A preliminary assessment of the palate and tongue for detecting organophosphorus and carbamate pesticide exposure in the degraded carcasses of vultures and other animals

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ABSTRACT

In many regions of the world, organophosphorus (OP) and carbamate (CM) pesticides are used to poison wildlife thought to be competing with human activities (e.g. hunting). Vultures may be secondarily poisoned or directly targeted, e.g. for muti or traditional medicine. Some OPs and CMs are so acutely toxic that animals will die with poisoned material still in their mouths - un-swallowed, before traces may have spread to other parts of the body. Even when death is more prolonged, the tissues in which residues have accumulated may deteriorate before the carcass is discovered, minimizing the chances of recovering viable samples for toxicological analyses that would conclusively identify poisoning as the cause of death. With all these factors in mind, we investigated the feasibility of detecting OP and CM pesticides in the oral cavity, with emphasis on the tongue and palate. A total of 60 degraded carcasses (n = 28 avian and 32 mammalian) recovered from various scenes of wildlife crime in Andalucía, southern Spain, where poisoning was suspected,

were submitted to the Center for Analysis and Diagnosis of Wildlife in Málaga for necropsy and toxicological analyses. Of these, 20 and 24 avian and mammalian tongues, respectively, could be recovered for analysis. Separately, the palate from one degraded Cinereous Vulture *Aegypius monachus* carcass was also opportunistically retrieved and analyzed following an incident of vulture mass-mortality in which nine Griffon Vultures *Gyps fulvus* also perished. Residues or presence of OPs and CMs were detected in one avian tongue (analyzed with food from the mouth) and four mammalian tongues. Our findings suggest avian tongues alone are not optimal, but canid tongues and those of larger mammals may lend themselves well to analysis. Detection of the OP chlorfenvinphos (3.39 mg/kg) in the Cinereous Vulture palate (the only part of the carcass in which residues were detected) indicates this is a promising sample. To our knowledge, this represents the first time that OP and CM pesticides have been detected in tongue and palate samples. We recommend further exploration of oral cavity samples, especially within the context of the risk that residues therein may pose to human health.

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Introduction

Vultures and other wildlife as well as domesticated animals around the world are being deliberately or secondarily poisoned at an alarming rate, primarily due to human-wildlife conflict (Ogada 2014). They are also being poisoned with pesticides for human consumption - either as food (Odino 2012, Ogada *et al.* 2015) or for use in traditional medicine (Mander *et al.* 2007, McKean *et al.* 2013, Saidu and Buij 2013, Ogada 2014, Ogada *et al.* 2015). Many of the organophosphorus (OP) and carbamate (CM) pesticides in question are so acutely toxic that animals die with the poison-laced material still in their mouths, sometimes before they can swallow it, and before residues can spread to, and be incorporated by, other parts of the body (Figure 1). Should this be the case, analysis of typically favored samples like stomach contents would not reflect that exposure had occurred (Mineau *et al.* 2011). And, even when a poisoning death is more protracted, conventionally-analyzed samples (i.e. soft tissues) could be degraded

between when the animal dies and the carcass is actually found, and/or parts of it may have been scavenged, thereby limiting its viability for toxicological analyses (Richards *et al.* 2014).

Figure 1: When the pesticides used to poison wildlife are acutely toxic, animals (such as this African White-backed Vulture *Gyps africanus*) may die with food in their mouths, and before residues can reach other parts of the body. Photo courtesy of Andre Botha.

A handful of studies have examined the feasibility of detecting OP and CM pesticides in talons, feet and beaks, which better withstand environmental degradation than soft tissues. Importantly, these also represent the first likely point of contact animals will have with these pesticides, as they paw at, step on, or grasp poisoned items prior to ingesting them. Simulating a dermal contact scenario, goslings were exposed to turf sprayed with the OP diazinon (*O*,*O* - diethyl *O* - (2-isopropyl-6-methyl-4-pyrimidinyl) phosphorothioate); residues were subsequently detected in their feet after these were removed and

weathered for seven days (Vyas *et al.* 2003). Similarly, eastern Screech-owls *Megascops asio* were exposed to baits laced with CM carbofuran (2,3 - dihydro-2,2-dimethyl-7-benzofuranyl methylcarbamate) and residues were detected in their talons after these had been weathered for 28 days post-exposure (Vyas *et al.* 2005). Residues of carbofuran and two of its primary metabolites (3-ketocarbofuran and 3-hydroxycarbofuran) were also identified in the highly weathered talons and beak of an African White-backed Vulture *Gyps africanus* recovered from an agricultural field in Kenya (Otieno *et al.* 2010, Otieno *et al.* 2012). We have also detected CMs (e.g., aldicarb) and OPs (e.g., chlorfenvinphos) in beaks and talons taken from the degraded carcasses of birds submitted to the Center for Analysis and Diagnosis of Wildlife (CAD) in Málaga, southern Spain, during routine wildlife forensic investigations (Richards *et al. in prep*). Nonetheless, despite their established utility and viability, it does not appear as yet that any of these 'alternative' samples are routinely collected, analyzed or considered when pesticide poisoning is suspected, even in the absence of other more 'conventional' samples like soft tissues.

After talons, feet and beaks, the oral cavity/mouth is the next and likely last point of contact with pesticides prior to death. Since pesticide-poisoned food rests on the tongue, we reasoned that residues might be detectible therein. We therefore sampled and analyzed the tongues from a selection of mammalian and avian carcasses that were degraded, and where pesticide poisoning was suspected to have caused death.

Here we report on our findings from both the analysis of tongues and the palate, offer further recommendations for refinement, and propose potential applications. Our aims were to a) assess the feasibility of detecting OP and/or CM pesticide residues in the tongue and palate; b) provide additional tools for determining cause of death in degraded carcasses, and, c) promote broader awareness and use of these and other 'alternative' samples in a wildlife forensic context. To our knowledge this is the first time that either the tongue or the palate has been analyzed for residues of OPs, CMs or any other class of pesticide, during the course of a wildlife forensics investigation or otherwise.

Materials and Methods

Carcass collection

A total of 60 carcasses (28 avian, of these 7 vultures; and 32 mammalian) in varying stages of decomposition or degradation, and where pesticide poisoning was suspected as the cause of death, were selected for this preliminary study (Tables 1a and 1b). Each carcass was collected according to specific forensic protocols (as detailed in Fajardo *et al.* 2015), during routine investigations under the Andalusian government's anti-poisoning/poaching strategy (described in Fajardo *et al.* 2012). The carcasses were submitted to the CAD for necropsy and toxicological analyses. All toxicologically viable samples (e.g., stomach contents, digestive tracts) were collected for analysis and for comparison with the tongues.

Table 1a: Summary of degraded bird carcasses (n = 28) recovered by species and weights of tongue samples recovered (n = 20)

Species	Carcasses	Tongues sampled	Tongue weights (g)
Buzzard, Eurasian *Buteo buteo*	1	1	1.1
Chicken, domestic *Gallus gallus*	1	1	0.1
Eagle, Bonelli's *Aquila fasciata*	1	0	Not obtained
Eagle, Booted *Aquila pennata*	3	2	0.78, 0.9
Eagle, Spanish Imperial *Aquila adalberti*	2	2	0.79, 2.41
Jackdaw, Eurasian *Corvus monedula*	2	2	0.15, 0.53
Kestrel, Eurasian *Falco tinnunculus*	1	0	Not obtained
Kite, Black *Milvus migrans*	5	4	0.49 - 1.72
Kite, Red *Milvus milvus*	1	0	Not obtained
Osprey *Pandion haliaetus*	1	1	0.46
Owl, Eurasian eagle *Bubo bubo*	2	2	2.34, 3.20
Owl, Tawny *Strix aluco*	1	0	Not obtained
Vulture, Cinereous *Aegypius monachus*	1	0	Not obtained
Vulture, Egyptian *Neophron percnopterus*	3	2	0.69, 1.01
Vulture, Griffon *Gyps fulvus*	3	3	3.48 - 5.98
TOTALS	**28**	**20**	

Table 1b: Summary of degraded mammalian carcasses (n = 32) recovered by species and weights of tongue samples recovered (n = 24)

Species	Carcasses	Tongues sampled	Tongue weights (g)
Badger, Eurasian *Meles meles*	1	1	5.0
Cat, domestic *Felis catus*	7	6	2.52 – 5.66
Dog, domestic *Canis lupus familiaris*	10	9	4.2 – 7.19
Fox, Red, European *Vulpes vulpes crucigera*	9	4	0.52 – 9.71
Genet, Common *Genetta genetta*	1	1	4.72
Hare, Iberian *Lepus granatensis*	1	1	5.4
Polecat, European *Mustela putorius*	1	1	0.55
Rabbit, European *Oryctolagus cuniculus*	1	1	2.07
Weasel, Least *Mustela nivalis*	1	1	0.35
TOTALS	**32**	**24**	

The tongue samples were cut from the mouth at their base - unless the carcass was in such poor condition that they could not be removed as an individual sample. Following an incident of mass vulture mortality in southern Spain in which nine Griffon Vulture *Gyps fulvus* carcasses were recovered with that of a Cinereous Vulture *Aegypius monachus* (see Fajardo *et al.* 2014 for details), we also opportunistically analyzed the palate of the Cinereous Vulture. The palates from the Cinereous vulture carcass (recovered in a degraded condition) and the Griffon Vulture carcasses were also excised (Figures 2a to 2f).

Figure 2a

Figure 2b.

Figure 2c.

Figure 2d.

Figure 2e.

Figure 2f.

Figure 2a : Cinereous Vulture head, showing the extent of carcass degradation.

Figure 2b: Ventral aspect of Cinereous Vulture head.

Figure 2c: Removal of the beak.

Figures 2d/2e: Palate being removed for processing and toxicological analysis.

Figure 2f: Cinereous Vulture head after complete removal of palate material.

Reproduced with permission from D. de la Bodega, SEO Birdlife (Fajardo *et al*. 2014).

Sample preparation and analysis for pesticide residues

Sample preparation, pesticides residue extraction and multi-screening methods were all adapted from those described by Zoun & Spierenburg (1989). Briefly, 5 g of each sample were ground in a mortar with 10 g of anhydrous sodium sulphate (Merck) and 60 ml of dichloromethane (Merck). After 10 minutes shaking, these were filtered (Whatman, nº1 paper) and rotavaporated to dryness (BÜCHI R-200). The sample was then reconstituted in 6 ml of ethanol (Panreac) and filtered through glass wool. Finally, sample cleanup was obtained via solid phase extraction (Extrabond C18 500 mg 3/ml, Sharlab S.L.). Aliquots (40 microliters) of these extracts were initially screened qualitatively, via thin-layer chromatography (TLC) at the CAD. If a positive result was obtained, i.e., indicating the presence of an OP or a CM, another aliquot (1 ml) was screened at the Laboratorio Analítico Bioclínico (LAB) in Almería (southern Spain). Each aliquot was analyzed for a suite of 278 pesticides (including OPs, CMs, organochlorines, strychnine and pyrethroids) using either gas chromatography–mass spectrometry (GC-MS/MS) with ion trap (IT) and triple quadrupole (QqQ) analyzers, or ultra-performance liquid chromatography mass spectrometry (UHPLC-MS/MS) with triple quadrupole (QqQ) analyzer. For GC-detectible pesticides, the limits of detection (LODs) ranged from 0.001 to 0.436 g L^{-1} and the limits of quantification (LOQs) ranged from 0.003 to 1.452 g L^{-1}. For LC-detectible pesticides, the LODs ranged from 0.003 to 1.048 g L^{-1} and the LOQs ranged from 0.011 to 3.494 g L^{-1} (Cazorla *et al.* 2011). All toxicological analyses were conducted in accordance with EU Directive 2002/657/CE (concerning the performance of analytical methods and the interpretation of results) and EU Regulation 1107/2009 CE (concerning the regulation of commercial insecticides). A list of the pesticides that were screened, and their individual limits of detection, is provided in Appendix 1 (online version only).

Results

Sixty carcasses were recovered for this preliminary study: 28 avian carcasses spanning 15 species (including three Egyptian Vultures *Neophron percnopterus*, three

Griffon Vultures and one Cinereous Vulture) and 32 mammalian carcasses of 10 species. Of these, 20 avian and 24 mammalian tongues were recovered for analysis. Tables 1a and 1b summarize the species represented, as well as the number of carcasses and tongues recovered from each. The range of weights of the tongues is also provided for consideration of the viability of this sample in relation to others (see Discussion).

Analysis of tongues relative to other carcass samples

From the 60 recovered carcasses, a total of 151 samples were retrieved, 78 from birds and 73 from mammals. Of these, 11 avian and 19 mammalian samples tested positive for an OP or CM pesticide. Eight of the positives were detected qualitative, i.e., via TLC, only. To better assess the viability of the tongue, we categorized pooled samples *including* the tongue from a given carcass separately from pooled samples that did not include it. These results are summarized in Table 2. We note that 11 livers (three from birds and eight from mammals) were neither included in the total sample count nor in our analysis, because these samples were only screened for anticoagulant rodenticides. The organochlorine p,p'-DDE was detected in five avian samples that were screened for the suite of compounds (and hence were included in the total sample tally), and is indicated where found with an asterisk in Table 2, however we did not further consider it in our analysis since its lone presence (absent DDE and other breakdown metabolites such as DDD) indicates environmental persistence from historical agricultural usage, rather than deliberate pesticide poisoning.

Twenty (20) avian and 24 mammalian tongues were retrieved and analyzed. No OP or CM pesticides were detected in any of the avian tongue samples, however the presence of carbofuran was detected in three of the mammalian tongues (two red foxes and one domestic dog; Table 3b). An additional six avian and six mammalian tongues were pooled with oral contents for analysis. Methamidophos was (qualitatively) detected in one of the avian pooled samples (Egyptian Vulture; Table 3a) and carbofuran was detected in one of the mammalian pooled samples (Red Fox; Table 3b). Two avian and two mammalian tongues pooled with other carcass components tested negative for pesticide residues.

To allow a more refined comparison of the tongue relative to other samples, Tables 3a and 3b list all carcass samples that tested positive for an OP and/or a CM pesticide, by species.

Table 2: Summary of all samples (n = 151) collected from avian carcasses (n = 28) and mammal carcasses (n = 32)

Sample type	Avian	Positive (key to abbreviations below)		Mammal	Positive
Insects recovered from carcasses	3	0		4	1: Car[a]
Digestive tract[b,*]	20	4: A(2), M(1,1[a])		2	0
Feather	1	0		N/A	N/A
Liver	0	0		1	0
Oral cavity/content	1	1: CPF		2	1: Car[a]
Organs, pooled[c,*]	12	1: CFV[a]		8	2: Car
Pellet	2	1: CFV[a]		N/A	N/A
Stomach contents	1	1: CPF		22	11: A (6), Car (4 (1[a])), M (1)
Talons/material held inside	4	1: A		N/A	N/A
Tongue only	20	0		24	3: Car
Tongue + oral contents	6	1 M[a]		6	1: Car
Tongue + various pooled samples[d]	2	0		2	0
Various pooled samples[e,f,*]	6	1: CFV[a,g]		2	0
TOTALS	**78**	**11**		**73**	**19**

a. QUAL = qualitative, i.e., detection by TLC only; b. Digestive tract includes: larynx, esophagus, trachea, crop, gizzard, proventriculus;
c. Pooled due to poor carcass condition; *p,p-DDE detected (n = 3 organs, pooled; 1 digestive tract and 1 various pooled samples);
d, e. In case of highly degraded/skeletonized carcass, *any* possibly viable samples were pooled for analysis; f. Tongues not included
g. Crop + insects recovered from carcass
A = aldicarb only or aldicarb and metabolites aldicarb sulfoxide or aldicarb sulfone; Car = carbofuran only or carbofuran and metabolite 3-hydroxycarbofuran; CFV = chlorfenvinphos; CPF = chlorpyrifos; M = methamidophos

Table 3a: Pesticides detected in samples (n = 11) from degraded avian carcasses (n = 4)

Species	Animals sampled (n)	Samples (n)	Sample description	Pesticide	Residues detected (mg g $^{-1}$)
Black Kite	1	3	Digestive tract (larynx, esophagus, trachea) Digestive tract (gizzard) Talon (skin from)	Aldicarb Aldicarb Aldicarb sulfoxide* Aldicarb sulfone*	50.25 0.04 0.38 0.29 0.01
Cinereous Vulture	1	3	Organs Pellet Digestive tract (crop) + insects from carcass	Chlorphenvinfos	QUAL QUAL QUAL
Black Kite	1	2	Stomach contents Oral cavity/contents = bait adhering to beak	Chlorpyrifos	2.03 2.96
Egyptian Vulture	1	3	Digestive tract (gizzard + proventriculus) Tongue + oral cavity/contents Digestive tract (larynx, esophagus, trachea)	Methamidophos	QUAL QUAL 6.57

*A metabolite of aldicarb

QUAL = qualitatively detected only, via thin layer chromatography

Table 3b: Range of pesticide residues detected in samples (n = 10) from degraded mammal carcasses (n = 19)

Species	Animals sampled	Samples	Sample description	Pesticide	Residues detected (mg g $^{-1}$)
Cat, domestic	3	3	Stomach contents	Aldicarb Aldicarb, Aldicarb sulfoxide	54 0.32, 0.03 15, 7.2
Dog, domestic	3	3	Stomach contents	Aldicarb Aldicarb, Aldicarb sulfoxide	66 12, 3.6 6, 30
	1	1	Stomach contents	Methamidophos	1.87
Cat, domestic	1	1	Organs, pooled	Carbofuran	0.34
Dog, domestic	2	4	Oral cavity/oral contents Stomach contents	Carbofuran	QUAL QUAL
			Stomach contents Tongue	Carbofuran, 3-hydroxy carbofuran	118, 0.03 0.03, 0.01
Red fox	3	7	Insects from carcass Organs, pooled	Carbofuran	QUAL 1.82
			Stomach contents (2) Tongues (2) Tongue + oral contents	Carbofuran, 3-hydroxy carbofuran	5.28, 0.01 4.03, 0.04 0.34, 0.02 0.06, 0.03 0.94, 0.02

Table 4 compares the residue levels detected in the tongues relative to other samples retrieved from the same carcass. Of the OPs and CMs screened for, only carbofuran and its 3-hydroxy metabolite were detected in mammalian (canid) tongue samples. Compounds were never only detected in the tongue rather than in other samples taken from the same carcass (but see results of the Cinereous Vulture palate, below). The residue levels of carbofuran detected in tongue samples were always the lowest relative to other analyzed samples. However, we note that levels of the 3-hydroxy metabolite detected in the tongue were sometimes similar to those found in the stomach contents.

Table 4: Comparison of residue levels of carbofuran and methamidophos detected in the tongue and other samples from the same carcass

Species	Animals sampled	Pesticide detected	Detected in	Residues (mg kg^{-1})
Red fox	3	Carbofuran, 3-hydroxy carbofuran	Tongue	0.34, 0.02
			Stomach contents	5.28, 0.01
			Tongue	0.06, 0.03
			Insects from carcass	QUAL
			Stomach contents	4.03, 0.04
			Tongue + oral contents	0.94, 0.02
			Organs	1.82, 1.2
Domestic dog	1		Tongue	0.03, 0.01
			Stomach contents	118, 0.03
Egyptian Vulture	1	Methamidophos	Tongue + oral contents	QUAL
			Digestive tract (gizzard + proventriculus)	QUAL
			Digestive tract ((larynx, esophagus, trachea)	6.57

Analysis of the Cinereous vulture palate

The analysis of this vulture palate arose under separate circumstances. The nine Griffon Vultures and one Cinereous Vulture found dead around a horse carcass in southern Spain were recovered for analysis (Fajardo *et al.* 2014). The carcass of the Cinereous Vulture was analyzed first, since the species is classified as 'Near Threatened' (Birdlife International 2015) and is therefore accorded a higher degree of protection than Griffon Vultures (CMA 2001). Time since death was estimated at between 15 and 20 days, and *the only parts* of the Cinereous vulture carcass initially deemed viable for analysis – the mummified organs - tested negative for any toxic compound. Therefore, cause of death was deemed 'inconclusive' at first. However, after residues of the OP chlorfenvinphos were detected in two of the Griffon Vultures, the Cinereous vulture carcass was re-examined, this time the palate was removed for analysis (Figures 2a to 2f), and residues of chlorfenvinphos (3.39 mg/kg) were detected therein. The detection of residues in the palate of this 'Near Threatened' species subsequently provided the impetus and regulatory justification required for further investigation into the circumstances surrounding the vulture mortality event, and five suspects were eventually apprehended and charged. The particulars of this case detailed in Fajardo *et al.* (2014).

Discussion

Tables 1a and 1b summarize the range of weights recorded for avian (0.1 – 5.98 g) and mammalian tongues (0.35 – 9.71 g). Our results indicate that the tongues of mammals (in this case, canids) are likely better suited for detecting OP and CM pesticides than those of birds because of their much greater surface area, which provides increased contact with residues and more sample for single or repeat analysis. However, we hasten to add that the viability of the avian tongue may also be species-dependent, since a sufficient sample may be recovered from larger birds like vultures (Table 1a). As such, this sample should be considered for opportunistic analysis in the absence of other samples. And, when available, the avian tongue can and should be pooled with mouth/oral contents to improve detection rates (as with the Egyptian vulture carcass (Table 3a)). At the CAD, we have on several occasions

detected toxic compounds in the oral cavity of birds. For example, carbofuran was detected in bait material retrieved from the mouth of a Black Kite *Milvus migrans* (0.3 mg/kg) whose carcass was so degraded that species identification was virtually impossible (CAD, unpublished report; Figure 3).

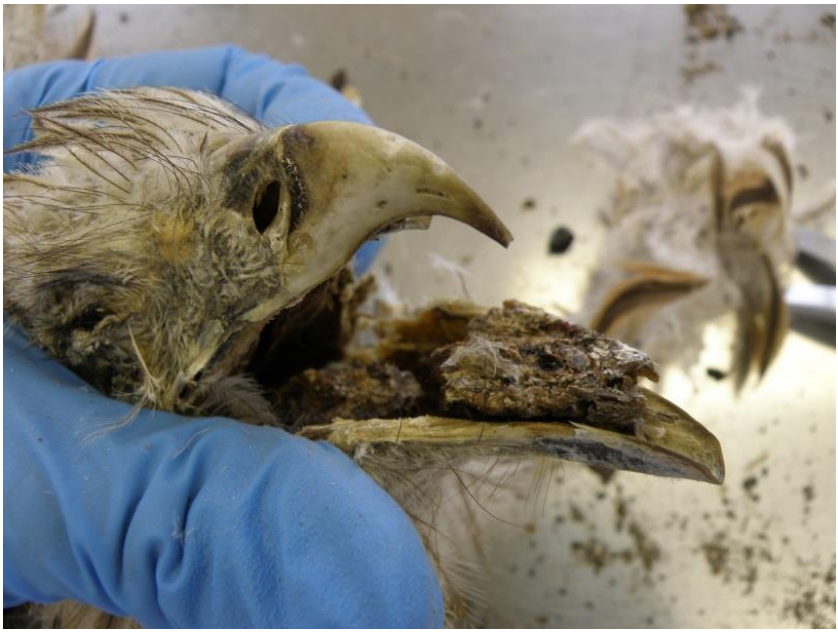

Figure 3: Degraded black kite *Milvus migrans* carcass with bait material in its mouth

Stomach contents are often favoured for toxicological analyses. Here, the stomach contents were retrieved from 22 of the 32 mammals and half of these tested positive for an OP or CM pesticide (Table 2, 3b). By contrast, one stomach content sample from the 28 avian recovered carcasses was deemed in sufficiently good condition for analysis – that of a Black Kite – and in which 2.03 mg kg^{-1} chlorpyrifos was detected (Table 3a). Interestingly, while the kite's tongue tested negative, residues

(2.96 mg kg^{-1}) were detected in the mouth and oral contents (Table 3a).

Although based on a single sample, the finding of chlorfenvinphos residues in the palate of the Cinereous vulture is promising and warrants pursuit in suspected wildlife poisoning cases, particularly when carcasses are highly degraded, and when other alternative and robust matrices such as talons are either unavailable or themselves in poor condition. The palate is shielded from environmental conditions that might degrade other parts of a carcass and destroy pesticide residues therein. Depletion of moisture during desiccation likely concentrates any residues present in the palate, and since the OP and CM compounds in question are highly toxic, a confirmatory finding of exposure in this sample provides conclusive evidence of ingestion and is also highly indicative that this led to the animal's death.

Generally, we suggest greater consideration of oral contents/palates/tongues during necropsy and toxicological analysis of avian and mammal carcasses. Further, we recommend collecting and analysing the tongues of mammalian scavengers that fall victim to pesticide poisoning, (e.g., lion, hyena, and bear) *regardless* of whether the carcass is degraded - to evaluate the viability of this sample in larger mammals than were assessed in the present study. We note that while avian tongues recovered from degraded carcasses may not be optimal samples, opportunistic collection of tongues from relatively fresh avian carcasses in which poisoning is suspected (especially in larger birds such as vultures), followed by controlled drying, may improve the viability of the sample, which would be beneficial in places with limited means of keeping carcasses (and soft tissues etc.) frozen, in addition to general space constraints. In this regard, the best option may be simply to collect the head (and talons/feet) for later analysis.

Finally, we strongly encourage greater analysis of oral contents/tongues/palates for reasons of utmost human safety. In several African countries, pesticides may be used to incapacitate or kill vultures for 'traditional medicine' purposes or 'muti' (Mander *et al.* 2007, McKean *et al.* 2013, Saidu & Buij 2013, Ogada 2014, Ogada *et al.* 2015). These vulture heads are then sold to people for personal consumption, and they are likely fresher than the carcasses analyzed in the present

study. It would therefore be highly relevant to examine vulture heads offered for purchase at markets to determine whether pesticide residues are present, and to assess potential risks for human health.

Questions about any of the sampling and analytical procedures are welcomed, and may be addressed to the corresponding authors.

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