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Interpreting the Historical Terrestrial Vertebrate Biodiversity of Cayman Brac (Greater Antilles, Caribbean) through Collagen Fingerprinting

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Abstract

Cayman Brac (Cayman Islands) lies within the Caribbean Islands Biodiversity Hotspot, an epicenter of high biodiversity and endemism. However, all endemic terrestrial mammals on the Cayman Islands are now extinct, following post-1500 AD human colonization of the islands. Introduced rodents and domesticated mammals now exclusively represent this facet of terrestrial fauna on the Cayman Islands, and are a likely cause of endemic species loss on the islands. Cayman Brac has numerous caves and rock fissures that offer protection to a naturally accumulated ensemble of vertebrate sub-fossil bone remains, documenting modifications in island biodiversity through the Holocene. In this study we showcase the first molecular faunal survey undertaken on sub-fossil remains from the Cayman Islands, using collagen fingerprinting for taxonomic identification of the cave skeletal deposits collected from a single cave system, Green Cave on Cayman Brac. Collagen type (I) extracts from 485 bone fragments were analyzed to determine faunal identity and assemblage composition. A total of 76% of the collagen fingerprint-yielding samples were mammalian in origin, 67% of which were identified as invasive murid rodents. Here, we present mass spectral biomarkers for the endemic terrestrial mammal fauna of Cayman Brac, including the extinct capromyid rodents, *Capromys* and *Geocapromys* (Rodentia: Capromyidae), alongside commentary on the composition of the sub-fossil bone assemblage between the five distinct depositional chambers that comprise Green Cave. Collagen (I) provides a key service in taxonomic identification and mapping of macroevolutionary trends and these results suggest a pivotal role for murid rodents in the competition and extinction of terrestrial endemic mammals from the Cayman Islands.

Key Words

Collagen fingerprinting; ZooMS; Species identification, Caribbean; Cayman Islands; Paleobiodiversity

Introduction

Extinction in the Caribbean

Insular ecosystems in the Caribbean have undergone considerable anthropogenic modifications over the last 7,000 years (Fitzpatrick and Keegan, 2007; Wilson, 2007; Fitzpatrick, 2015). Humans have altered habitats causing a localized mass extinction in the Caribbean Islands Biodiversity Hotspot (CIBH) (Brooks *et al.*, 2002; Steadman *et al.*, 2005; Fitzpatrick and Keegan, 2007; Wilson, 2007; Upham, 2017). The CIBH archipelago comprises over 1,000 habitat-rich islands and is one of the world's greatest centers for biodiversity and endemism that is under unprecedented threat (Myers *et al.*, 2000) (Figure 1). In the last 500 years, 90% of primary vegetation in the CIBH has been affected by deforestation and urban encroachment, and over 40 endemic species have become extinct (Brooks *et al.*, 2002; Anadón-Irizarry *et al.*, 2012). The vertebrate class most affected by this extinction event in the CIBH are mammals, with more than 100 volant and non-volant species identified as becoming extinct or extirpated during the Holocene (~11,700 years ago–present) (Steadman *et al.*, 2005; Steadman *et al.*, 2007; Turvey *et al.*, 2007; Turvey and Fritz, 2011). The specific drivers of these events are difficult to quantify, although direct (e.g. habitat transformation, hunting) and indirect human activities (e.g. species introduction) are suggested to have been major factors driving the CIBH extinctions (Giovas *et al.*, 2012; Cooke *et al.*, 2017). As a consequence, this biodiversity hotspot is the source of the highest levels of recent mammal species loss in the world (Dávalos and Turvey, 2012).

Figure 1: The location of the Cayman Islands within the Caribbean Island Biodiversity Hotspot (CIBH; solid red line). Adapted from Smith *et al.* (2004).

Located within the CIBH, the Cayman Islands (British Overseas Territory) consist of three main islands: Grand Cayman, Little Cayman and Cayman Brac. These islands are unique in their geographical and temporal isolation within the Caribbean, being ~200 km (~125 mi) from any other substantive landmass. Remarkably, the Cayman Islands have had little to no anthropogenic influences until 1503, following their discovery by Columbus (Stokes and Keegan, 1996; Drewett *et al.*, 2000). Historical records of the Cayman Islands from early European settlers speak of ample natural resources comparable to that of other Caribbean islands that are known to have hosted indigenous populations (Drewett *et al.*, 2000). Additionally, whilst the distance required to reach the Cayman Islands from neighboring islands is notably the longest journey in the Caribbean, Drewett *et al.* (2000) conclude that it is “not significantly greater than from Jamaica to Cuba, St Vincent to Barbados or Venezuela to Los Roques”—all crossings known to have been made by indigenous people in prehistory. Yet, targeted surveys and excavations made by Stokes and Keegan (1996) and Drewett *et al.* (2000), in addition to faunal surveys from Patton (1966), Morgan (1994a) and this study, have revealed no evidence of human occupation in the Prehistoric Period. Furthermore, there is no record of permanent settlement on the Cayman Islands until the middle of the 17th century (Williams, 1992; Smith, 2000), thousands of years after humans first settled other Caribbean islands (Fitzpatrick and Keegan, 2007). Hereafter, humans have played a dominant role influencing terrestrial vertebrate composition through habitat alteration, predation and invasive species introduction; the latter of which is suggested to have been a key factor in Cayman biodiversity loss over the last five hundred years (Morgan, 1994a).

Cayman Island Caves

The Cayman Islands are composed of carbonate platforms that have been shaped through eustatic sea level change since the Miocene (Jones, 1992). Cayman Brac, the larger (~38 km²) of the two sister islands to Grand Cayman, has been subject to repeated periods of cave development over the last 30 million years (Ma), resulting in numerous Karst limestone systems and cavities that typify the island (Jones, 1992). These cave systems are host to a natural, temporally accumulated sub-sample of the Cayman Brac vertebrate community through the deposition of bones via transportation, natural traps, carnivore (e.g. *Tyto alba*, barn owl) accumulation and habitation (Morgan, 1994a). Many of the Cayman cave sub-fossils are also encased in calcareous flowstone, with these factors working synergistically to limit morphological identification, thereby reducing the usefulness of large parts of the assemblages for morphology-based faunal surveys.

The cave sub-fossil assemblages document modifications in island biodiversity spanning the Holocene, encompassing the timeline traversing pre-human interaction through to the modern day. However, the sub-fossil remains on Cayman Brac, and indeed all of the Cayman Islands, have been vastly understudied. Primary investigations into the vertebrate skeletal deposits from cave systems on the Cayman Islands began with Moyne (1938), who target-collected large sub-fossils from the surface of some caves on Grand Cayman and Cayman Brac, never collecting microvertebrate remains or excavating stratigraphic layers beneath present cave floors. Later studies on Cayman Island fauna are scarce, with the vast majority focusing mainly on mammals (Morgan and Woods, 1986; Morgan, 1994a; Morgan, 1994b; MacPhee *et al.*, 1999). Moreover, all previous sub-fossil examinations on Cayman Brac have relied exclusively on bone morphology to undertake species identification. Yet, such a process of identification neglects to utilize the wealth of fragmented and heavily calcified material that represents much of the Island fossil vertebrate assemblages and has, until now, remained an untapped resource for biodiversity data.

The extinct endemic terrestrial (i.e. non-volant, non-marine) mammal fauna of Cayman Brac consist of three undescribed species previously identified from the sub-fossil deposits: the large capromyid rodent *Capromys* sp., the smaller capromyid *Geocapromys* sp., and one species of the evolutionarily distinct and globally extinct shrew-like eulipotyphlan, *Nesophontes* sp. (Morgan, 1994a) (see Supplementary Table S1). However, the terrestrial mammal fauna in present day Cayman Brac is wholly represented by three species of introduced rodents: the black rat, *Rattus rattus*; the brown or Norway rat, *Rattus norvegicus*; and the house mouse, *Mus musculus* (Morgan, 1994b), alongside introduced domesticates. This extensive modification in island biodiversity on Cayman Brac is therefore particularly apparent within the Rodentia, but it should also be noted that certain bats (four species) and birds (nine species) have

become extinct or extirpated on Cayman Brac in modern times (Morgan, 1994a; GISD, 2016). Fortunately, all taxonomically described herpetofaunal species have thus far escaped extinction on Cayman Brac, though the two native amphibian species *Osteopilus septentrionalis* and *Eleutherodactylus planirostris* may be under threat by the possible introduction of *Rhinella marina* (the cane toad) which is particularly famed for its opportunistic feeding and prolific breeding habits (Echternacht *et al.*, 2011). Similarly, there have been six reptile species introduced within the last 70 years (Echternacht *et al.*, 2011), joining the 14 endemic and native species known to Cayman Brac (Supplementary Table S1).

Despite the high number of introduced species on Cayman Brac, little research has been undertaken to investigate their impact, regardless of the fact that invasive species introductions are thought to be the leading cause of endemic species loss on both the Cayman Islands and other islands in the Caribbean. Biodiversity surveys and observational methods of sampling are no longer viable for extinct fauna, and morphological identification requires both expertise and relatively non-fragmentary material, particularly when dealing with post-cranial elements (e.g. Andrews, 1990). Rather, faunal identification of sub-fossils through biomolecular techniques such as collagen fingerprinting remains a useful alternative. Such an approach has the potential to yield clearly defined molecular-based species identification alongside presence/abundance data to compare with modern day Cayman Brac. These data are vital in the assessment of past and present island biodiversity, which can be utilized to further measure the overall health of the extant ecosystem.

Collagen Fingerprinting

Bone collagen type I (hereafter 'collagen (I)'), has a complex four-tiered hierarchical organization, locked within mineral hydroxyapatite. Collagen (I) survives both high temperatures and long-term burial far longer than that of other biologically informative proteins, such as ancient DNA (aDNA). Successfully extracted from ancient camel bone of ~3.5 Ma (Rybczynski *et al.*, 2013), collagen (I) is the most abundant protein in animals and is therefore often selectively enriched in archaeological bones due to faster degradation of other bone proteins (Schmidt-Schultz and Schultz, 2004). Taxonomic identification via collagen fingerprinting can often provide greater confidence than visual identification can provide (e.g. Buckley and Kansa, 2011), particularly when identifying fragmentary material or microvertebrate mammal remains from owl pellets—a notoriously challenging task, even when pellets are fresh (Matisoo-Smith and Allen, 2001; Guimaraes *et al.*, 2016).

The Cayman Brac cave systems offer refuge from environmental extremes (temperature fluctuations, humidity, salinity etc.) with subterranean systems aiding the preservation of vertebrate remains far beyond that which would be expected from a typical site in the tropics (Harvey *et al.*, 2016). This excellent preservation permits the survival of

informative biomolecules such as collagen (I), from which faunal identification (e.g. Buckley *et al.*, 2014), temporal frameworks (Harvey *et al.*, 2016) and phylogenetic information (e.g. Buckley, 2013) can be deduced. Collagen (I) chains are large and evolve at a slow rate, allowing soft-ionization methods of mass spectrometry (MS) to identify discrimination in the mass-to-charge (m/z) ratios of distinct collagen peptides, or ‘biomarkers’, between taxa across the animal kingdom. It is due to the relative resistance of collagen to degradation and its amino acid sequence variability between (Buckley *et al.*, 2010; Buckley *et al.*, 2017) and occasionally within genera (Buckley *et al.*, 2016), that collagen (I) provides a key service in taxonomic identification and the reconstruction of molecular-based phylogenies and mapping of macroevolutionary trends.

This study aims to assess the ability of a high-throughput form of collagen fingerprinting (Zooarchaeology by Mass Spectrometry; ZooMS), to determine the faunal identity of 485 partial, fragmented and/or flowstone-encased bone remains from Green Cave, Cayman Brac, in the absence of morphological expertise. Within this remit, ZooMS, supported by peptide sequencing using liquid chromatography tandem mass spectrometry (LC-MS/MS), was deployed on modern and ancient specimens—including Caribbean representatives from mammalian, reptilian, avian and amphibian clades—to acquire collagen (I) peptide mass fingerprints (PMFs), and verify species collagen (I) biomarker sequences in the creation of a ‘reference library’ for Cayman Brac sub-fossil vertebrate assemblages. Such a reference library is also applicable for use across the Cayman Islands, throughout the Caribbean and key biomarkers are likely to be applicable elsewhere globally. This research presents the use of ZooMS in evaluating the faunal composition of a Cayman Island sub-fossil assemblage and explores the potential for applying the ZooMS methodology to other archaeological and paleontological sites in the Caribbean and beyond.

Materials and Methods

Location

The Cayman Islands are sites of great scientific interest due to their historical isolation yet present-day ease of access. Cayman Brac (19°43’N, 79°47’W) has been chosen for this study due to its relatively low levels of urbanization (in comparison to Grand Cayman) and numerous cave systems, the majority of which remain relatively untouched, unexplored and unexcavated. This study focuses on Green Cave (19°43’9” N, 79°44’4” W), chosen because of its five large, distinct chambers and the relatively large quantity of sub-fossil remains contained within each. The cliff entrance to Green Cave is on the south face of the limestone plateau termed ‘The Bluff’, 22 m (+/- 1–5 m) above sea level, which connects to a series of passages and chambers that ends in a large chamber that opens to the top of The Bluff. The chambers do not exceed 1.5 m in height and the whole cave system developed through solutional widening of joint systems

within a single bedding plane. Chamber 1 is positioned closest to the cave entrance on The Bluff; chamber 5 extends northerly, towards the center of the island. The cave system was mapped in 3D using a portable LiDAR scanner (Z&F Imager 5006i), and converted to plan view (Figure 2).

Figure 2: Map of Green Cave (Cayman Brac) depicting each of the five chambers and site of a 60 x 75 cm excavation (*).

Sample Collection

A total of 485 bone samples collected from Green Cave were examined in this study (Supplementary Table S2). Bone material was collected from the cave floor surface sediments of each of the five chambers. These sediments (Table 1) consist of partially cemented fine-grained colluvial deposits containing rounded limestone clasts, shell fragments and bones. The limestone clasts are most likely rounded from chemical weathering that is typical of sediments formed on karstified limestones in Mediterranean and tropical climates (Wright and Wilson, 1987). The amount of sample material collected was dependent upon two factors: (i) relative chamber size, and (ii) accessibility of the chambers (i.e. where roof height restricted collection). Additionally, a small pit was excavated against the eastern wall of chamber 5, the northernmost chamber (see Figure 2), between two prominent stalagmite buttresses. The pit measured approximately 60 cm E–W by 75 cm N–S and was hand-excavated down to the limestone cave floor, 35 cm below the current cave floor. Remains were mapped and removed from each depositional context for analysis (Table 1).

Table 1: Summary of depositional contexts representing natural stratigraphic layers of the pit excavated in chamber 5, Green Cave (Cayman Brac).

Context	Depth (cm)	Description
0	0–5	Unconsolidated surface deposits consisting of fine silts with rounded limestone clasts and fragments of mollusc and crustacean shells. Skeletal remains collected from loose surface sediments
1	5–15	Solid stalagmite floor (no skeletal collection possible)
2	15–20	Cemented stone and shell deposits
3	20–25	<i>Terra rossa</i> sediments with root penetration
4	25–35	Shelly breccia
–	>35	Limestone floor to cave (solid geology)

ZooMS Collagen Fingerprinting

Chemicals were procured according to Buckley *et al.* (2014) and collagen (I) peptide mass fingerprints (PMFs) were obtained using methods modified from Buckley *et al.* (2016) whereby 485 sub-fossil bone fragments from Green Cave (a selection comprising examples from each chamber and context) were demineralized in 1 mL 0.6 M hydrochloric acid (HCl) at 4 °C for 18 h to extract soluble collagen (I). Where heavy

geological matrix encased bone samples, 1 mL 0.6 M HCl was added for several minutes until the calcareous matrix finished reacting. The acid was then replaced with 1 mL fresh 0.6 M HCl, and samples were treated as above. Larger bone samples were drilled to remove ~25 mg of matrix-free bone powder. Following demineralization, the supernatant was ultrafiltered at 3700 rpm for 30 min using 96-well 30 kDa molecular weight cut-off (MWCO) plates, washed with 0.8 mL 50 mM ammonium bicarbonate (ABC), re-suspended in 100 μ L 50 mM ABC and digested with 0.3 μ g sequencing-grade trypsin for 18 h at 37 °C. Digested samples were diluted into 10 mg/mL α -cyano-4-hydroxycinnamic acid matrix solution and 2 μ L spotted onto a 384-well Bruker Ultraflex target plate and air-dried following Buckley *et al.* (2016). A Bruker Ultraflex II MALDI mass spectrometer (MS) was utilized at up to 5000 laser shots per plate spot over a m/z range of 700–3700 and against five calibrant peptides. Resultant PMFs were analyzed using mMass software (v5.5.0). Where PMFs from the soluble collagen did not denote a clear faunal identification, the acid-insoluble pellets that remained following demineralization were then treated with 300 μ L 50mM ABC and gelatinized at 65 °C for 18 h to extract insoluble collagen (I) before being analyzed as above and fractionated following Buckley *et al.* (2009).

Reference specimens (*Nesophontes major*, *Capromys pilorides*, *Geocapromys brownii*, *Dasyprocta punctata*, *Macrotus waterhousii*, *Erophylla sezekorni*, *Eptesicus fuscus*, *Tadarida brasiliensis*, *Agalychnis callidryas*, *Bufo viridis*, *Larus canus* and *Cyclura* sp.; see Supplementary Table S3) were demineralized in 1 mL 0.6 M HCl at 4 °C for 6 h, briefly centrifuged, and the supernatant applied to singular 10 kDa MWCO ultrafilter units. Samples were washed twice (0.5 mL) and collected (100 μ L) with 50 mM ABC prior to digestion with 0.4 μ g trypsin at 37 °C for 18 h. Reference samples were then purified and fractionated via C18 solid phase extraction pipette tips following methods detailed in Buckley *et al.* (2009) whereby samples were acidified to 0.1% trifluoroacetic acid (TFA), washed twice on the C18 pipette tip column with 100 μ L 0.1% TFA and transferred to a stepped gradient of 10% and 50% acetonitrile (ACN) in 0.1% TFA concentrations to elute the peptides. Fractions were dried and re-suspended in 10 μ L 0.1% TFA before 1 μ L of each fraction was applied to the target plate and analyzed using MS as above.

LC-MS/MS

Liquid chromatography tandem mass spectrometry (LC-MS/MS; Waters nanoAcquity UPLC system coupled to a Thermo Scientific Orbitrap Elite mass spectrometer) was carried out on reference samples through methodology modified from Buckley *et al.* (2015), whereby 10% and 50% fractions were combined, dried and re-suspended in 5% ACN + 0.1% formic acid (FA). Samples were then concentrated on a pre-column (20 mm x 180 μ m) and separated on a 1.7 μ m Waters nanoAcquity Ethylene Bridged Hybrid (BEH) C18 analytical column of 75 mm x 250 μ m i.d., using a gradient starting from 99% buffer A/1% buffer B and finishing on 75% buffer A/25% buffer B in 45 min at 200 nL min⁻¹, where buffer A = 0.1% FA in H₂O and buffer B = 0.1% FA in ACN. Resultant MS/MS

data files were initially searched against publically available protein sequence database, SWISS-PROT, to ensure all samples predominantly contained the collagen (I) protein. MS/MS data files were then subjected to standard searches of all significant protein families using MASCOT (v2.5.1; Cottrell and London, 1999) against a custom (local) database of collagen (I) sequences created through manual collagen (I) sequence acquisition of relevant species from 'Protein BLAST' (Basic Local Alignment Search Tool; BLAST/P) (see Supplementary Table S4 for species list). Standard error tolerant searches, detailed in Buckley *et al.* (2015), were undertaken to highlight any potential sequence variations between the MS/MS data and the collagen (I) sequences in the custom database. Proposed homologous species-specific peptide sequences for each reference specimen were manually examined for quality based upon assigned ion scores and the assessment of fragment ion spectra. Standard MASCOT 'decoy' searches defined in Buckley *et al.* (2015) were then employed to further interrogate ion fragmentation matches. False positive peptide matches were assessed and assigned ion scores and matched peptides were then filtered to remove peptides with an ion score lower than the highest score for any false positive match. Fragment ion spectra were checked against the relevant PMF and where a peak was visible ($M+H^+$ m/z), the peptide and sequence was recorded as a biomarker for this species. Collagen (I) biomarkers were labeled following the system outlined in Buckley *et al.* (2009) whereby unique coding refers a peptide to a specific section in the collagen (I) sequence. For example, peptide COL1A1T47 represents collagen (I) $\alpha 1$ tryptic peptide number 47 (see Buckley, 2016 for complete sets described for cow, sheep and pig).

Results

Collagen (I) Peptide Biomarkers

Of the 485 sub-fossil bone samples selected for ZooMS analysis from Green Cave (Cayman Brac), a total of 260 (54%) generated collagen (I) peptide mass fingerprints (PMFs) that were suitable for biomolecular identification—a reasonable result considering the tropical climate of the deposition locality. The remainder of the samples produced 'failed' fingerprints, characterized by <10 peaks above m/z 1500 following a signal-to-noise (S/N) filter of 5.0, following Harvey *et al.* (2016).

Almost all samples in this study can be separated initially into vertebrate class (Mammalia, Amphibia, or Reptilia [birds and reptiles]) using a single peptide biomarker, COL1A1T47. This biomarker is known to be present at m/z 1105 in the PMFs of terrestrial mammals (amino acid sequence GVQGPPGPAGPR) (Buckley *et al.*, 2014) and its homologous peptide is observed at m/z 1108 or m/z 1134 in the modern amphibians tested (sequences GAQGPPGPQGAR and GAQGPPGPQGPR respectively) and at m/z 1162 in the majority of reptiles and birds of taxonomic relevance to this study (sequence GVQGPPGPQGPR; Supplementary Figures S1–3). Unexpectedly in iguanids, both the reference sample of the ground iguana *Cyclura* sp. and Sample 71 (suspected iguanid)

display the COL1A1T47 biomarker at m/z 1105—identical in mass to terrestrial mammals (Supplementary Figure S2). LC-MS/MS data confirms the peptide sequence for iguanids is GVQGPAGPPGPR, which is isobaric to the terrestrial mammal version of the biomarker, but not identical in sequence. Another prominent PMF biomarker common to both *Cyclura* sp. and Sample 71 (the iguanids) can be viewed at m/z 1393, representing peptide COL1A1T28 and sequence GEPGPAGLPGPAGER. This peptide is also common to the avian reference taxon from Reptilia, *Larus canus* (common gull), and we conclude this as a viable marker with which to separate the Reptilia (including iguanids) from Mammalia in this study, although it is worthy of noting that outside of this study area not all Reptilia share the same peptide sequence for COL1A1T28, and hence can have different masses (e.g. *Python bivittatus*, Supplementary Table S4.7).

Through the process of PMF examination coupled with LC-MS/MS data analysis we propose that 17 collagen (I) peptide biomarkers are the minimum required to distinguish all vertebrate fauna on Cayman Brac, which includes the refinement of all terrestrial mammals to species-level (Figure 3). (See also Supplementary Table S4 and Supplementary Figures S5–S18 for details of the peptide sequences for these biomarkers.)

Figure 3: Flow chart depicting the minimum collagen (I) peptide biomarkers required for the identification of all ancient bone samples in this study. Note: outside of this study these biomarker pathways must be used with caution (see text). *Species-specific biomarkers from Buckley *et al.* (2016). NB: the COL1A2T43 biomarker for iguanids remains unidentified from the PMF and LC-MS/MS data due to a lack of published sequence information and thus is not included.

All extinct and extant terrestrial mammals (non-marine, non-volant) on Cayman Brac can be refined to species level assuming the minimum biomarkers required for this task are visible in the PMF. Additionally, the chiropterans (volant taxa) can be confidently separated out at order level. However, it should also be noted that other vertebrate fauna present outside of the Cayman Islands might have the same marker pathways as those used here. For example, we use m/z 1105 + 1453 + 2705 as a set of markers specific to all chiropterans in this study but are well aware that they are observed in fauna found outside of the Cayman Islands (e.g., some carnivores as well as pig; Buckley *et al.*, 2009; Buckley, 2016). Additionally, in this assemblage all samples identified as Reptilia, with the exception of *Cyclura* sp., display a peak at m/z 1162, although this peptide is highly variable in sequence on a global level and is not present at m/z 1162 in all birds and reptile PMFs (see Supplementary Table S4.2).

Faunal Identifications

Biomarker acquisition permitted the identification of Cayman Brac bone samples down to the following lowest-level designations: species ($n=159$), genus ($n=28$), family ($n=10$), order ($n=1$) and class ($n=62$) (Figure 4). Therefore, the majority (61%) of successfully fingerprinted sub-fossil bones are identifiable to species level. Of these, 198 are mammals (65 *Capromys* sp.; 132 Muridae; 1 Chiroptera), 40 samples are Amphibia and 22 are Reptilia (reptiles/birds). There appear to be no occurrences of the introduced Central American red agouti, *Dasyprocta punctata*, nor the endemic and extinct hutia, *Geocapromys* sp., or basal eutherian, *Nesophontes* sp., in the assemblage. There are also no specific identifications of the brown rat (*R. norvegicus*) or the house mouse (*M. musculus*) from the Green Cave sub-fossil remains, though their presence within the ten samples identified only to 'Muridae', cannot be ruled out. This is due to an absence of species-specific biomarkers in the PMFs, most likely as a consequence of poorer collagen integrity in these samples.

Figure 4: Chart showing faunal composition of the ancient vertebrate remains in Green Cave as established through ZooMS analysis.

Species-Specific Biomarkers

Capromyid rodents, *Geocapromys brownii* and *Capromys pilorides* (reference species), differ subtly in their collagen fingerprints. Though most peaks appear to match in the PMFs of the two species, a single homologous biomarker, COL1A2T54, identified here at m/z 1235 (GEAGAAG**P**AGPAGPR) and m/z 1239 (GEAGAAG**T**AGPAGPR) in *G. brownii* and *C. pilorides* respectively, can be used to separate these two closely related species (Figure 5). This mass shift of +4 Da has occurred through the amino acid substitution of proline (P) to threonine (T)—both amino acids that are common in collagen (I) and a substitution that is frequently observed (e.g. Buckley *et al.*, 2009). When comparing the reference PMF for *C. pilorides* to the PMFs of the identified capromyid rodents from Cayman Brac, a peak at m/z 1239 is visible in all 65 of the ancient samples. Following the in-depth work of Morgan (1994a) only two species of hutia are known from Cayman Brac: *Capromys* sp. which is thought to be derived from *C. pilorides*, and *Geocapromys* sp. which is thought to be derived from a Cuban form. We therefore conclude the identity of the 65 ancient samples as the extinct, endemic and undescribed Cayman Island *Capromys* sp.

Figure 5: MALDI-MS peptide mass fingerprints showing 10% ACN fractionations of collagen (I) extracted from reference samples of *C. pilorides* (top) and *G. brownii* (bottom) following tryptic digest. Inset: Species-specific homologous biomarker at m/z 1239 in *C. pilorides* and m/z 1235 in *G. brownii*.

Faunal Composition in Green Cave

The majority of specimens from the Green Cave sub-fossil assemblage are murid rodents (introduced; extant) and capromyid rodents (endemic; extinct), with these two families dominating differing chambers of Green Cave. This shift in faunal composition can be observed as the cave system is followed from the entrance to the deepest chamber, chamber 5. At the cave entrance and within chamber 1 murid rodent remains are dominant, with deeper chambers becoming more concentrated in the remains of *Capromys* sp. (Figure 6). Therefore, the entrance chamber and chamber 1 are composed primarily of species that are currently introduced and extant on the island, whereas deeper chambers (chambers 2–5) mainly host remains of extinct capromyid rodents. The one exception to this rule is chamber 3, where a relatively large proportion of remains are identified as Reptilia and less than half are capromyid rodents. Additionally, the proportion of positive compared to failed PMFs varies within each specific cave locality (Supplementary Figure S4). The majority of samples in the entrance chamber and chambers 1–4 were successful in producing PMFs, whereas samples excavated from chamber 5, including each excavated stratigraphic context (see Table 1), were mostly unsuccessful. All of the samples in the deepest contexts of chamber 5 (context 3, 20–25 cm; and context 4, 25–35 cm) failed to produce PMFs and these trends were also recognized in pilot analyses which included the same cave system by Harvey *et al.* (2016).

Figure 6: Faunal composition within each distinct region of Green Cave. Percentages expressed as a proportion of the total identifications for each chamber (declared in brackets). Contexts 3 and 4 of chamber 5 are omitted as there are no identifications in these regions due to poor PMFs.

Discussion

Due to their isolated nature, islands serve as unique ‘model systems’ for both ecological and evolutionary study (Gillespie *et al.*, 2008; Fitzpatrick and Erlandson, 2018), and this very quality renders them susceptible to endemic species loss. Our approach samples both surficial and sub-surface deposits from Green Cave, Cayman Brac, to constrain temporal changes in the fossil assemblage and also maps the samples spatially within the cave system to resolve any environmental and taphonomic controls on deposition and preservation respectively. The method sub-samples the entire bone assemblage from specific cave locations, rather than focusing on portions of the assemblage that may only be identified using morphological characters. This holistic approach makes use

of all samples collected, as opposed to a fraction of the potential in the absence of morphological characteristics and/or morphology experts.

Faunal Identifications

Collagen (I) analyses of faunal assemblages from the Caribbean have not been undertaken previously until this current study. The ZooMS mass spectrometric approach allows visual discrimination between collagen peptides mass fingerprints (PMFs) to aid in zooarchaeological identification and the technique is demonstrated here as effective in achieving faunal identity in bone remains from Green Cave, Cayman Brac (Figure 3; Supplementary Figures S1–3). Successful PMF interrogation has enabled the categorization of all collagen-containing fragmented, degraded and/or matrix-encased bone samples into a definitive order, family, genera or species (Figure 4).

Mammalia

Collagen fingerprints of all Cayman terrestrial mammal remains can be identified to species level through the determination of a set of biomarkers defining each specific taxon (Figure 3). This ‘biomarker database’ allows the cataloguing of all terrestrial mammal species on Cayman Brac, which offers key ecological insights considering terrestrial mammals alone contribute to 76% of the fingerprinted Green Cave assemblage. Refining identification to species level classification is possible in closely related hutia species, *C. pilorides* and *G. brownii* (Figure 5). Here, the presence of at least one amino acid substitution within the ~2,000 residue sequence of collagen (I) is not altogether unexpected given that the two taxa diverged in the Late Miocene (~4.2 Ma) (Fabre *et al.*, 2014). However, it is not always the case that mammalian species can be separated through collagen (I) analysis following such divergence timeframes. For example, harp seals and grey seals (both of the family Phocidae) diverged approximately 6–7 Ma, (Flynn *et al.*, 2005), yet have collagen PMFs that cannot be readily differentiated (Buckley and Collins, 2011); whereas *R. rattus* and *R. norvegicus* are estimated to have diverged just ~2 Ma (Verneau *et al.*, 1998), and whose sequences contain at least one amino acid substitution (Buckley *et al.*, 2016). Although viable estimations can be made with regards to divergence timeframes versus the number of evolution-induced collagen (I) sequence variations, these exceptions to the rule suggest that either one of these factors cannot necessarily be accurately determined by the other. Here, other aspects of biology, such as generation time, may come into play when estimating the frequency of residue substitutions through time in collagen (I).

Chiroptera

A total of eight bat species are known from Cayman Brac, although only four inhabit the island today. All other species have been extirpated from the island including the region’s only endemic form, an undescribed sub-species of *Eptesicus fuscus* (*E. fuscus* sp.

nov.; Family: Vespertilionidae), now extirpated from Cayman Brac though remains present on Grand Cayman. Despite bat skeletal remains making up almost 3% (31 of 1096) of a sub-fossil assemblage during a previous study of a cave system on Cayman Brac (Morgan, 1994b), only a single bat, Sample 174, was identified from Green Cave and whose PMF did not match either of the two Cayman Brac reference chiropterans tested, *Macrotus waterhousii* and *Erophylla sezekorni* (Family: Phyllostomidae). Indeed, all the peptide biomarkers identified in Sample 174 matched the published collagen (I) sequence for *E. fuscus* (BLAST/P). Sample 174 also shares more identical biomarker sequences with the globally widespread genus *Myotis* sp., also of the Vespertilionidae family, than it does to either of the two Cayman Brac phyllostomids (LC-MS/MS data; see Supplementary Table S4), supporting its identification as vespertilionid. In order to test this hypothesis, bone samples of *E. fuscus* and also of *Tadarida brasiliensis* (Family: Molossidae, the third bat family present on Cayman Brac) were acquired for collagen fingerprinting (Supplementary Table S3). The PMF of Sample 174 matches that of reference sample *E. fuscus* (Figure 7), whereas at least two biomarkers can be seen to differ between *E. fuscus* and *T. brasiliensis* (Supplementary Table S4). We therefore conclude that Sample 174 is a specimen of the extirpated vespertilionid bat, *E. fuscus* sp. *nov.* As well as highlighting an excellent application of ZooMS, this result provides evidence that Green Cave was a likely roosting location for *E. fuscus* sp. *nov.* prior to the species' demise on the island. Such knowledge of this local extinction could be used in arguments for the reintroduction of this species; such has been the case with European beaver (*Castor fiber*) in Britain in the 16th century (Raye, 2015). Due to the extirpation of half of all the bat species known to Cayman Brac, ZooMS faunal screening for bat remains may be effective in the support of future initiatives towards ecological restoration on this island.

Figure 7: MALDI-MS peptide mass fingerprints showing 10% ACN fractions of collagen (I) extracted from ancient bone fragment, Sample 174, *Eptesicus fuscus sp. nov* (top), and a modern reference sample of *E. fuscus* (bottom) following tryptic digest. Visible biomarkers are labeled in each case (see also Supplementary Table S4).

Amphibia

The collagen (I) PMF from the amphibian reference sample, *Bufo viridis* (European green toad) shows a clear peak at m/z 1134 (Supplementary Figure S3), representing biomarker COL1A1T47 and sequence **GA**QGP**PGP****Q**GPR as derived by LC-MS/MS. Where the COL1A1T47 biomarker is highly conserved at m/z 1105 (**GV**QGP**PGP****A**GPR) in all known terrestrial mammals (Buckley *et al.*, 2014), this peptide biomarker appears to be less conserved in amphibians. Only two species of amphibian are native to Cayman Brac, *Eleutherodactylus planirostris* (greenhouse frog, Leptodactylidae) and *Osteopilus septentrionalis* (Cuban tree frog, Hylidae). The reference species of tree frog analyzed, *Agalychnis callidryas* (Family: Hylidae), displays the homologous peptide peak at m/z 1108 (Supplementary Figure S3), representing sequence **GA**QGP**PGP****Q****G**AR and highlighting a further amino acid substitution in this species. All 40 samples identified as Amphibia from the Green Cave assemblage have identical PMFs, with the COL1A1T47 biomarker visible at m/z 1134. Therefore these samples are not likely to represent the hylid tree frog, *O. septentrionalis*, and are more likely to be *E. planirostris* (Anura: Leptodactylidae). The possibility that the Green Cave amphibian remains are from the introduced cane toad *Rhinella marina* (Anura: Bufonidae), though unlikely, cannot be ruled out at this stage as this bufonid frog species would be expected to have a

biomarker at m/z 1134 as with *B. viridis*. Where *R. marina* has become well established on Grand Cayman in recent times (Edgar, 2010), it has been rarely recorded on Cayman Brac (Echternacht *et al.*, 2011), though as yet its full distribution has not been documented. Ruling out the presence of highly invasive *R. marina* in this cave system would be an important ecological clarification for future studies of this ilk. Overall, this lower level of sequence specificity for a key collagen (I) biomarker that is otherwise highly conserved in the mammals means that the potential for taxonomic refinement to species-level in both species of Amphibia on Cayman Brac is high, and indeed for many amphibian species beyond the confines of this island.

Reptilia

Identification of the Reptilia requires care due to the sequence variant for collagen (I) biomarker peptide COL1A1T47 (GVQGPAGPAGPR) in iguanids (*Cyclura* sp. and Sample 71), which is isobaric to the homologous peptide biomarker in mammals (GVQGPAGPAGPR). There are two iguanid species on Cayman Brac, the endemic and critically endangered *Cyclura nubila caymanensis* and the introduced *Iguana iguana*. Understanding which of these two species Sample 71 represents would require the acquisition of reference bone samples from each of these two species, and the result may change what we know about the ecology and distribution of these species on Cayman Brac so could be a valuable analysis for a future study. With this one exception, all other samples of Reptilia (birds/reptiles) from Green Cave display the COL1A1T47 biomarker at m/z 1162. This single biomarker is useful in separating the Reptilia from other vertebrate clades in this assemblage, but cannot alone be used to distinguish between birds and non-avian reptiles (with the exception of iguanids). Reptilia specimens from Green Cave exhibit some, though not all, of the avian biomarkers suggested by Buckley *et al.* (2009), though some of these biomarkers are also expressed in Sample 71, a definitive reptile. It is therefore likely that some of these previously suggested avian biomarkers are also common to many reptiles and further work is required to separate this complex vertebrate order using collagen (I) analysis.

Cayman Brac hosts a diverse repertoire of birds and reptiles, with a total of 32 and 23 species respectively (Seidel and Franz, 1994; Bradley and Rey-Millet, 2013). Acquiring reference samples of each would be an extensive task (particularly as two avian species are globally extinct; Morgan, 1994a) and perhaps would not be the most efficient solution to deriving biomarkers to separate the Cayman birds and reptiles. Here, collagen sequence analysis on Cayman Brac samples, matched to a greater variety of easily accessible modern reference specimens than the scope of this study permits, could be useful in separating the birds from the reptiles, and perhaps also for refinement into family, genus and/or species identity. When such biomarkers are in place, a more detailed understanding of Reptilia composition in the cave system could be achieved, and any ecological causes and effects better understood.

It is important to note that proposed biomarkers are not necessarily present as the tallest (i.e. most abundant) peaks in a PMF as the relative abundance of a peptide relates to its chemistry [as defined by its amino acid sequence], and consequently how it behaves following ionisation during MALDI-MS. Where collagen (I) concentrations in a bone are reduced due to degradation—as is common in older samples—PMFs are typically poorer, with fewer peaks, a reduction in biomarker visibility and peaks present at lower relative abundance, thereby limiting biomarker resolution. The speed of collagen (I) degradation through time is also related to whole bone surface area, with smaller bone elements from microvertebrates being more vulnerable to faster degradation than larger bones (Collins *et al.*, 2002). Additionally, chemical and/or biological degradation of collagen (I) through time is driven by collagen molecule hydrolysis, reducing the probability of high molecular weight peptides survival over shorter length peptides, as modeled by Collins *et al.* (1995).

Faunal Composition

Noticeable differences can be seen in the spatial distribution of taxa within the Green Cave system (Figure 6). Deeper cave chambers (4–5) are comprised primarily of the large, extinct capromyid *Capromys* sp. whereas chambers nearer the cave entrance host a majority of smaller extant taxa (murid rodents, amphibians and birds/reptiles). There are two explanations to such an arrangement. Firstly, smaller animals are more likely to have been deposited into the cave system through barn owl pellet regurgitation rather than cave habitation (Morgan, 1994a), and as such, barn owls are known to roost on ledges, tucked into the entrance chambers of caves as opposed to deep inside cave systems (Andrews, 1990; Madsen, 2000). A second explanation pertains to the relative age of the bones in the cave systems on Cayman Brac, whereby remains deposited in deeper chambers are typically older than those of entrance chambers. This tendency is assumed here due to the proportions of successful PMFs in each cave region (Supplementary Figure S4), and is also supported by radiocarbon dates achieved in a previous study by Harvey *et al.* (2016) on Green Cave faunal remains, reporting dates of 609 ± 26 radiocarbon years before present (yr BP) (chamber 2), 928 ± 26 yr BP (chamber 3), 1588 ± 26 yr BP (chamber 4) and 1166 ± 34 yr BP (chamber 5). Therefore, the distribution of fauna across the cave system displayed using the criterion of collagen (I) survival alone is perhaps an artifact of preservation bias—a trend not unknown from the literature (Andrews, 1990; Lam and Pearson, 2005). Ultimately, it is probable that larger bone remains in Cayman Brac, such as that of capromyid rodents, will have recoverable collagen (I) for longer than the bones of smaller species of the same age simply as a consequence of their greater surface to volume ratio (Collins *et al.*, 2002; Buckley and Collins, 2011). Of course, frequent bioturbation caused by murid rodents and Caribbean land crabs (Family: Gecarcinidae) in the cave system ensures deeper chambers do not definitively contain older samples than entrance caves, which is likely to explain why murid rodents, reptiles/birds and the solitary bat specimen have been recovered from chamber 5.

Green Cave, although one of the larger cave systems on Cayman Brac, may not be the preferred habitat for some species on the island, both past and present. This study has not uncovered any specimens of the extinct eutherian, *Nesophontes* sp., nor has it found any examples of the introduced rodents, *R. norvegicus*, *M. musculus* and *D. punctata*. In the case of the former, Morgan (1994a) found remains of *Nesophontes* in only three caves on Cayman Brac from a total of eight that were excavated; all three of which are located on the north-east coast of the island. Perhaps these species did not inhabit Green Cave, or perhaps they were not present in the hunting range of ancient *Tyto alba*.

The Central American red agouti, *D. punctata*, is not normally recognized for its cave-dwelling behavior though has been found in cave fossil assemblages in Brazil where it has likely been deposited through the act of large predators or scavengers (Victor *et al.*, 2013). With the absence of such predatory species on the Cayman Islands, it is to be expected that *D. punctata* remains would not commonly be found in Cayman cave assemblages, nor were they uncovered in the thorough searches achieved by Morgan (1994a) on both Cayman Brac and Grand Cayman. To date, this species remains unreported on Cayman Brac, and therefore its absence in the assemblage was expected. Due to similar trophic levels, it has been speculated that *D. punctata* would compete with hutia species, *Capromys*, directly for food and space (Borroto-Páez and Woods, 2012), yet the loss of *Capromys* and *Geocapromys* species on Cayman Brac in the likely absence of *D. punctata* requires a separate hypothesis. Elsewhere in the Caribbean, introduced dogs have been known to attack both the Cuban Demarest's hutia (*C. pilorides*) and the Bahamian hutia (*G. ingrahami*), evidenced by observed fur remains in excreta (Borroto-Páez and Woods, 2012). Incidentally, dogs have also been blamed for population reduction of the endemic iguanids *Cyclura nubila caymanensis* (Lewis, 1940), and *C. nubila lewisi* (Morgan, 1994a), both of which remain critically endangered today (IUCN, 2018). However, remains of canids on the Cayman Islands have neither been recovered from this study nor that of Morgan (1994a), which suggests that the extinction of the Cayman Island hutia species is likely linked to the introduction of invasive murid rodents. The presence of *Rattus rattus* in the assemblage alongside the absence of the other two prevalent murid rodents on Cayman Brac, *R. norvegicus* and *M. musculus*, was not an anticipated result. Interestingly, it has been observed on islands in New Zealand that *M. musculus* directly competes with the two *Rattus* sp. for food and space, with the former being subordinate to the rat species (Yom-Tov *et al.*, 1999). The high percentage of *Rattus* sp. in the assemblage therefore explains the lack of *M. musculus*. However, the absence of *R. norvegicus* in the presence of *R. rattus* remains unusual. Research by Moors (1990) shows that *R. rattus* is statistically more likely to inhabit an island when that locality lacks *R. norvegicus*, suggesting competition between the two species that favors *R. norvegicus*. In all cases, it seems the ecology behind murid rodent distribution on Cayman Brac cannot yet be clarified but could be crucial in the understanding of how biodiversity has changed since their introduction to these islands.

Collagen Analysis: The Wider Context

The sub-fossil record on the Cayman Islands displays a faunal legacy that traces biodiversity through time. The cave systems provide molecular sanctuaries, preserving vertebrate bone remains beyond the period of human occupation on these islands. Currently, these proteomic tools remain underexplored despite their proven usefulness in both terrestrial (e.g. Buckley *et al.*, 2017) and marine (e.g. Harvey *et al.*, 2018) realms. Our findings here demonstrate collagen analysis as valuable in the reconstruction of paleofaunal assemblage data and in the understanding of how ecosystems have changed as a function of anthropogenic influences. Importantly, this case study presents collagen biomarkers and species-level identifications for a suite of vertebrate families never before studied through collagen fingerprinting, demonstrating the versatility of this technique. This study also provides a comprehensive collagen (I) biomarker 'catalogue' for the identification of terrestrial fauna from Cayman Brac, refining taxonomic identification to species level in terrestrial mammals. Such biomarkers are directly applicable for species identification across the Cayman Islands, into other areas of the Caribbean and beyond. Finally, collagen analysis can harness a wealth of information from sub-fossil assemblages, including the extraction of faunal identity that can be constrained within a temporal framework (Harvey *et al.*, 2016), the applications of which are extensive and wholly relevant to similar threatened environments worldwide. At present, the main limitation of collagen (I) sequencing by soft-ionisation mass spectrometry is the lack of reference collagen (I) PMFs and sequences, a concern also highlighted by Buckley (2013). Overcoming this challenge requires the expansion of publically available protein sequence databases, such as BLAST/P, which in turn depends upon a wider adoption of collagen-based analyses across interdisciplinary fields of science. Despite this hindrance, collagen analysis provides ample opportunities to gain new perspectives on past human impacts to assist in the direct protection of our future. Work in this area is critically important as we begin to formally recognize a departure from the Holocene epoch, into the Anthropocene (e.g. Smith and Zeder, 2013; Dirzo *et al.*, 2014; Waters *et al.*, 2016).

Conclusions

Current global conservation strategies largely ignore data provided by paleontological and sub-fossil records, despite the fact that they supply an invaluable long-term perspective on ecosystem alterations, such as foreign species invasions. The Caribbean island of Cayman Brac is a remarkable example of an island model system with an array of cave systems that have collected faunal remains through time. The island has been subjected to a multitude of naturally occurring experimental controls (geographical isolation; lack of prehistoric occupation), and a combination of these factors has created a unique research opportunity to improve our understanding of island palaeobiodiversity spanning the boundary of human occupation of the region. The assessment of the sub-fossil assemblage in Green Cave (Cayman Brac) has found the

majority of vertebrate remains to be introduced and invasive murid rodents, far outnumbering the remains of any other endemic or native species, either extinct or extant. In view of the recent anthropogenically-caused extreme loss of biodiversity on the Cayman Islands, this study showcases the use of a molecular surveying technique to uncover new information on species presence, composition and diversity. A knowledge of past trends diagnosed from fossil assemblages could be critical in the management of environments that are facing similar threats posed by invasive species.

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Declaration of conflicting interests

The authors declare that they have no competing interests.

Author Contributions

Conceived and designed the experiments: VLH, PLM and MB. Performed the experiments: VLH and MB. Analyzed the data: VLH and MB. VLH wrote the manuscript and designed figures (with the exception of Figure 2 produced by ATC and WIS), with author contributions from MB, VME, PLM, ATC, and WIS. Collected the samples and scanned the cave system: MB, VME, PLM, ATC and WIS.

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