotland, UK on the 21st of January 2020. Following calibration of the mass spectrometer, background spectra were recorded using blank DART Quickstrip mesh directly before introduction of lion bone samples. Captive-bred samples were analysed first (using 3 DART Quickstrips consecutively) directly followed by wild samples (using 1 DART Quickstrip).

DART-MS analyses were performed on a DART-SVP ion source (IonSense Inc, Sangus, MS, USA) interfaced to a micrOTOF QII Quadrupole Time-of-Flight mass spectrometer (Bruker Daltonics, Bremen, Germany). The mass spectrometer was calibrated by infusing ESI low concentration tune mix (Agilent) prior to DART analysis. The reference tune mix included, betaine, hexamethoxyphosphazine,hexakis(2,2-difluoroethoxy)phosphazine, hexakis(1H, 1H, 3H-tetrafluoropropoxy)phosphazine, hexakis(1H, 1H, 5H-octafluoropentoxy)phosphazine, hexakis(1H, 1H, 7H-dodecafluoroheptoxy)phosphazine, hexakis(1H, 1H, 9H-perfluorononyloxy)phosphazine, hexakis(1H, 1H,4H-hexafluorobutyloxy)phosphazine, hexakis(1H, 1H, 6H-decafluorohexyloxy)phosphazine, hexakis(1H, 1H, 8H-tetradecafluorooctyloxy)phosphazine, ranging from m/z 118.0862 to 2721.8948. Additionally, on DART source, quinine ($[M+H]^+$ = 325.1911) was loaded on the DART Quickstrip and spectra were collected in positive ion mode between sample data acquisition.

The optimised parameters ranges for DART were the following; helium ionization gas (99.997%) at a flow rate of 3 L/min with nitrogen (99.998%) as standby gas, DART grid voltage of 600 V, ionisation temperature of 500 °C and linear rail speed of 1 mm/min. The MS system was operated in positive-ion mode, acquiring profile MS data in a mass range of 100–1000 m/z, using oTOF control software. Collision cell energy was set at 10 eV and quadrupole energy at 5 eV with a PrePulse Ion storage of 10 µs. Data preview and integration was achieved by Compass DataAnalysis 4.2 (Bruker Daltonics, Bremen, Germany) and centroided peakslits were exported for further analysis in Mass Mountaineer.

Analysis

Analysis of DART-generated spectra was carried out using the mass spectrum elemental composition and classification software Mass Mountaineer (Mass Mountaineer Version 5.0, 2018 https://diabloanalytical.com/ms-software/mass-mountaineer/). As DART is an ambient ionisation source a background ambient spectrum was taken using DART Quickstrip mesh without bone samples. The background spectrum was subtracted from the sample spectra and the spectra were searched for common contaminants, including cleaning solutions, plastics, and products of recent decomposition (e.g. putrescine compounds), which were also removed from sample spectra. Kernel Discriminant Analysis (KDA) was used to test for separation between the sample classes of captive and wild.

KDA is an extension of Principal Component Analysis (PCA) that maps features into a higher-dimensional space using a non-linear function, and thus allows separation of points that may not be linearly separated in two-dimensions (Souza 2010). KDA is a supervised

learning technique which uses class membership of samples in the training set in order to maximise class separation (Mass Mountaineer 2018). The ions (i.e. mass peaks) selected for the classification of captive and wild bone samples by KDA had an abundance threshold of at least 10%. Classification accuracy was assessed by leave-one-out cross-validation (LOOCV) which successively omits each sample from the training set and thus compares each sample in turn against the whole of the training set (Cody et al. 2012).

Fisher's ratios were calculated to determine the discriminating power of mass peaks (i.e. features), of over 10% abundance, for the difference between captive and wild classes. Higher Fisher's ratios for a class relative to other classes indicate that the feature has a higher discriminating value for that class. Fisher's ratios are calculated as follows:

$$Fisher^{'}sRatio = \frac{(m_1 - m_2)^2}{v_1 + v_2}$$

where m_1 and m_2 are the means for a feature in class 1 and class 2, and v_1 and v_2 are the variances for class 1 and class 2 respectively. m_1 and v_1 represent the in-class mean and variance for the feature, and m_2 and v_2 represent the between-class mean and variance for the feature (Mass Mountaineer 2018).

Potential compound compositions were calculated for selected mass peaks with higher discriminating value (i.e. greater differences in Fisher's ratios) using the 'Composition' function of Mass Mountaineer. We chose mass peaks for which the Fisher's ratio for the captive class was approximately double-or-greater than the wild class (Table 5 in Appendix 3). Potential compound formulae generated by Mass Mountaineer were searched for in the online databases Chemspider (http://www.chemspider.com) and PubChem (https://pubchem.ncbi.nlm.nih.gov/) and, where possible, tentative compound names were assigned.

We also searched spectra for matches to masses of commonly used veterinary tranquilizers and sedatives (Table 2) provided to us by a member of the lion bone trade industry.

Results

When wild samples from each individual lion, taken in duplicate, were considered as discrete classes duplicates largely overlaid each other in the KDA; indicating that the method produced replicable spectra (99.32% variance covered on 3 Principal Components and 66.67% classification accuracy using leave-one-out cross-validation LOOCV) (Fig. 1). Replicates showed close matching of spectra (Fig. 2) and major features in those spectra were replicated.

Kernel Discriminant Analysis (KDA) showed clear separation between the spectra of captive-bred and wild lion bone samples (Fig. 3) with 97.50% classification accuracy using LOOCV. Three principal components accounted for 100% of the variance. A randomly selected captive-bred spectrum was excluded from the training set and was used as an unknown; it was successfully classified with KDA (Fig. 3).

Identification of the composition of mass peaks is not required for classification. However, we tentatively attempted to identify potential compounds that may be responsible for the key diagnostic mass peaks for the difference between captive-bred and wild lion bone spectra. At a level of 10% relative abundance peaks were selected based upon their Fisher's ratios (Table 5 in Appendix 3) and were tentatively assigned compound names (Table 1).

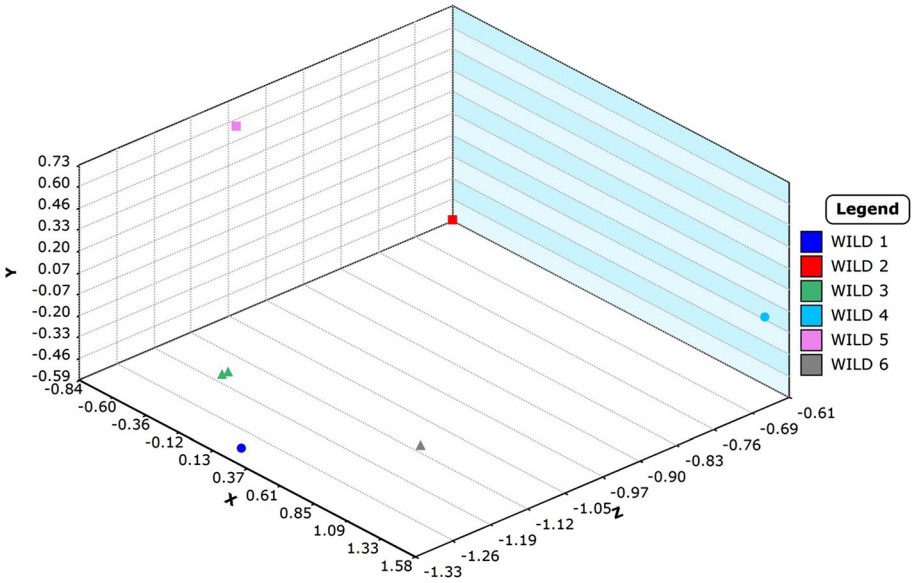

Fig. 1 Kernel Discriminant Analysis for wild lion samples; 6 individuals in duplicate (duplicate samples for WILD 1,2,4,5 & 6 are effectively superimposed). 99.32% variance covered on 3 Principal Components; 66.67% classification accuracy using LOOCV

We found no matches within the captive-bred spectra mass peaks for the masses of commonly used veterinary tranquilizers and sedatives (Table 2).

Discussion

The visual similarity of captive-bred lion bone to wild lion bone creates complications for the regulation and investigation of potentially illegal lion bone trade. To date, there has been no analytical method developed for accurately determining captive-bred lion bone from wild lion bone. We therefore conducted a preliminary investigation to determine whether Direct Analysis in Real Time (DART) mass spectrometry has potential to be used as a tool to distinguish between lion bone from captive-bred and wild sources. We found that DART analysis was able to reveal clear separation between a batch of captive-bred and a batch of wild lion bone mass spectra. This finding has relevance for discussions about the lion bone trade as the indistinguishability of captive from wild sources has been suggested to present a significant barrier to regulating lion bone trade and generating accurate intelligence concerning captive-bred or wild origins of lion products in the global market. We suggest that, with suitable techniques and resources, captive-bred and wild lion bones could be chemically distinguishable.

A well-acknowledged (but not sole) condition for farmed sources of wildlife to be considered non-detrimental is that its system of trade should not be vulnerable to the laundering of wild products (Dutton et al. 2013). In order to prevent laundering attempts, physical inspections, including verified 'chain of custody' for skeletons from farm to airport, have been undertaken for lion skeletons exported from South Africa as part of the 2017 and

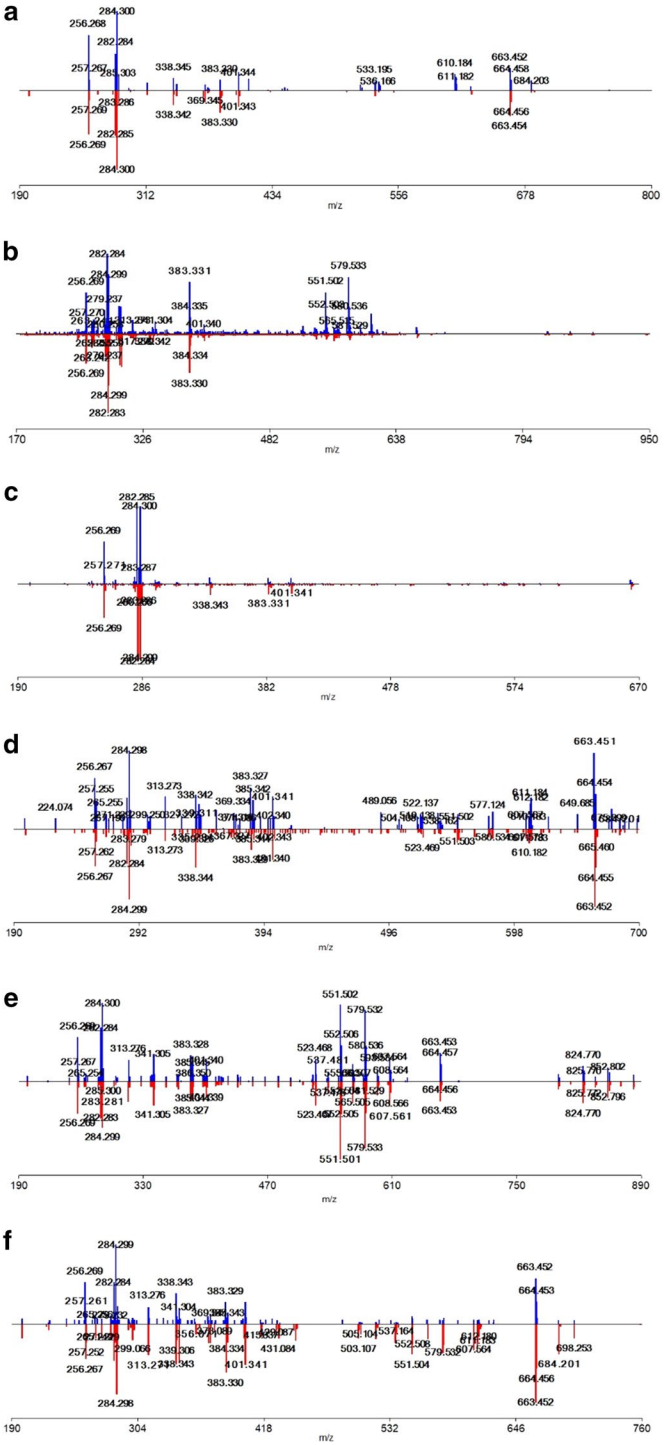

Fig. 2 Wild lion bone spectra duplicate matching (spectra = blue, top; duplicate = red, below). **a** Wild 1; **b** Wild 2; **c** Wild 3; **d** Wild 4; **e** Wild 5; **f** Wild 6

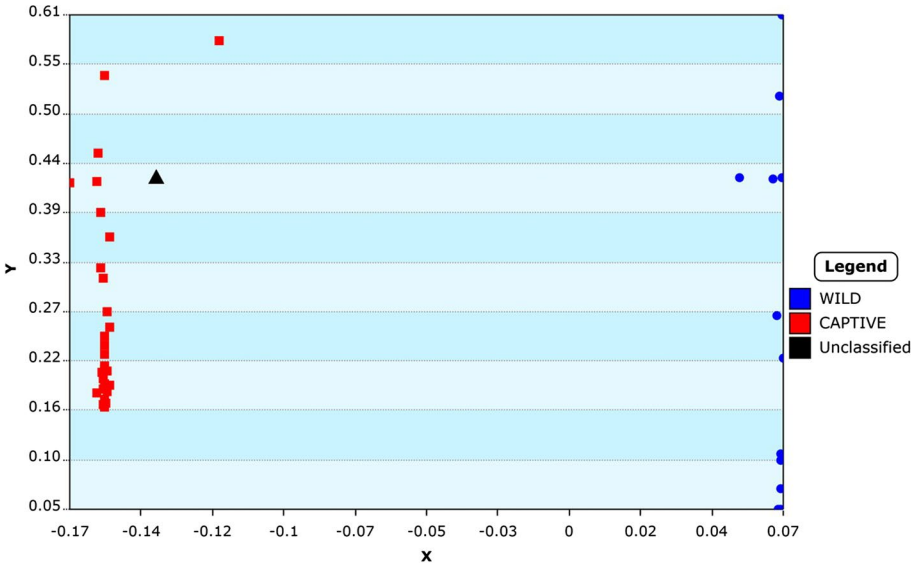

Fig. 3 Kernel Discriminant Analysis for separation between captive-bred and wild lion bone. Captive n = 28; Wild n = 12 (6 samples in duplicate). 100% variance covered on 3 Principal Components; 97.50% classification accuracy using LOOCV. 'Unknown' captive-bred sample (black) correctly grouped with other captive-bred samples

2018 export quotas (Williams et al. 2021). Although such measures largely prohibit any substitutions of skeletons after the first instance of inspection they do not provide surety of the captive-bred origin of skeletons (Williams et al. 2021). Our preliminary results, and others (e.g. Hutchinson and Roberts 2020), indicate that chemical methods, such as DART mass spectrometry, could provide such a measure. In addition, the relative contributions of wild and captive-bred lion parts in seizures and global end-markets are unknown and concerns have been raised that a conservation-significant contribution to overall trade may be made from wild animals (EIA 2017; Everatt et al. 2019). DART analysis shows potential to be able to provide information about the captive-bred origins of such lion products in illegal trade markets and seizures.

As a proof of concept this study used commercially captive-bred lion bone from South Africa and museum samples of wild-collected lion bone from Anglo-Egyptian Sudan (historical) in the early twentieth century. We were certain of the wild and captive origins of all samples in the study. Although DART analysis revealed clear separation between wild and captive-bred samples we cannot be certain that this separation is solely due to the wild or captive origin of the lions as additional variables could influence the result. Amongst these is the geographical area from which the samples originated. Indeed, bone, and other tissues, have been used to geo-locate biological samples through geographically variable chemical composition (although differences used in this way are often O and H isotope ratios e.g. Juarez 2008; Sugiyama et al. 2018). We searched spectra for, and removed, peaks generated by common contaminant compounds, it is thus unlikely that treatments such as cleaning solutions or plastic packaging lead to the observed differences. An additional, not-inconsiderable factor is the age of the bone sample and subsequent state of decomposition which may have influenced the observable difference between the two sample types. Although we treated common products of recent decomposition as contaminants and

Table 1 Tentative assignments of mass peaks for differentiation of captive-bred lion bone from wild lion bone, identified through Fisher's ratios (Table 5 in Appendix 3)

Mass (m/z)	Formula	Name
199.176*	$C_{11}H_{23}N_2O$	1-Ethoxyimino-2,2,6,6-tetramethylpiperidin-1-ium
257.267	$C_{15}H_{33}N_2O$	2-(Decanoylamino)-N,N,N-trimethylethanaminium
279.237	$C_{18}H_{31}O_2$	Linoleate
280.258	$C_{18}H_{34}NO$	2-(Adamantan-2-yloxy)-N,N,N-triethylethanaminium
281.254*	$C_{17}H_{33}N_2O$	1-[(Decyloxy)methyl]-2-ethyl-3-methyl-1H-imidazol-3-ium
283.286	$C_{16}H_{35}$	3-{4-[{2-[Ethyl(dimethyl)ammonio]ethyl}(dimethyl)ammonio]butyl}-1-methyl-1H-imidazol-3-ium
299.260*	$C_{18}H_{35}O_3$	3-Hydroxyoctadecanoate
399.335*	$C_{26}H_{43}N_2O$	3-Hexadecyl-1-(2-oxo-propyl)-3H-benzoimidazol-1-ium
519.138	$C_{24}H_{19}N_6O_8$	1-Benzyl-4-[(E)-2-hydroxy-2-(4-methoxyphenyl)-1-(2,4,6-trinitrophenyl)vinyl]-1H-1,2,4-triazol-4-ium
523.472	$C_{35}H_{59}N_2O$	3-(12-Hydroxydodecyl)-1-[13-(3-pyridinyl)tridecyl]pyridinium
536.166	$C_{28}H_{22}N_7O_5$	1-Methyl-4-{[4-({2-nitro-4-[(6-nitro-4-quinolinyl)amino]benzoyl}amino)phenyl]amino}pyridinium
551.502	$C_{37}H_{63}N_2O$	3-(14-Hydroxytetradecyl)-1-[13-(3-pyridinyl)tridecyl]pyridinium
552.503	$C_{36}H_{62}N_3O$	3-(13-Aminotridecyl)-1-[13-(1-oxido-3-pyridinyl)tridecyl]pyridinium
565.515	$C_{38}H_{65}N_2O$	3-(14-Hydroxytetradecyl)-1-[14-(3-pyridinyl)tetradecyl]pyridinium
580.536		Unidentified compound
684.203*		Unidentified compound

Potential elemental compositions were limited to Mass Mountaineer default settings for calculating possible formulae of organic compounds: C (0 to 50), H (0 to 100), O (0 to 10), and N (0 to 10)

*Absent (0 abundance) in captive-bred bone

Table 2 Commonly used veterinary tranquilizers and sedatives in commercial lion-husbandry

Name	Formula	Mass (m/z)
Ketamine	$C_{13}H_{16}ClNO$	237.725
Tiletamine	$C_{12}H_{17}NOS$	223.335
Zolazepam	$C_{15}H_{15}FN_4O$	286.304
Xylazine	$C_{12}H_{16}N_2S$	220.334
Medetomidine	$C_{13}H_{16}N_2$	200.279
Midazolam	$C_{18}H_{13}ClFN_3$	325.767

removed them from analysis we cannot be certain of the influence of longer-term decomposition on captive-bred-wild sample differences.

The peaks identified from their Fisher's ratios as diagnostic for the separation between captive-bred and wild lion bone were large, complex compounds (Table 1). Of those tentatively assigned compounds several, containing imidazolium, pyridinium, and triazolium (Table 1), were of interest due to the use of imidazole, pyridine, and triazole derivatives in pharmaceutical drugs (including antibiotics and tranquilizers), and pesticides (e.g. Borgers 1980; Ross et al. 1980; Shimizu et al. 2000; Kharb et al. 2011). However, such derivatives are also naturally occurring biological molecules (Shimizu et al. 2000). In addition, a number of diagnostic compounds were found to occur in wild but *not* captive bones (Table 1). Therefore, we do not think it likely that man-made chemical additives are the main reason for the differing chemical compositions of captive-bred and wild lion bones. Nevertheless, we expected the different diets of captive-bred and wild lions to have an influence on body tissue chemical composition, and dietary studies across a range of organisms demonstrate the use of body tissue chemical composition to reflect dietary differences (e.g. Ambrose 1991; Cerling and Harris 1999; Katzenberg 2008). Our preliminary results suggest that amongst selected compounds with highly different Fisher's ratios there are absences in captive-bred lion bone that were found in wild lion bone. We cannot explain these differences. However, it is possible that the more restricted diet of captive-bred lions had an effect. Future work could beneficially focus on detailed examination of dietary specifics in the differentiation of wild and captive-bred origin lion bone.

The development and use of chemical profiling analyses in the monitoring of CITES-associated wildlife trade generally lags behind DNA-based techniques (Ogden and Mailley 2016). Nevertheless, we suggest that with further development DART mass spectrometry is likely to prove a useful tool in the regulation and investigation of lion bone trade. Although there is a long way to go before the use of DART analysis as a forensic method for lion bone trade, this study demonstrated that DART analysis can differentiate between a batch of captive-bred South African lion bones and bones of separate, wild origin. The development of robust forensic methods is difficult and often lengthy (Ogden and Mailley 2016) but for now DART analysis appears to be able to provide useful intelligence and research information for the lion bone trade. Future implementation of DART analysis into export regulation protocol as a screening method could be used to identify skeletons with chemical compositions that may be worthy of further investigation and verification of captive-bred origin. We searched captive-bred spectra for mass peaks of commonly used tranquilizers and veterinary sedatives (Table 2), we found no matches. This is unsurprising as we did not consider the metabolic products of these veterinary chemicals in detail, which can be complex and poorly understood (Tranquilli et al. 2013). Mass spectrometry is a key technique in the identification of drug metabolites (Zhu et al. 2011) and DART has been

successfully used in the identification of trace amounts of drug metabolites (Sisco et al. 2016). However, our primary research objective was not to identify specific chemical markers, rather to broadly differentiate between captive-bred and wild lion bone spectra. The identification of specific chemical markers in captive-bred lion bone, of which we believe veterinary drugs are likely candidates, would greatly aid in the detection of captive-bred lion bone. In addition, the mandatory introduction of suitable markers by lion breeders and keepers should also be considered. We thus suggest that refining knowledge of chemical markers in captive-bred lion bone is an important area upon which future research effort could focus.

DART analysis provides significant operational benefit over other chemical techniques (such as stable isotope analysis) due to the speed with which mass spectra can be generated (in this study a DART Quickstrip of 12 samples was analysed in approximately two minutes). Time was added to our method by the powdering of bone. Powdering was deemed necessary to present a more homogenous sample than introduction of a whole bone piece; which is likely to be prone to high levels of variation in thickness and surface composition. Preparation times of powdered bone samples were approximately 10 min per sample, including waiting time for the evaporation of ethanol from the DART Quickstrip mesh. Even with the added sample preparation time, DART is a suitable method for rapid result generation from lion bone. In addition, further attention is being given to the development of detection and analysis equipment that can be used for 'in-the-field' (i.e. decentralised) wildlife trade investigations (Morrison et al. 2018; Masters et al. 2019). The development of robust, portable DART technology (e.g. Wells et al. 2008) further increases the potential utility of such an approach to support lion bone trade investigations, especially in key locations of concern for the lion bone trade which may lie in regions of weaker laboratory capacity for the investigation of wildlife crime (Williams et al. 2017b; Ogden and Mailley 2016).

Conclusion

In response to a lack of analytical techniques to differentiate between captive-bred and wild sources of lion bone in trade we tested the potential of Direct Analysis in Real Time (DART) mass spectrometry to differentiate between captive-bred and wild lion bone samples. We found that DART analysis was able to differentiate between two batches of visually indistinguishable lion bone powders; one from contemporary South African captive-bred lion bones and the other from historical Sudanese wild lion bones. We suggest that DART analysis shows good potential as an emerging tool for the investigation of the origin of lion bones in trade. At present the future of the legal trade in lion skeletons from South Africa is uncertain. However, regardless of the future legality of captive-bred lion bone trade, we anticipate a continued requirement for effective methods of captive-bred and wild differentiation; either in the regulation of legal trade or investigation of illegal trade.

Appendix 1

See Table 3.

Table 3 Accession numbers of 29 captive-bred lion bone samples at the National Zoological Gardens of South Africa used for DART analysis

Accession numbers for captive-bred lion bones
BN1052
BN1046
BN1034
BN1042
BN1496
BN1035
BN1045
BN1485
BN1051
BN1047
BN1037
BN1036
BN1044
BN1038
BN1033
BN1032
BN1054
BN1041
BN1039
BN1040
BN1048
BN1490
BN1492
BN1050
BN1043
BN1494
BN1488
BN1049
BN1053

Appendix 2

See Table 4.

Table 4 Oxford University Museum of Natural History historic wild lion bone samples used for DART analysis

Object number	Taxon	Object Name	Locality	Precise location	Date collected	Sex	Physical description	DART sample number
ZC-14182	*Panthera leo*	Skull	Sudan	Anglo-Egyptian Sudan	1902–1912	Unknown	Skull with right ramus of jaw	2
ZC-14185	*Panthera leo*	Skull	Sudan	Anglo-Egyptian Sudan	1902–1912	Unknown	Skull with jaw	5
ZC-14186	*Panthera leo*	Skull	Sudan	Anglo-Egyptian Sudan	1902–1912	Unknown	Skull with jaw	1
ZC-14188	*Panthera leo*	Skull	Sudan	Anglo-Egyptian Sudan	1902–1912	Unknown	Skull with jaw	3
ZC-14189	*Panthera leo*	Skull	Sudan	Anglo-Egyptian Sudan	07/05/1905	Female	Skull with jaw	6
ZC-14190	*Panthera leo*	Skull	Sudan	Anglo-Egyptian Sudan	05/05/1905	Female	Skull with jaw	4

Appendix 3

See Table 5.

Table 5 Fisher's ratios for difference between captive–bred and wild lion bone samples calculated at 10% abundance threshold

Class index	Class name	Fisher's ratio	Abundance	Variance
199.1764069	–	–		
0	WILD	494.667523	15,523	5,740,816
1	CAPTIVE	935.818658	0	0
224.0738068	–	–		
0	WILD	0	13,513	0
1	CAPTIVE	0	0	0
229.1502991	–	–		
0	WILD	100.364687	0	0
1	CAPTIVE	83.481721	13,741.1765	9,511,833.91
245.2312012	–	–		
0	WILD	0	0	0
1	CAPTIVE	0	12,100	0
247.245697	–	–		
0	WILD	248.70471	0	0
1	CAPTIVE	172.528972	12,771.4286	7,239,183.673
256.2683105	–	–		
0	WILD	7.428018	51,539	86,436,053.5
1	CAPTIVE	6.036091	39,427.3793	200,285,829.9
257.26651	–	–		
0	WILD	7.018523	16,253	62,322,439.33
1	CAPTIVE	19.976187	11,085.7143	629,795.9184
263.2414856	–	–		
0	WILD	39.045456	16,450	722,500
1	CAPTIVE	15.098694	14,071.4286	6,467,755.102
265.2565002	–	–		
0	WILD	4.080715	14,564.5	14,329,668.92
1	CAPTIVE	2.70345	18,295.4545	57,774,979.34
267.1901855	–	–		
0	WILD	0	13,127	0
1	CAPTIVE	0	0	0
271.2286072	–	–		
0	WILD	1024.084797	17,839.5	6320.25
1	CAPTIVE	1024.918582	0	0
277.2210083	–	–		
0	WILD	0	0	0
1	CAPTIVE	0	16,200	0
279.1705017	–	–		
0	WILD	0	15,444	0
1	CAPTIVE	0	0	0

Table 5 (continued)

Class index	Class name	Fisher's ratio	Abundance	Variance
279.2367859	–	–		
0	WILD	6.385084	21,779.5	58,993,132.75
1	CAPTIVE	13.73945	17,316.6667	17,251,388.89
280.2583923	–	–		
0	WILD	1.451757	11,133.3333	815,555.5556
1	CAPTIVE	11.156153	10,900	0
281.2542114	–	–		
0	WILD	309.377798	24,700	31,360,000
1	CAPTIVE	850.192718	0	0
282.2840881	–	–		
0	WILD	2.879713	66,704.0833	579,072,355.9
1	CAPTIVE	2.886329	81,250.8621	574,861,538
283.2862854	–	–		
0	WILD	0.027961	19,932.4545	53,685,652.07
1	CAPTIVE	0.04513	20,270.3704	14,844,307.27
284.2997131	–	–		
0	WILD	7.711279	89,426.3333	211,309,397.4
1	CAPTIVE	6.048276	68,737.1379	592,868,918.1
285.0177917	–	–		
0	WILD	0	12,741	0
1	CAPTIVE	0	0	0
285.302887	–	–		
0	WILD	3.423868	19,157	17,317,462.83
1	CAPTIVE	4.096744	16,754.1667	10,393,315.97
297.2461853	–	–		
0	WILD	477.188082	36,750	5,062,500
1	CAPTIVE	658.063688	18,000	0
299.0657959	–	–		
0	WILD	0	19,075	0
1	CAPTIVE	0	0	0
299.260498	–	–		
0	WILD	128.726333	28,054	95,770,394
1	CAPTIVE	344.712732	0	0
301.0643921	–	–		
0	WILD	0	16,988	0
1	CAPTIVE	0	0	0
309.2788086	–	–		
0	WILD	0	0	0
1	CAPTIVE	0	11,300	0
311.2598877	–	–		
0	WILD	112.169325	0	0
1	CAPTIVE	96.734658	13,200	6,196,250
313.2727051	–	–		
0	WILD	3.809944	26,598	92,385,346.29
1	CAPTIVE	3.25468	33,848	151,227,451.7

Table 5 (continued)

Class index	Class name	Fisher's ratio	Abundance	Variance
314.2771912	–	–		
0	WILD	245.482551	0	0
1	CAPTIVE	221.619756	12,350	1,672,500
317.2698975	–	–		
0	WILD	0	10,000	0
1	CAPTIVE	0	0	0
321.3160095	–	–		
0	WILD	963.21163	0	0
1	CAPTIVE	595.352823	11,450	2,102,500
323.262085	–	–		
0	WILD	0	0	0
1	CAPTIVE	0	16,600	0
325.2687988	–	–		
0	WILD	1003.913804	0	0
1	CAPTIVE	829.090214	13,800	1,000,000
326.3428955	–	–		
0	WILD	891.125072	0	0
1	CAPTIVE	381.026337	12,900	6,250,000
327.2539978	–	–		
0	WILD	0	0	0
1	CAPTIVE	0	10,100	0
327.2720947	–	–		
0	WILD	0	16,988	0
1	CAPTIVE	0	0	0
335.2907104	–	–		
0	WILD	0	11,010	0
1	CAPTIVE	0	0	0
336.2547913	–	–		
0	WILD	0	0	0
1	CAPTIVE	0	11,600	0
337.2730103	–	–		
0	WILD	114.983589	0	0
1	CAPTIVE	94.494314	13,626.6667	8,753,955.556
338.3445129	–	–		
0	WILD	1.53228	30,063.25	257,171,939.9
1	CAPTIVE	1.199443	38,099.72	549,576,200.2
339.2909851	–	–		
0	WILD	52.072662	0	0
1	CAPTIVE	40.078295	58,711.36	495,270,655.6
339.3109131	–	–		
0	WILD	102.162332	17,850	8,753,850
1	CAPTIVE	22.963303	33,199.6667	207,607,066.9
339.3388977	–	–		
0	WILD	640.810913	13,725	0
1	CAPTIVE	391.254553	22,600	1,960,000

Table 5 (continued)

Class index	Class name	Fisher's ratio	Abundance	Variance
340.2948914	–	–		
0	WILD	92.250171	0	0
1	CAPTIVE	79.770433	17,473.6842	12,945,096.95
341.0227051	–	–		
0	WILD	0	12,741	0
1	CAPTIVE	0	0	0
341.3035889	–	–		
0	WILD	17.153188	28,070.7143	135,611,587.6
1	CAPTIVE	35.443278	15,241.1765	11,817,716.26
342.310791	–	–		
0	WILD	0	18,147	0
1	CAPTIVE	0	0	0
352.3568115	–	–		
0	WILD	1024.968886	0	0
1	CAPTIVE	1024.650075	18,150	2500
353.2622986	–	–		
0	WILD	128.642658	0	0
1	CAPTIVE	90.471937	16,666.6667	22,775,555.56
355.2528992	–	–		
0	WILD	255.6921	0	0
1	CAPTIVE	137.487671	15,100	19,166,666.67
355.2802124	–	–		
0	WILD	127.788735	24,324	0
1	CAPTIVE	77.715061	15,180	10,539,600
356.071106	–	–		
0	WILD	0	13,295	0
1	CAPTIVE	0	0	0
357.3005981	–	–		
0	WILD	427.058485	0	0
1	CAPTIVE	230.292815	15,775	12,446,875
360.3265076	–	–		
0	WILD	0	0	0
1	CAPTIVE	0	13,000	0
367.3268127	–	–		
0	WILD	1.438044	17,417.5	47,052,740.25
1	CAPTIVE	2.667532	15,475	16,818,125
369.2341919	–	–		
0	WILD	177.088731	0	0
1	CAPTIVE	117.519312	14,755.5556	15,580,246.91
369.3454895	–	–		
0	WILD	1.822486	17,931	23,174,926.5
1	CAPTIVE	1.07154	23,771.92	351,148,147.6
370.3526917	–	–		
0	WILD	742.999133	0	0
1	CAPTIVE	194.50195	22,400	47,610,000

Table 5 (continued)

Class index	Class name	Fisher's ratio	Abundance	Variance
371.3164063	–	–		
0	WILD	0	14,286	0
1	CAPTIVE	0	0	0
373.0892944	–	–		
0	WILD	0	10,116	0
1	CAPTIVE	0	0	0
374.0856018	–	–		
0	WILD	0	13,513	0
1	CAPTIVE	0	0	0
383.2466125	–	–		
0	WILD	176.022388	0	0
1	CAPTIVE	115.990865	20,422.2222	30,657,283.95
383.3089905	–	–		
0	WILD	222.135765	0	0
1	CAPTIVE	130.115963	18,857.1429	28,302,448.98
383.3299866	–	–		
0	WILD	11.292015	35,800	314,185,733.5
1	CAPTIVE	23.906249	19,988.8889	22,145,432.1
384.3351135	–	–		
0	WILD	366.718276	17,842	5,165,252
1	CAPTIVE	481.264513	0	0
385.3417053	–	–		
0	WILD	18.964344	22,111.8333	60,244,434.47
1	CAPTIVE	42.077025	13,491.6667	6,437,430.556
386.3457031	–	–		
0	WILD	13.404296	11,861.5	2,727,452.25
1	CAPTIVE	5.766002	13,300	7,840,000
391.2893982	–	–		
0	WILD	0	15,058	0
1	CAPTIVE	0	0	0
397.3198853	–	–		
0	WILD	0	13,899	0
1	CAPTIVE	0	0	0
399.3351135	–	–		
0	WILD	446.125435	15,812	7,209,225
1	CAPTIVE	919.003369	0	0
401.3435974	–	–		
0	WILD	4.393809	27,484.2	151,569,123
1	CAPTIVE	3.069039	40,935.72	595,975,012.4
402.3396912	–	–		
0	WILD	21.099731	14,032.5	6,602,330.25
1	CAPTIVE	10.675194	19,190.9091	37,389,917.36
411.4000854	–	–		
0	WILD	0	14,539	0
1	CAPTIVE	0	10,600	0

Table 5 (continued)

Class index	Class name	Fisher's ratio	Abundance	Variance
415.0372925	–	–		
0	WILD	0	15,896	0
1	CAPTIVE	0	0	0
425.3422852	–	–		
0	WILD	678.232399	0	0
1	CAPTIVE	645.315288	11,033.3333	228,888.8889
429.0870056	–	–		
0	WILD	0	11,272	0
1	CAPTIVE	0	0	0
429.3714905	–	–		
0	WILD	0	0	0
1	CAPTIVE	0	14,300	0
431.0842896	–	–		
0	WILD	0	18,208	0
1	CAPTIVE	0	0	0
432.0798035	–	–		
0	WILD	0	10,116	0
1	CAPTIVE	0	0	0
437.3510132	–	–		
0	WILD	980.865554	0	0
1	CAPTIVE	680.521262	13,200	1,960,000
439.3474121	–	–		
0	WILD	85.021148	0	0
1	CAPTIVE	65.975509	16,666.6667	23,578,888.89
445.3673096	–	–		
0	WILD	0	0	0
1	CAPTIVE	0	13,100	0
446.1213074	–	–		
0	WILD	0	21,098	0
1	CAPTIVE	0	0	0
451.3485107	–	–		
0	WILD	483.009379	0	0
1	CAPTIVE	373.007232	12,500	2,385,000
453.353302	–	–		
0	WILD	61.042662	0	0
1	CAPTIVE	44.694887	22,814.2857	77,968,843.54
454.3604126	–	–		
0	WILD	400.435913	0	0
1	CAPTIVE	365.480993	11,060	730,400
455.3648987	–	–		
0	WILD	131.389376	0	0
1	CAPTIVE	104.027179	14,938.4615	11,168,520.71
465.3575134	–	–		
0	WILD	72.456875	0	0
1	CAPTIVE	57.890394	19,247.619	32,163,446.71

Table 5 (continued)

Class index	Class name	Fisher's ratio	Abundance	Variance
466.3645935	–	–		
0	WILD	0	0	0
1	CAPTIVE	0	10,500	0
467.3705139	–	–		
0	WILD	46.842267	0	0
1	CAPTIVE	35.485521	46,653.4231	371,767,877.2
468.3747864	–	–		
0	WILD	80.416727	0	0
1	CAPTIVE	65.870389	17,230	20,381,100
469.3612061	–	–		
0	WILD	1017.173183	0	0
1	CAPTIVE	943.339707	11,400	250,000
479.3622131	–	–		
0	WILD	88.197345	0	0
1	CAPTIVE	66.618892	17,464.7059	28,004,636.68
481.3711853	–	–		
0	WILD	83.505703	0	0
1	CAPTIVE	64.046374	20,350	37,669,166.67
482.3829956	–	–		
0	WILD	0	0	0
1	CAPTIVE	0	10,300	0
483.3699036	–	–		
0	WILD	229.467346	0	0
1	CAPTIVE	180.992698	12,150	4,307,500
489.0563965	–	–		
0	WILD	0	21,235	0
1	CAPTIVE	0	0	0
491.3699036	–	–		
0	WILD	498.31783	0	0
1	CAPTIVE	436.423507	11,475	936,875
493.3811951	–	–		
0	WILD	49.195914	0	0
1	CAPTIVE	37.945101	32,538.3462	159,525,506.6
494.3898926	–	–		
0	WILD	146.439447	0	0
1	CAPTIVE	117.722382	14,783.3333	9,101,388.889
495.3893127	–	–		
0	WILD	94.336279	0	0
1	CAPTIVE	74.720647	18,382.3529	23,508,512.11
495.4046021	–	–		
0	WILD	93.605749	0	0
1	CAPTIVE	73.71548	18,176.4706	23,808,858.13
503.1065979	–	–		
0	WILD	0	18,497	0
1	CAPTIVE	0	0	0

Table 5 (continued)

Class index	Class name	Fisher's ratio	Abundance	Variance
504.1076965	–	–		
0	WILD	0	12,741	0
1	CAPTIVE	0	0	0
505.104187	–	–		
0	WILD	0	13,006	0
1	CAPTIVE	0	0	0
507.3703003	–	–		
0	WILD	324.911024	0	0
1	CAPTIVE	278.28293	11,283.3333	1,641,388.889
509.3848877	–	–		
0	WILD	197.168098	0	0
1	CAPTIVE	150.959789	12,277.7778	5,850,617.284
519.1381836	–	–		
0	WILD	319.783443	17,501	1,651,225
1	CAPTIVE	620.617193	10,900	0
520.5014038	–	–		
0	WILD	0	0	0
1	CAPTIVE	0	10,300	0
521.4511719	–	–		
0	WILD	221.147073	0	0
1	CAPTIVE	163.687847	13,662.5	7,407,343.75
522.1367798	–	–		
0	WILD	0	18,533	0
1	CAPTIVE	0	0	0
523.4718018	–	–		
0	WILD	8.298666	21,185.75	63,512,816.19
1	CAPTIVE	18.372601	15,625	12,435,625
533.1945801	–	–		
0	WILD	0	11,702	0
1	CAPTIVE	0	0	0
536.1657715	–	–		
0	WILD	4.920255	18,181.6667	24,900,656.89
1	CAPTIVE	36.169174	15,800	0
537.1638184	–	–		
0	WILD	0	10,694	0
1	CAPTIVE	0	0	0
537.4810181	–	–		
0	WILD	854.695896	13,983.5	865,830.25
1	CAPTIVE	1007.161429	0	0
538.1621094	–	–		
0	WILD	865.58892	11,137.5	507,656.25
1	CAPTIVE	1008.490792	0	0
547.468689	–	–		
0	WILD	96.287302	0	0
1	CAPTIVE	77.463056	15,341.1765	14,849,480.97

Table 5 (continued)

Class index	Class name	Fisher's ratio	Abundance	Variance
548.4746094	–	–		
0	WILD	0	0	0
1	CAPTIVE	0	10,300	0
549.4857178	–	–		
0	WILD	52.351399	0	0
1	CAPTIVE	39.342647	33,545.7083	177,687,835.9
550.4893188	–	–		
0	WILD	98.960271	0	0
1	CAPTIVE	81.36309	16,270.5882	14,464,429.07
551.5015259	–	–		
0	WILD	4.677523	53,415.3333	1,199,762,890
1	CAPTIVE	9.175364	34,249.7917	237,381,687.7
552.5032959	–	–		
0	WILD	10.952528	28,142.8333	139,108,302.1
1	CAPTIVE	20.483431	17,890	27,461,900
553.5032959	–	–		
0	WILD	851.949692	11,059	552,049
1	CAPTIVE	1006.821486	0	0
563.4750977	–	–		
0	WILD	246.292214	0	0
1	CAPTIVE	223.987971	11,862.5	1,422,343.75
563.4927979	–	–		
0	WILD	221.233865	0	0
1	CAPTIVE	207.645712	11,377.7778	957,283.9506
565.4874878	–	–		
0	WILD	370.48652	0	0
1	CAPTIVE	265.556074	12,960	4,478,400
565.5145264	–	–		
0	WILD	71.318095	20,696.5	17,905,214.25
1	CAPTIVE	230.943078	12,100	0
566.5073242	–	–		
0	WILD	884.016391	10,633	400,689
1	CAPTIVE	1010.672636	0	0
573.4857788	–	–		
0	WILD	394.919334	0	0
1	CAPTIVE	343.167404	11,260	1,210,400
575.5028076	–	–		
0	WILD	44.855013	0	0
1	CAPTIVE	33.476104	36,665.1538	254,684,070.6
576.5050049	–	–		
0	WILD	85.866945	0	0
1	CAPTIVE	70.366712	17,805.2632	20,332,077.56
577.1240234	–	–		
0	WILD	0	16,602	0
1	CAPTIVE	0	0	0

Table 5 (continued)

Class index	Class name	Fisher's ratio	Abundance	Variance
577.5181274	–	–		
0	WILD	30.008172	10,626.5	20.25
1	CAPTIVE	21.272936	62,463.6071	919,240,604.8
578.5205078	–	–		
0	WILD	64.658353	0	0
1	CAPTIVE	51.946069	29,743.4783	83,708,544.42
579.5328979	–	–		
0	WILD	72.060451	70,986.75	446,919,288.2
1	CAPTIVE	117.405088	13,709.0909	7,324,462.81
580.5358276	–	–		
0	WILD	41.523891	29,906.3333	126,733,981.6
1	CAPTIVE	123.545929	12,100	0
581.5288086	–	–		
0	WILD	791.590654	11,316	839,056
1	CAPTIVE	998.821019	0	0
589.4833984	–	–		
0	WILD	1023.670665	0	0
1	CAPTIVE	1010.241181	11,100	40,000
591.4968872	–	–		
0	WILD	86.350138	0	0
1	CAPTIVE	64.326104	18,152.9412	32,664,844.29
592.5021973	–	–		
0	WILD	501.388443	0	0
1	CAPTIVE	451.247786	11,375	716,875
593.5101929	–	–		
0	WILD	182.533883	0	0
1	CAPTIVE	149.048477	13,570	5,666,100
593.5333252	–	–		
0	WILD	394.382685	15,093.3333	5,326,838.889
1	CAPTIVE	624.886576	0	0
601.5162964	–	–		
0	WILD	109.079565	0	0
1	CAPTIVE	79.417804	13,342.8571	15,239,591.84
603.5314941	–	–		
0	WILD	52.876154	0	0
1	CAPTIVE	38.806967	36,491.1739	228,252,278.8
604.5349731	–	–		
0	WILD	95.106533	0	0
1	CAPTIVE	75.793148	18,800	23,674,117.65
605.5426025	–	–		
0	WILD	84.440844	0	0
1	CAPTIVE	71.52418	18,415	18,131,275
608.5656128	–	–		
0	WILD	510.455258	13,364.6667	1,930,598.222
1	CAPTIVE	655.013801	0	0

Table 5 (continued)

Class index	Class name	Fisher's ratio	Abundance	Variance
609.4887695	–	–		
0	WILD	0	0	0
1	CAPTIVE	0	12,200	0
612.5499268	–	–		
0	WILD	681.351434	0	0
1	CAPTIVE	668.110166	10,633.3333	82,222.22222
617.510376	–	–		
0	WILD	288.790034	0	0
1	CAPTIVE	277.503659	10,571.4286	393,469.3878
619.5269775	–	–		
0	WILD	247.653897	0	0
1	CAPTIVE	228.050446	12,050	1,260,000
626.2260132	–	–		
0	WILD	0	15,830	0
1	CAPTIVE	0	0	0
642.4993286	–	–		
0	WILD	0	0	0
1	CAPTIVE	0	11,600	0
649.6848755	–	–		
0	WILD	0	18,533	0
1	CAPTIVE	0	0	0
656.5111084	–	–		
0	WILD	981.32276	0	0
1	CAPTIVE	683.005018	11,850	1,562,500
663.4520264	–	–		
0	WILD	1.77635	59,474.5	1,032,452,323
1	CAPTIVE	2.187012	46,699.6296	601,173,474.3
664.4577026	–	–		
0	WILD	0.925652	29,827	286,433,890
1	CAPTIVE	1.433254	25,454.5455	103,563,388.4
665.4603271	–	–		
0	WILD	203.172127	10,105	0
1	CAPTIVE	56.667332	11,900	1,025,000
675.6990967	–	–		
0	WILD	0	13,127	0
1	CAPTIVE	0	0	0
677.7145996	–	–		
0	WILD	0	25,482	0
1	CAPTIVE	0	0	0
684.2030029	–	–		
0	WILD	58.018505	20,854	149,590,608.7
1	CAPTIVE	287.610803	0	0
688.1956787	–	–		
0	WILD	0	13,513	0
1	CAPTIVE	0	0	0

Table 5 (continued)

Class index	Class name	Fisher's ratio	Abundance	Variance
691.7000122	–	–		
0	WILD	0	13,127	0
1	CAPTIVE	0	0	0
698.255188	–	–		
0	WILD	494.667523	15,523	5,740,816
1	CAPTIVE	935.818658	0	0
738.630188	–	–		
0	WILD	0	0	0
1	CAPTIVE	0	10,000	0
740.633728	–	–		
0	WILD	1019.796954	0	0
1	CAPTIVE	969.360676	11,200	160,000
766.6524048	–	–		
0	WILD	329.593048	0	0
1	CAPTIVE	294.086201	10,916.6667	1,091,388.889
824.7703247	–	–		
0	WILD	501.842599	23,094	12,355,225
1	CAPTIVE	938.074049	0	0
825.7698975	–	–		
0	WILD	825.934481	14,197	1,079,521
1	CAPTIVE	1003.501123	0	0
848.7681885	–	–		
0	WILD	0	0	0
1	CAPTIVE	0	15,300	0
850.7814941	–	–		
0	WILD	0	0	0
1	CAPTIVE	0	14,100	0
852.8024902	–	–		
0	WILD	937.491034	19,190	763,876
1	CAPTIVE	1016.565328	0	0
853.802002	–	–		
0	WILD	1016.982579	11,635	23,716
1	CAPTIVE	1024.282225	0	0
874.7836304	–	–		
0	WILD	0	0	0
1	CAPTIVE	0	10,700	0
876.7979126	–	–		
0	WILD	0	0	0
1	CAPTIVE	0	17,300	0
877.8029175	–	–		
0	WILD	0	0	0
1	CAPTIVE	0	10,300	0
880.8275146	–	–		
0	WILD	0	10,086	0
1	CAPTIVE	0	0	0

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Author contributions P.C: conceptualization; analysis and investigation; writing (first draft); writing (review & editing). A.L: conceptualization; supervision; writing (review & editing). D.K: analysis and investigation; writing (review & editing). V.L.W: sample collection; writing (review & editing). D.W.M: supervision; writing (review & editing). R.O: conceptualization; supervision; writing (review & editing).

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Data availability Summary data and sample identification numbers are included as appendices and access to full spectra data may be requested directly from the authors.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Consent for publication All authors consent to publication.

Ethical approval Bone samples were transferred from South Africa to the UK under CITES scientific institution licence numbers: South Africa, ZA034 (National Zoological Gardens of South Africa); UK, GB031 (SASA).

References

Ambrose SH (1991) Effects of diet, climate and physiology on nitrogen isotope abundances in terrestrial foodwebs. J Archaeol Sci 18(3):293–317

Bauer H, Chapron G, Nowell K, Henschel P, Funston P, Hunter LT, Packer C (2015) Lion (Panthera leo) populations are declining rapidly across Africa, except in intensively managed areas. Proc Natl Acad Sci USA 112(48):14894–14899

Bauer H, Packer C, Funston PF, Henschel P, Nowell K (2016) *Panthera leo* (errata version published in 2017). The IUCN Red List of Threatened Species 2016: e.T15951A115130419. https://doi.org/10.2305/IUCN.UK.2016-3.RLTS.T15951A107265605.en. Accessed 14 Sept 2019

Borgers M (1980) Mechanism of action of antifungal drugs, with special reference to the imidazole derivatives. Rev Infect Dis 2(4):520–534

Brandis KJ, Meagher PJ, Tong LJ, Shaw M, Mazumder D, Gadd P, Ramp D (2018) Novel detection of provenance in the illegal wildlife trade using elemental data. Sci Rep 8(1):1–8

Broad S, Mulliken T, Roe D (2014) The nature and extent of legal and illegal trade in wildlife. The trade in wildlife. Routledge, London, pp 25–44

Bulte EH, Damania R (2005) An economic assessment of wildlife farming and conservation. Conserv Biol 19(4):1222–1233

Cerling TE, Harris JM (1999) Carbon isotope fractionation between diet and bioapatite in ungulate mammals and implications for ecological and paleoecological studies. Oecologia 120(3):347–363
CITES (2016) Committee 1: Conservation of and the trade in the African Lion. CoP17 Com. 1. 29. https://cites.org/sites/default/files/eng/cop/17/Com_I/E-CoP17-Com-I-29.pdf. Accessed 29 Apr 2020
Coals P, Burnham D, Loveridge A, Macdonald DW, Sas-Rolfes MT, Williams VL, Vucetich JA (2019) The ethics of human–animal relationships and public discourse: a case study of lions bred for their bones. Animals 9(2):52
Cody RB, Laramée JA, Durst HD (2005a) Versatile new ion source for the analysis of materials in open air under ambient conditions. Anal Chem 77(8):2297–2302
Cody RB, Laramée JA, Nilles JM, Durst HD (2005b) Direct analysis in real time (DART) mass spectrometry. JEOL News 40(1):8–12
Cody RB, Dane AJ, Dawson-Andoh B, Adedipe EO, Nkansah K (2012) Rapid classification of White Oak (Quercus alba) and Northern Red Oak (Quercus rubra) by using pyrolysis direct analysis in real time (DART) and time-of-flight mass spectrometry. J Anal Appl Pyrol 95:134–137
Dalton DL, Kotzé A, McEwing R, De Bruyn M, Mnisi C, Mwale M (2018) A tale of the traded cat: development of a rapid real-time PCR diagnostic test to distinguish between lion and tiger bone. Conserv Genet Resour 12:29–31
Damania R, Bulte EH (2007) The economics of wildlife farming and endangered species conservation. Ecol Econ 62(3–4):461–472
Davidson Z, Valeix M, Van Kesteren F, Loveridge AJ, Hunt JE, Murindagomo F, Macdonald DW (2013) Seasonal diet and prey preference of the African lion in a waterhole-driven semi-arid savanna. PLoS ONE 8(2):e55182
DEA (2017) Lion export quota for 2017 communicated to the CITES Secretariat in line with CITES requirements. https://www.environment.gov.za/mediarelease/lionexportquota_communicatedtocitessecretariat. Accessed 29 Apr 2020
De Telegraaf (2019) Honderden kilo's leeuwenbotten onderschept. https://www.telegraaf.nl/nieuws/1492487166/honderden-kilo-s-leeuwenbotten-onderschept. Accessed 3 Oct 2019
Dutton AJ, Gratwicke B, Hepburn C, Herrera EA, Macdonald DW (2013) Tackling unsustainable wildlife trade. Key Top Conserv Biol 2(5):74–91
EAGLE (2017) The EAGLE Network Annual Report 2017. EAGLE Network. http://www.eagle-enforcement.org/data/files/eagle-network-annual-report-2017.pdf. Accessed 17 July 2019
EAGLE (2018) The EAGLE Network Annual Report 2018. EAGLE Network. http://www.eagle-enforcement.org/data/files/eagle-network-annual-report-2018.pdf. Accessed 17 July 2019
EIA (2017) The lion's share: South Africa's trade exacerbates demand for tiger parts and derivatives. The Environmental Investigation Agency. https://eia-international.org/wp-content/uploads/The-Lions-Share-FINAL-1.pdf. Accessed 17 Jan 2020
EMS (2018) The extinction business: South Africa's 'Lion' Bone Trade. EMS & Ban Animal Trading. https://emsfoundation.org.za/wp-content/uploads/THE-EXTINCTION-BUSINESS-South-Africas-lion-bone-trade.pdf. Accessed 29 April 2020
Espinoza EO, Lancaster CA, Kreitals NM, Hata M, Cody RB, Blanchette RA (2014) Distinguishing wild from cultivated agarwood (*Aquilaria* spp.) using direct analysis in real time and time of-flight mass spectrometry. Rapid Commun Mass Spectrom 28(3):281–289
Everatt KT, Kokes R, Pereira CL (2019) Evidence of a further emerging threat to lion conservation; targeted poaching for body parts. Biodivers Conserv 28(14):4099–4114
Fischer C (2004) The complex interactions of markets for endangered species products. J Environ Econ Manag 48(2):926–953
Gore ML, Braszak P, Brown J, Cassey P, Duffy R, Fisher J, Machalaba C (2019) Transnational environmental crime threatens sustainable development. Nat Sustain 2(9):784–786
Grange AH, Sovocool GW (2011) Detection of illicit drugs on surfaces using direct analysis in real time (DART) time-of-flight mass spectrometry. Rapid Commun Mass Spectrom 25(9):1271–1281
Hayward MW, Kerley GI (2005) Prey preferences of the lion (Panthera leo). J Zool 267(3):309–322
He Y, Wang J, Wang M, Zhang J (2018) Discrimination of wild and domestic deer musk using isotope ratio mass spectrometry. J Mass Spectrom 53(11):1078–1085
Hemley G (1994) International wildlife trade: a CITES sourcebook. Island Press
Hinsley A, King E, Sinovas P (2016) Tackling illegal wildlife trade by improving traceability: a case study of the potential for stable isotope analysis. The geography of environmental crime. Palgrave Macmillan, London, pp 91–119
Hogg CJ, Dennison S, Frankham GJ, Hinds M, Johnson RN (2018) Stopping the spin cycle: genetics and bio-banking as a tool for addressing the laundering of illegally caught wildlife as 'captive-bred.' Conserv Genet Resour 10(2):237–246

Hutchinson A, Roberts DL (2020) Differentiating captive and wild African lion (Panthera leo) populations in South Africa, using stable carbon and nitrogen isotope analysis. Biodivers Conserv 29:2255–2273
Jimenez-Bustamante D, Rentería LPD (2018) Laundering of Illegal Wild Fauna in Mexico: case study of a pair of desert monitors Varanus griseus griseus (Daudin, 1803). Green crime in Mexico. Palgrave Macmillan, Cham, pp 127–136
Juarez CA (2008) Strontium and geolocation, the pathway to identification for deceased undocumented Mexican border-crossers: a preliminary report. J Forensic Sci 53(1):46–49
Katzenberg MA (2008) Stable isotope analysis: a tool for studying past diet, demography, and life history. Biol Anthropol Hum Skelet 2:413–441
Kays R, Feranec RS (2011) Using stable carbon isotopes to distinguish wild from captive wolves. Northeast Nat 18(3):253–264
Kharb R, Sharma PC, Yar MS (2011) Pharmacological significance of triazole scaffold. J Enzyme Inhib Med Chem 26(1):1–21
Kim HJ, Jee EH, Ahn KS, Choi HS, Jang YP (2010) Identification of marker compounds in herbal drugs on TLC with DART-MS. Arch Pharmacal Res 33(9):1355–1359
Lancaster C, Espinoza E (2012) Analysis of select Dalbergia and trade timber using direct analysis in real time and time-of-flight mass spectrometry for CITES enforcement. Rapid Commun Mass Spectrom 26(9):1147–1156
Lyons JA, Natusch DJ (2011) Wildlife laundering through breeding farms: illegal harvest, population declines and a means of regulating the trade of green pythons (Morelia viridis) from Indonesia. Biol Conserv 144(12):3073–3081
Macdonald DW, Laurenson MK (2006) Infectious disease: inextricable linkages between human and ecosystem health. Biol Conserv 131(2):143
Mass Mountaineer (2018) Mass Mountaineer Guide. Version 5.0. Diablo Analytical. https://diabloanalytical.com/ms-software/mass-mountaineer/
Masters A, Ogden R, Wetton JH, Dawnay N (2019) Defining end user requirements for a field-based molecular detection system for wildlife forensic investigations. Forensic Sci Int 301:231–239
Meier-Augenstein W (2017) Stable isotope forensics: methods and forensic applications of stable isotope analysis. Wiley
Miller SM, Harper CK, Bloomer P, Hofmeyr J, Funston PJ (2014) Evaluation of microsatellite markers for populations studies and forensic identification of African lions (Panthera leo). J Hered 105(6):856–866
Morrison J, Watts G, Hobbs G, Dawnay N (2018) Field-based detection of biological samples for forensic analysis: established techniques, novel tools, and future innovations. Forensic Sci Int 285:147–160
Nilles JM, Connell TR, Stokes ST, Dupont Durst H (2010) Explosives detection using direct analysis in real time (DART) mass spectrometry. Propellants Explos Pyrotech 35(5):446–451
Ogden R, Dawnay N, McEwing R (2009) Wildlife DNA forensics—bridging the gap between conservation genetics and law enforcement. Endang Species Res 9(3):179–195
Ogden R, Mailley J (2016) A review of wildlife forensic science and laboratory capacity to support the implementation and enforcement of CITES. In: Review commissioned by the Secretariat of the Convention on International Trade in Endangered Species of Wild Fauna and Flora and review undertaken by the United Nations Office on Drugs and Crime (Vol. 37)
Outhwaite W (2018) The legal and illegal trade in African lions; a study in support of decision 17. TRAFFIC, Cambridge, p 241
Pavlovich MJ, Musselman B, Hall AB (2018) Direct analysis in real time—mass spectrometry (DART-MS) in forensic and security applications. Mass Spectrom Rev 37(2):171–187
Price ER, McClure PJ, Jacobs RL, Espinoza EO (2018) Identification of rhinoceros keratin using direct analysis in real time time-of-flight mass spectrometry and multivariate statistical analysis. Rapid Commun Mass Spectrom 32(24):2106–2112
Republic of South Africa. In the Matter between National Council of The Society for Prevention of Cruelty to Animals and Minister for Environmental Affairs, Director-General Department of Environmental Affairs, South African Predator Association. Case number: 86515/2017. 2019. https://nspca.co.za/wp-content/uploads/2019/08/Lion-Bone-judgment-1.pdf. Accessed 12 Aug 2019
Rosen GE, Smith KF (2010) Summarizing the evidence on the international trade in illegal wildlife. EcoHealth 7(1):24–32
Ross JH, Krieger RI (1980) Synthesis and properties of paraquat (methyl viologen) and other herbicidal alkyl homologs. J Agric Food Chem 28(5):1026–1031
Shimizu S, Watanabe N, Kataoka T, Shoji T, Abe N, Morishita S, Ichimura H (2000) Pyridine and pyridine derivatives Ullmann's encyclopedia of industrial chemistry. Wiley, New York
Sisco E, Forbes TP, Staymates ME, Gillen G (2016) Rapid analysis of trace drugs and metabolites using a thermal desorption DART-MS configuration. Anal Methods 8(35):6494–6499

Smoluch M, Mielczarek P, Silberring J (2016) Plasma-based ambient ionization mass spectrometry in bioanalytical sciences. Mass Spectrom Rev 35(1):22–34
Souza C (2010) Kernel Principal Component Analysis in C#. http://crsouza.com/2010/01/19/kernel-principal-component-analysis-in-c/. Accessed 21 Feb 2020
Sugiyama N, Fash WL, France CA (2018) Jaguar and puma captivity and trade among the Maya: stable isotope data from Copan, Honduras. PLoS ONE 13(9):e0202958
Tranquilli WJ, Thurmon JC, Grimm KA (eds) (2013) Lumb and Jones' veterinary anesthesia and analgesia. Wiley, New York
UN (2018) The state of knowledge of crimes that have serious impacts on the environment. United Nations Environment Programme. https://wedocs.unep.org/bitstream/handle/20.500.11822/25713/knowledge_crime_envImpacts.pdf?sequence=1&isAllowed=y Accessed 6 Jan 2020
van Uhm D (2018) Wildlife and laundering. In: Spapens T, White R, Van Uhm D, Huisman W (eds) Green crimes and dirty money. Routledge, London
Volpato G, Fontefrancesco MF, Gruppuso P, Zocchi DM, Pieroni A (2020) Baby pangolins on my plate: possible lessons to learn from the COVID-19 pandemic. Springer, Berlin
Warchol GL (2004) The transnational illegal wildlife trade. Crim Justice Stud 17(1):57–73
Wells JM, Roth MJ, Keil AD, Grossenbacher JW, Justes DR, Patterson GE, Barket DJ (2008) Implementation of DART and DESI ionization on a fieldable mass spectrometer. J Am Soc Mass Spectrom 19(10):1419–1424
Williams VL, Newton DJ, Loveridge AJ, Macdonald DW (2015) Bones of contention: an assessment of the South African trade in African lion Panthera leo bones and other body parts. TRAFFIC & WildCRU, Cambridge, Oxford
Williams VL, Loveridge AJ, Newton DJ, Macdonald DW (2017a) A roaring trade? The legal trade in Panthera leo bones from Africa to East-Southeast Asia. PLoS ONE 12(10):e0185996
Williams VL, Loveridge AJ, Newton DJ, Macdonald DW (2017b) Questionnaire survey of the pan-African trade in lion body parts. PLoS ONE 12(10):e0187060
Williams VL, 't Sas-Rolfes MJ (2019) Born captive: a survey of the lion breeding, keeping and hunting industries in South Africa. PLoS ONE 14(5):e0217409
Williams VL, Coals PG, de Bruyn M, Naude VN, Dalton DL, Kotzé A (2021) Monitoring compliance of CITES lion bone exports from South Africa. PLoS ONE 16(4):e0249306
Zhu M, Zhang H, Humphreys WG (2011) Drug metabolite profiling and identification by high-resolution mass spectrometry. J Biol Chem 286(29):25419–25425
Zimmerman ME (2003) The black market for wildlife: Combating transnational organized crime in the illegal wildlife trade. Vand J Transnat'l L 36:1657

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