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STABLE ISOTOPE ANALYSES OF AFRICAN GREY PARROTS:

A FORENSIC ISOTOPE APPROACH

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437594

A Dissertation submitted to the Faculty of Science, University of the Witwatersrand, in fulfilment of the requirements for the degree of Master of Science

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Declaration

I declare that this dissertation is my own unaided work, and was conducted under the supervision of Prof. Craig T. Symes, Prof. Colleen T. Downs and Dr Stephan M. Woodborne. Submission is for the degree of Master of Science at the University of the Witwatersrand, Johannesburg. The work has not been submitted before for any degree or examination at any other university.

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Abstract

Stable isotope analyses have been used to infer diets of organisms, define trophic partitioning, and infer geographic origins of species. It has further been applied to forensic ecology to infer the origins of deceased humans and illegally traded animal (elephant ivory and rhinoceros horn) and plant (cycads, coca, and cannabis) material. However, no research has focused on the isotope analysis of avian material in forensic ecology. African grey parrots *Psittacus erithacus* are one of the most traded species in the world, with the trade often being illegal, and the origins of confiscated or deceased specimens being unknown. The aim of this study was to determine if stable isotopes (δ^{13}C, δ^{15}N, and δ^{2}H) in African grey parrot feathers could be used to determine the wild or captive origins of birds. African grey parrot feathers (primary, body, and tail) differed isotopically so standardising isotope values of African grey parrot feathers to a single feather type was recommended, to maintain consistent sampling and allow for comparisons to be drawn between different feather types. African grey parrot feathers from unknown origins can be identified as wild or captive using δ^{13}C and δ^{2}H values, but not δ^{15}N values. Known wild and captive feathers possibly differ isotopically from one another because of dietary and location differences. Wild African grey parrots inhabit and feed in isotopically depleted C3 forests compared to captive African grey parrots which are usually fed C4 based foods with more positive isotope values. Wild African grey parrot δ^{2}H isotope values were the most negative in the central region of their native distribution. The ability to differentiate wild from captive African grey parrots, as well as infer basic origins (East from West Africa) may improve the monitoring of the illegal trade as well as help in tracing illegally traded parrots.

For Shane, Tracy, Trent, and Layla (The family)

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Thank you to my supervisor Craig Symes for the opportunity to conduct such interesting research for my M.Sc. and for the continual guidance. Hopefully this research will help in monitoring and tracking the illegal trade of African grey parrots. To my co-supervisors Stephan Woodborne, thank you for the input involving isotope analysis and the understanding of the science and Colleen Downs, thank you for the constructive input towards the work and for the access to the deceased parrots. To Mike Butler, thank you for helping with hydrogen isotope analysis. Many thanks goes out to those who helped by supplying feathers, both captive and museum specimens, with special thanks to Greg Davies from The Ditsong National Museum of Natural History (Pretoria) and Paul Sweet and Peter Capainolo from The American Museum of Natural History (New York).

Thank you to my family, for the undying support, motivation, funding, and the occasional push in the right direction. I hope you have learnt a bit about stable isotope analysis and ecology through all the reading and discussions. Without each of you this goal would have never been achievable and for this I will always be grateful. The opportunity that you have given me will no doubt benefit me for the rest of my life. However, it is not only the qualification that I can flaunt but the many lessons I have learnt along the way. To Layla, thank you for the continual company and for being such a patient soul.

Lastly, to all those working hard and fighting for the protection of these incredible creatures, your time and efforts will always make a difference. The illegal trade of African grey parrots and other avian species may never be stopped but certainly each improvement to monitoring and tracking the trade will make a difference.

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Preface

African grey parrot (*Psittacus erithacus* and *P. timneh*) populations are threatened by habitat loss, hunting, and are possibly one of the most heavily traded animal species. They are a desirable species of avifauna as a result of their extreme intelligence, mimicry, and suitability as pets. Many countries within their native distribution have placed bans or quotas on the trade of this species, but the illegal trade persists. Even within countries that are signatories to national and international trade agreements and regulations, corruption is still rife, making it difficult to monitor and track African grey parrot trade. The actual number of African grey parrots that are taken from the wild annually and the conditions that these parrots have to endure to reach their new locations are not entirely understood. It is thus beneficial for any improvements to be made to the current methods of monitoring and documenting the regions from where African grey parrots are traded. As well as suggesting the numbers of African grey parrots captured and traded. This research, focusing on the stable isotope analyses of African grey parrot feathers was intended to add a new forensic approach to the current methods of trade monitoring and control.

This dissertation comprises five chapters, a general introduction chapter, three data chapters, and a conclusion chapter. Chapter 1 focuses on an introduction of the study species, African grey parrots, with a brief explanation of the taxonomy, distribution, ecology, and trade of this species. A detailed explanation of stable isotope analysis precedes the introduction to African grey parrots and how isotope analysis is used in ecological studies. Each data chapter (2–4) is structured with the intent to publish, containing its own methods, results, discussion, and reference sections, allowing it to be read independently. For this reason repetition is often unavoidable. The data chapters are co-authored papers by Jarryd Alexander, Craig T. Symes, Stephan M. Woodborne, Mike Butler and Colleen T. Downs. Chapter 5 concludes and summarises the three data chapters.

Chapter 1: General introduction

"He didn't like to see animals in captivity. When he looked into their eyes, something in their eyes looked back at him." **- Rick Yancey**



Introduction

African grey parrots (*Psittacus erithacus* and *P. timneh*) are traded as a single taxonomic entity and are one of the most popular traded bird and animal species worldwide (Mulliken 1995; Melo and O'Ryan 2007; Tamungang and Cheke 2012; Tamungang *et al.* 2013). Their popularity in the international trade market is a result of their incredible intelligence, longevity, and ability to mimic human speech (Pepperberg 1981; Tamungang *et al.* 2013; Olah *et al*. 2016). This unrelenting and unregulated or illegal trade, as well as habitat loss, is causing notable declines in the global population (Juste 1995; Mulliken 1995; Fahlman 2002; Amuno *et al.* 2007; Melo and O'Ryan 2007; Tamungang and Cheke 2012; Tamungang *et al.* 2013; 2014; BirdLife International 2014a; 2014b; Annorbah *et al.* 2016; Olah *et al*. 2016).

Taxonomy

African grey parrots are endemic to Africa, being one of 24 parrot species occurring in the Afrotropical region (Perrin 2012). Two species of African grey parrot were formerly recognised; one in the Gulf of Guinea on Bioko, Príncipe, and Säo Tomé Islands (*Psittacus principes*) and one on mainland Africa (*Psittacus erithacus*) (Forshaw and Cooper 1989). The mainland species is comprised of two subspecies; the Red-tailed African grey parrot (*Psittacus erithacus erithacus*) and Maroon-tailed African grey parrot (*Psittacus erithacus timneh*) (Forshaw and Cooper 1989). However, recent studies have suggested that the mainland subspecies be recognised as two different species; the Congo African grey parrot (*Psittacus erithacus*) and the Timneh African grey parrot (*Psittacus timneh*) (Fig. 1) (Melo and O'Ryan 2007; Perrin 2012) and that the birds on Príncipe Island (*viz. P. principes*) comprise geographically isolated *P. erithacus* and *P. timneh*, most of which belong to the *P. timneh* lineage (Melo and O'Ryan 2007). The mainland species and the island populations are

all threatened by the international trade (Mulliken 1995; Melo and O'Ryan 2007; CITES 2014; BirdLife International 2014a; 2014b). *Psittacus erithacus* is generally larger and darker than *P. timneh*, with red tail feathers compared to the maroon tail fathers of *P. timneh* (Fig. 1) (Melo and O'Ryan 2007; BirdLife International 2014a; 2014b). The call of these species is also distinctly different (BirdLife International 2014b). The parrots from the Gulf of Guinea also show size variation from parrots in mainland Gabon, adjacent to the island of Príncipe (Melo and O'Ryan 2007). African grey parrots, like other psittacines undergo what is known as divergent primary moult; moult which does not start at the first primary feather, but rather at the central primaries P5 or P6. Moult thereafter commences first inwards from P5/P6 to P1 and later outwards from P6/P7 to P10 (Howell 2010; Mallet-Rodrigues 2012; Pyle 2013). This pattern of whole feathers and moulting feathers maintains flight (Mallet-Rodrigues 2012).



Figure 1: Congo African grey parrot (*Psittacus erithacus*) (Left) and Timneh African grey parrot (*Psittacus timneh*) (Right).

Distribution

The ranges of *Psittacus erithacus* and *P. timneh* overlap in eastern Côte d'Ivoire (Fig. 2) (BirdLife International 2014a; 2014b). The range of *P. erithacus* includes; south-east Côte d'Ivoire, southern Ghana, Togo, Benin, Nigeria, south-west Cameroon, southern Central African Republic, Equatorial Guinea, Gabon, Congo, northern Angola, Democratic Republic of the Congo, Rwanda, Burundi, western and central Uganda, northern Tanzania, and western Kenya (Fig. 2) (Mulliken 1995; Forshaw 2006; Eniang *et al.* 2008; Tamungang *et al.* 2013; BirdLife International 2014a), whilst *P. timneh* occurs in northern Guinea-Bissau, southern Mali, south-west Guinea, Sierra Leone, Liberia, and western and central Côte d'Ivoire (Fig. 2) (Tamungang *et al.* 2013; BirdLife International 2014b). The island inhabiting African grey parrot populations occur on the Bioko, Príncipe, and São Tomé Islands in the Gulf of Guinea (Melo and O'Ryan 2007; BirdLife International 2014a).

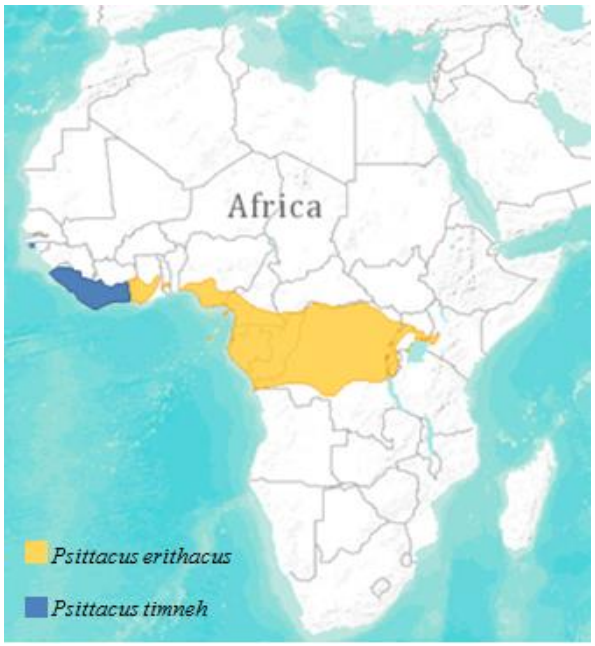


Figure 2: Distribution of *Psittacus erithacus* and *Psittacus timneh*, showing the region of overlap in Côte d'Ivoire (BirdLife 2014a; 2014b).

African grey parrots inhabit mostly lowland forest, but are also observed in highland forests, wooded savannas, mangrove swamps, marshes, and agricultural land; such as crop fields and cocoa (*Theobroma cacao*) and oil palm (*Elaeis guineensis*) plantations (Chapman *et al.* 1993; Juste 1995; May 2001; Tamungang and Ajayi 2003; Forshaw 2006; Amuno *et al.* 2007; Melo and O'Ryan 2007; Tamungang and Cheke 2012; Tamungang *et al.* 2013; 2014). However, African grey parrots prefer inhabiting lower altitude forests below 800 m (Forshaw 2006; Tamungang *et al.* 2014) and open vegetation areas for feeding (May 2001; Tamungang *et al.* 2014).

Ecology

Lowland and highland forests contain the dietary requirements for granivorous-frugivorous parrots (Chapman *et al.* 1993). African grey parrots favour seeds and nuts, but will also ingest fruit, flowers, and cambium (Chapman *et al.* 1993; Juste 1995; Tamungang and Ajayi 2003; Forshaw 2006, Amuno *et al.* 2007; Tamungang and Cheke 2012; Tamungang *et al.* 2013; 2014). Some of the plant species on which they feed are agricultural; such as oil palms, wheat (*Triticum* spp.), sugar cane (*Saccharum officinarum*), and sorghum (*Sorghum bicolor*) (Juste 1995; Forshaw 2006; Tamungang and Cheke 2012). African grey parrots have also been observed practicing geophagy (May 2001; Forshaw 2006; Tamungang and Cheke 2012; Tamungang *et al.* 2013). Parrots worldwide are recorded practicing geophagy and possibly do so in an attempt to obtain minerals and nutrients not readily available in a seed diet (Diamond *et al.* 1999).

Many of these food sources vary seasonally in Africa, such as African grape (*Psuedospondias microcarpa*) fruits (Chapman *et al.* 19993), generally coinciding with the wet season that moves along a north-south gradient (Tamungang and Cheke 2003; Ossa *et al.* 2012; Larson *et al.* 2013). African bats, e.g. Straw-coloured fruit bat (*Eidolon helvum*) (Ossa

et al. 2012) and birds, including African grey parrots (Chapman *et al.* 1993; Tamungang and Cheke 2003; Amuno *et al.* 2007; Yohannes *et al.* 2007; Larson *et al.* 2013; Tamungang *et al.* 2014) have adapted to track these locally changing food sources (Chapman *et al.* 1993; Juste 1995; Amuno *et al.* 2007; Ossa *et al.* 2012; Tamungang and Cheke 2012; Larson *et al.* 2013; Tamungang *et al.* 2014). African grey parrots may not migrate on large intra-African scales, but do move between local feeding sites within a home range averaging 10 km^2 (Tamungang and Cheke 2012). Differences in vegetation, geological, and even climatic composition may occur throughout these home ranges.

Trade, protection and other threats

African grey parrots are under threat from several factors; trade, habitat destruction, trapping for food and to prevent crop damage, and disease and predation (Fahlman 2002; Amuno *et al.* 2007; Melo and O'Ryan 2007; Martin *et al.* 2014; Tamungang *et al.* 2014; Olah *et al.* 2016). These parrots are CITES II listed since 1981; meaning that their trade is required to be regulated to prevent overutilization of the species. African grey parrots are also listed as vulnerable since 2012 by the IUCN (Mulliken 1995; BirdLife International 2014a; 2014b; CITES 2014). Legal and illegal trade occurs in several of the countries throughout the native distribution of these parrots. In 1995 Mulliken produced a report documenting the trade of African grey parrots in and out of South Africa, which included information on the trade in the countries of the African grey parrot's distribution. The global population of African grey parrots was estimated at *c*. 600 000 individuals in 1995 (Mulliken 1995) and that since being CITES II listed in 1981 approximately *c.* 500 000 African grey parrots were traded from Africa (Mulliken 1995). Martin *et al.* (2014) suggest that approximately 1.7–2 million African grey parrots could have been taken from the wild since 1975. Meaning at least *c.* 35 000 parrots per year were removed from the wild up until 1995, which increased to *c.* 40 000 parrots in 2007 (Melo and O'Ryan 2007) and *c.* 43 500 in 2014 (Martin *et al.* 2014). These

numbers may be underrepresented as *c.* 21 % of the global population is believed to be traded annually (Tamungang *et al.* 2003, BirdLife International 2014a). However, it appears that the global population has declined since 1995, to a combined estimate of *c.* 350 000 *P. erithacus* and *P. timneh* (BirdLife International 2014a; 2014b). Suggesting that even though the species is CITES II listed, with bans and moratoria in place (Mulliken 1995; Melo and O'Ryan 2007; CITES 2014; Martin *et al.* 2014), the trade has not been reduced. Almost half of the countries in which African grey parrots occur have shown population declines, some of which are not even major contributors to the international trade (Mulliken 1995; Melo and O'Ryan 2007; Eniang *et al.* 2008; BirdLife International 2014a; CITES 2014; Martin *et al.* 2014; Annorbah *et al.* 2016). Previously Togo, Ghana, Guinea, and Côte d'Ivoire were considered the major traders in African grey parrots; however, the numbers of traded parrots from these countries were very close to the total populations expected to occur in these countries (Mulliken 1995), which suggested the illegal capturing and import of parrots from other African countries where bans or moratoria were in place (Mulliken 1995). Cameroon, Democratic Republic of Congo, and Congo have recently become the trade strongholds of Africa, yet CITES quotas are not near the annual numbers of traded parrots coming from these countries (CITES 2014). Even though the numbers of traded parrots presented by Mulliken (1995) suggest that the global population may be at risk, these numbers are thought to be drastically underrepresented (May 2001; Fahlman 2002; Tamungang *et al.* 2014; Martin *et al.* 2014). It is estimated that *c.* 33 % of African grey parrots die of stress or injury during the trapping processes (Tamungang *et al.* 2014) and that 40–60 % of the parrots in transport die from starvation, water depravation, self-inflicted injuries, and injuries from other struggling parrots (May 2001; Fahlman 2002; Martin *et al.* 2014). This increases the concern about the survival of this species as more birds may be trapped and transported than the numbers ultimately reported.

The second greatest threat to African grey parrot populations is deforestation or destruction of suitable habitats (Fahlman 2002; Amuno *et al.* 2007; Melo and O'Ryan 2007; Tamungang *et al.* 2014; Martin *et al.* 2014; Olah *et al.* 2016). This emphasises that the largest threat to African grey parrots is caused by man (Olah *et al.* 2016). Deforestation in the native range of African grey parrots occurred at a rate of *c.* 8 % between 2000 and 2010 (FAO 2012). However tropical African forests are the main habitats where African grey parrots can survive (Tamungang *et al.* 2013; 2014), so any decline in habitat has a negative influence on parrot populations. Tropical forest loss is detrimental to African grey parrot survival as they not only supply the parrots with seasonal food sources, but African grey parrots prefer nesting in very tall trees (Juste 1995; Tamungang *et al.* 2013). Together anthropogenic influences; trade and habitat loss account for *c.* 63 % of the threats to African grey parrots (Tamungang *et al.* 2014) with the remaining *c.* 37 % of threats being natural causes; predation, disease, and food source availability (Tamungang *et al.* 2014).

Forensic isotope analysis

Numerous studies have used stable isotope analyses to determine the origin[1] of deceased humans (Beard and Johnson 2000) and of traded animal material; African elephant (*Loxodonta africana*) ivory and white (*Ceratotherium simum*) and black (*Diceros bicornis*) rhinoceros horn (Vogel *et al.* 1990; van der Merwe *et al.* 1990; Hall-Martin *et al.* 1993; Koch *et al.* 1995; Ehleringer *et al.* 2007), drugs, including heroin, cocaine, and marijuana (Ehleringer *et al.* 1999; 2000; 2007), and plants, e.g. cycads (*Encephalartos* spp.) (Retief 2012). These studies focused on determining an estimated origin of analysed material and all show promise for forensic ecology. However, no isotope analysis has been performed on deceased birds of unknown geographic origin. This lack of applying a forensic approach to isotope analysis has created a gap in the methods and tools that could be used for avian

[1] Origin – pertains to the accurate location/region of an organism, specimen, sample etc. i.e. South Africa.

conservation. However, isotope analysis has been used successfully to suggest the migratory origins of birds in North America, Europe (Chamberlain *et al.* 1997; Hobson and Wassenaar 1997; Hobson 1999; Wassenaar and Hobson 2000; Kelly *et al.* 2002; Hobson *et al.* 2004a; 2004b), and Africa (Chamberlain *et al.* 2000; Yohannes *et al.* 2005; 2007; Reichlin *et al.* 2010; Symes and Woodborne 2010; Wakelin *et al.* 2010; Yohannes *et al.* 2011; Hobson *et al.* 2012a; 2012b; Reichlin *et al.* 2013). The focus though has mainly been on migratory connectivity rather than forensic ecology. By using the same stable isotope analyses for determining migratory connectivity of birds, it may be possible to develop a system which could be used to determine the origin or provenance[2] of traded birds. The following sections will explain in depth the factors which alter isotope values and how these differences can be applied to determining geographic origin or provenance of species in a forensic manner.

Stable isotopes

All elements are made up of nucleus configurations, though some elements, e.g. carbon, nitrogen, and hydrogen are made up of additional and different nuclei configurations referred to as isotopes. All isotopes are naturally occurring, though some are less common than others and some are radioactive. Those which are less common contain additional neutrons, making them slightly heavier than the more common and lighter isotopes. This increased mass slows the influence of chemical reactions, e.g. physical and biological processes resulting in different ratios of the heavier to lighter isotopes in material (Hoefs 2009). Isotopes are expressed in delta-value notation (δ) as the value reflects the ratio of light to heavy/common to less-common isotopes in a material in relation to a known standard, e.g. *Belemnitella americana* from the Cretaceous PeeDee formation, South Carolina (PeeDee Belemnite) for carbon ($^{13}C/^{12}C$) (Hoefs 2009). The δ values calculated are represented as parts per thousand (per mil, ‰) using the equation:

[2] Provenance – pertains to the terms of origin, being either captive or wild bred.

$$\delta_{sample}\ (‰) = [(R_{sample}/R_{standard}) - 1] \times 1000$$

with R = isotopic ratio of heavier to lighter isotopes for sample and standard, e.g. $^{13}C/^{12}C$ (Peterson and Fry 1987; Hoefs (2009).

Carbon

The stable carbon isotope ($\delta\ ^{13}C$) values within the tissues of organisms have been used to determine geographic origin and dietary composition (DeNiro and Epstein 1978; Peterson and Fry 1987; Hall-Martin *et al.* 1993; Ehleringer *et al.* 1999; Hobson 1999; Chamberlain *et al.* 2000; Kelly 2000; Hobson 2005; Hobson *et al.* 2012b). Carbon isotope values differ as a result of physiological and biological factors and these differences are what help infer the origins of organisms. These techniques have been utilised for at least three decades and have provided valuable insight.

Carbon isotope differences of plants

Carbon found in the plant tissues is derived from carbon dioxide (CO_2) during photosynthesis (Tieszen *et al.* 1983; Kennedy and Krouse 1989), which occurs along three different photosynthetic pathways; the Calvin cycle (C3), Hatch and Slack cycle (C4), and Crassulacean Acid Metabolism (CAM), each with different degrees of fractionation resulting in different $\delta\ ^{13}C$ values (Tieszen *et al.* 1983; Schleser and Jayasekera 1985; Kennedy and Krouse 1989; Peterson and Fry 1989). Fractionation is the processes in which the lighter isotope molecules, i.e. ^{12}C, are favoured and assimilated faster than the heavier ^{13}C molecules (Dansgaard 1964; McKechnie *et al.* 2004; Becker *et al.* 2007; Marshall *et al.* 2007; Smith *et al.* 2008). C3 plants (trees, shrubs, and temperate grasses) show the most negative $\delta\ ^{13}C$ values compared to C4 plants (sub- and tropical grasses, maize, sorghum, and sugar cane), and CAM plants (succulents) as fractionation through the C3 pathway is the greatest (Peterson and Fry 1987; Kennedy and Krouse 1989). Atmospheric CO_2 has a $\delta\ ^{13}C$ isotope

value of -8 ‰ (Peterson and Fry 1987; Marshall *et al.* 2007). As CO_2 diffuses in through the stomatal openings it experiences a fractionation of *c*. 4.4 ‰, as the heavier ^{13}C molecules enter at a slower rate than the ^{12}C molecules, resulting in a $\delta\,^{13}C$ value of *c.* -12.4 ‰. Carbon is further discriminated by the carboxylating enzyme; rubisco by up to 29 ‰, resulting in an isotopic value of up to -37 ‰ (Fogel and Cifuentes 1993; Marshall *et al.* 2007). The actual $\delta\,^{13}C$ value of C3 plants lies between the value of stomatal diffusion discrimination and rubisco discrimination, usually *c.* -26.5 ‰ but can be between -37 ‰ and -24 ‰ (Peterson and Fry 1987; Kennedy and Krouse 1989; Marshall *et al.* 2007; Still and Powell 2010). ^{13}C is discriminated less in the C4 pathway, usually in the range of -9 ‰ to -16 ‰ (Kennedy and Krouse 1989; Fogel and Cifuentes 1993; Marshall *et al.* 2007; Still and Powell 2010). Fractionation through the stomatal opening remains the same as with C3 plants, yet the more positive $\delta\,^{13}C$ value in C4 plants is a result of less ^{13}C discrimination (*c.* -6 ‰) by the C4 carboxylating enzyme; phosphoenolpyruvate resulting in a $\delta\,^{13}C$ value of *c.* -2 ‰, if fractionation through the stomata is excluded (Peterson and Fry 1987; Fogel and Cifuentes 1993; Marshall *et al.* 2007). CAM plants have a similar photosynthetic pathway and rely on the same carboxylating enzymes as C4 plants, thus are isotopically difficult to distinguish from C4 plants (Marshall *et al.* 2007), yet the CAM $\delta\,^{13}C$ values may lie within the values of C4 and C3 $\delta\,^{13}C$ values, ranging from -20 ‰ to -10 ‰ (Kennedy and Krouse 1989). This discrimination through the different photosynthetic pathways causes the initial difference in $\delta\,^{13}C$ values between C3, C4, and CAM plants (Reichlin *et al*. 2010). The global distribution of C3, C4, and CAM plants creates isotopically distinct regions and the tissues of organisms within these regions usually reflect similar isotope values, depending on their vegetation source. Additional geographic, environmental, and anthropogenic factors may further alter the $\delta\,^{13}C$ values at an ecological scale (Rubenstein and Hobson 2004).

Geographic influence on $\delta^{13}C$ values

The geography of a region influences $\delta^{13}C$ values of plants and animals, be it differences in latitude, altitude, or distance from coastal regions. Several studies have shown that $\delta^{13}C$ of bird feathers are correlated with latitude; as latitude increases so $\delta^{13}C$ values become more negative (Chamberlain *et al.* 1997; Kelly 2000; Pain *et al.* 2004). In contrast their $\delta^{13}C$ values increase with increased altitude (Kelly 2000; Rubenstein and Hobson 2004; Hobson 2005). However, at low altitudes in high latitudes $\delta^{13}C$ values may be similar to $\delta^{13}C$ values at high altitudes in low latitudes (Kelly *et al.* 2002). Lastly with increased distance from marine ecosystems, $\delta^{13}C$ values become more negative (Hobson *et al.* 1999a). This is possibly as a result of marine environments generally having more positive isotope signatures than terrestrial environments (Hobson 2005). Climate changes with geographic location and influences the immediate environment, which further influences the $\delta^{13}C$ values in that area (Hall-Martin *et al.* 1993; Koch *et al.* 1995). Xeric regions; being drier and more dominated by C4 and CAM plants tend to have more positive $\delta^{13}C$ values than mesic regions with increased precipitation and C3 dominance (Ehleringer *et al.* 1999; Hobson 1999; Chamberlain *et al.* 2000; Suits *et al.* 2005; Hobson 2005; 2007; Yohannes *et al.* 2007; Still and Powell 2010). In regions where tree cover is increased, $\delta^{13}C$ values may also be more negative than regions without increased tree cover. This is a result of reduced contact with atmospheric CO_2 and the increased uptake of already $\delta^{13}C$ negative CO_2 from decomposed leaf litter (Schleser and Jayasekera 1985; van der Merwe *et al.* 1995; Hobson 2007; Schoeninger 2010).

Anthropogenic influences on $\delta^{13}C$ values

Anthropogenic influences alter vegetation type and ultimately the $\delta^{13}C$ values of the vegetation (Hobson *et al.* 1993; Evans *et al.* 2003; Hobson 2005). These anthropogenic

influences can occur as an increase or decrease in water availability; resulting in more negative or positive $\delta^{13}C$ values respectively due to water stress factors (Ehleringer *et al.* 1999). Changes in vegetation type, from C3 to C4 vegetation as well as removal of tree cover; results in changes of $\delta^{13}C$ values observed in organisms feeding in the altered environment (Evans *et al.* 2003; Hobson 2005; 2007). These changes may also result in the introduction or removal of food sources in an environment resulting in nutritional stress and the subsequent changes in $\delta^{13}C$ values (Hobson and Clark 1992a; Hobson *et al.* 1993). Lastly increased human development and urbanisation has caused an increase in pollution, mainly in the form of CO_2 through the burning of fossil fuels, so atmospheric ^{13}C has become more depleted, resulting in more negative $\delta^{13}C$ values over time (Keeling 1979; Friedli *et al.* 1986; Hilton *et al.* 2006; Verburg 2007); this is known as the Suess effect.

Physiological influences on $\delta^{13}C$ values by consumers

$\delta^{13}C$ is transferred from plant material to primary consumers, and the diet of the consumer generally reflects the dominant vegetation of the environment it inhabits; however, not all species feed on the dominant vegetation (DeNiro and Epstein 1978; Peterson and Fry 1987; van der Merwe *et al.* 1990; Hobson and Clark 1992a; Hall-Martin *et al.* 1993). There is little fractionation of ^{13}C between producer and consumer tissue (DeNiro and Epstein 1978; Tieszen *et al.* 1983; Peterson and Fry 1987; Kennedy and Krouse 1989) though feathers may show a slight ^{13}C enrichment of *c.* 1 ‰ compared to diet (Mizutani *et al.* 1992; Chamberlain *et al.* 1997; Cherel *et al.* 2005). $\delta^{13}C$ values of organisms can reflect different isotope values to their food sources during times of nutritional stress, reproduction, moult, and increased metabolic activity (Kennedy and Krouse 1989; Hobson and Clark 1992a; 1992b; Bearhop *et al.* 2002; Hobson 2005). During times of nutritional stress or demand birds could draw on nutrient pools stored in their bodies from previously incorporated food sources, which can reflect different $\delta^{13}C$ values depending on the isotopic difference in food sources utilised

(Hobson and Clark 1992b; Mizutani *et al.* 1992; Bearhop *et al.* 2002). Different tissues source different proportions of biochemical fractions; carbohydrates, proteins, and lipids of which some such as lipids may reduce $\delta^{13}C$ values by up to 3 ‰ (DeNiro and Epstein 1978; Tieszen *et al*. 1989; Mizutani *et al.* 1992; Gannes *et al.* 1997; Cherel *et al.* 2005; Hobson 2005; Wassenaar and Hobson 2006).

Stable carbon isotope analysis can help determine which environments organisms occupy, as their diet should reflect the vegetation types influenced by geography, climate, and anthropogenic factors. This however depends on whether the study species feeds on the dominant vegetation type. The physiological influences on $\delta^{13}C$ must be taken into account to avoid misinterpreting the $\delta^{13}C$ values. With further analysis more precise origin or provenance may be determined, e.g. by comparing $\delta^{13}C$ values in canopy covered areas to grasslands (Schoeninger 2010).

Nitrogen

Stable isotope analysis of nitrogen ($\delta^{15}N$) is usually measured at the same time as that of $\delta^{13}C$ isotope analysis, due to their similar masses. Plants take in both atmospheric- and soil-N_2, which is passed on through the trophic levels (DeNiro and Epstein 1981; Peterson and Fry 1987; Kennedy and Krouse 1989). It is usually observed that $\delta^{15}N$ values will fractionate *c.* 3–5 ‰ per shift in trophic level (DeNiro and Epstein 1981; Peterson and Fry 1987; Kennedy and Krouse 1989; Mizutani *et al.* 1992; Gannes *et al.* 1997; Kelly 2000). Thus, $\delta^{15}N$ values can also be used to delineate diet and estimate origins of organisms (Vogel *et al.* 1990; van der Merwe *et al.* 1990; Hall-Martin *et al.* 1993; Ehleringer *et al.* 1999; Chamberlain *et al.* 2000; Rubenstein and Hobson 2004).

Fractionation of ^{15}N occurs as a result of several factors, including environmental (Peterson and Fry 1987; Vogel *et al.* 1990; van der Merwe *et al.* 1990; Hall-Martin *et al.*

1993; Evans 2001; Amundson *et al.* 2003) and anthropogenic influences (Hobson 2005, 2007; Pardo and Nadelhoffer 2010; Ossa *et al.* 2012), trophic level shifts (DeNiro and Epstein 1981; Peterson and Fry 1987; Kennedy and Krouse 1989; Mizutani *et al.* 1992; Gannes *et al.* 1997), and physiological factors (Mizutani *et al.* 1992; Hobson and Clark 1992b; Hobson *et al.* 1993; Gannes *et al.* 1997; Kelly 2000; Bearhop *et al.* 2002; Evans *et al.* 2003).

$\delta^{15}N$ differences between plants

Immediate differences in organic N are the first factors which define the $\delta^{15}N$ values of plants and other organisms at higher trophic levels. Plants assimilate N_2 in different forms between their roots and leaves; nitrate (NO_3) is assimilated at both the roots and leaves whereas ammonium (NH_4) is only assimilated at the roots (Evans 2001; Amundson *et al.* 2003). $\delta^{15}N$ is synthesised from both NO_3 and NH_4 and pooled within the plant (Evans 2001). $\delta^{15}N$ values of the plant tissue may also become more negative, depending if the plant is host to fungal symbiants or not (Evans 2001). These symbiants help plants acquire N_2 however the fungus favours ^{14}N and translocates depleted ^{15}N to the rest of the plant (Evans 2001).

Geographic influence on $\delta^{15}N$ values

The soil concentration of N will increase the discrimination of $\delta^{15}N$ between soil and plant (Ehleringer *et al.* 2000; Evans 2001; Amundson *et al.* 2003; Rubenstein and Hobson 2004), resulting in plants having slightly more negative $\delta^{15}N$ values compared to the soils in which they are found (Amundson *et al.* 2003). $\delta^{15}N$ isotopic signatures for different regions are created by climatic influences as with $\delta^{13}C$ (Vogel *et al.* 1990; van der Merwe *et al.* 1990; Ehleringer *et al.* 2000; Chamberlain *et al.* 2000; Kelly 2000; Amundson *et al.* 2003; Yohannes *et al.* 2005; 2007; Hobson *et al.* 2012b). In regions of high rainfall $\delta^{15}N$ values become more negative, thus drier/xeric areas tend to have more positive $\delta^{15}N$ values (Vogel

et al. 1990; van der Merwe *et al.* 1990; Hall-Martin *et al.* 1993; Chamberlain *et al.* 2000; Amundson *et al.* 2003; Yohannes *et al.* 2005; 2007; Hobson *et al.* 2012b). The more positive δ^{15}N values of organisms could also be a result of the abundance of C4 and CAM plants that grow in drier/xeric regions which organisms may be feeding on resulting in more positive δ^{15}N values than organisms feeding on C3 plants (Vogel *et al.* 1990; van der Merwe *et al.* 1990; Chamberlain *et al.* 2000; Hobson 2005; Yohannes *et al.* 2005; Hobson *et al.* 2012b).

Anthropogenic influences on δ^{15}N values

δ^{15}N values can also be influenced by anthropogenic effects, mainly by addition of fertilisers to soils, irrigation, and landscape changes. Fertilisers, which are predominantly N-based, increase δ^{15}N values of local soils (DeNiro and Epstein 1981; Pardo and Nadelhoffer 2010; Ossa *et al.* 2012). Areas with increased irrigation may have more negative δ^{15}N values, as δ^{15}N is reduced with increased rainfall. Lastly land cleared, be it through burning, grading, or deforestation can also influence δ^{15}N values of soils. Soils which are exposed to the atmosphere, through tilling, burning, or vegetation clearing come into contact with more atmospheric-N and their δ^{15}N values become more positive. Fire additionally removes ^{15}N depleted matter, creating an area with more positive δ^{15}N values (Hobson 2007; Pardo and Nadelhoffer 2010). These factors all alter δ^{15}N values of the soil and/or vegetation of an area. However this is prior to consumption by organisms.

Physiological influences on δ^{15}N by consumers

δ^{15}N values can also differ between organisms, as a result of physiological effects. It is usually observed that δ^{15}N values will fractionate *c.* 3–5 ‰ per shift in trophic level (DeNiro and Epstein 1981; Peterson and Fry 1987; Kennedy and Krouse 1989; Mizutani *et al.* 1992; Gannes *et al.* 1997; Kelly 2000; Vanderklift and Ponsard 2003; Caut *et al.* 2009). This enrichment occurs as the heavier ^{15}N molecules are favoured less than the lighter ^{14}N

molecules in dietary absorption and thus the ratio between $^{15}N/^{14}N$ decreases with each shift upwards in trophic position (Gannes *et al.* 1997; Kelly 2000). ^{15}N enrichment may also occur between species with the same ecology, as a result of larger individuals feeding on different prey, or having access to different food sources (Chamberlain *et al.* 2000; Kelly 2000; Evans *et al.* 2003; Pain *et al.* 2004). Therefore it is important to have knowledge about the ecology of the organism being analysed, so as to have an initial idea of what the $\delta^{15}N$ values should be in relation to the food base (Mizutani *et al.* 1992; Kelly 2000; Evans *et al.* 2003; Pain *et al.* 2004). Along with understanding the ecology of the sampled organism it is key to also have knowledge on the state of the organism; nutritional and water stress, moult, and reproduction as these may all affect the assimilation and fraction of $\delta^{15}N$ within the body (Peterson and Fry 1987; Vogel *et al*. 1990; van der Merwe *et al.* 1990; Hobson and Clark 1992b; Hobson *et al.* 1993; Kelly 2000; Bearhop *et al.* 2002; Vanderklift and Ponsard 2003; Caut *et al.* 2009). Nutritionally stressed individuals have shown more positive $\delta^{15}N$ values, as they experience amino acid hydrolysis, where ^{14}N stores are used up and ^{15}N remains in the tissues (Hobson and Clark 1992b; Hobson *et al.* 1993; Kelly 2000). It must be understood that fractionation and assimilation of $\delta^{15}N$ does differ among taxa and species (Vanderklift and Ponsard 2003). Water stressed organisms also have more positive $\delta^{15}N$ values (Peterson and Fry 1987; Vogel *et al.* 1990; van der Merwe *et al.* 1990; Hobson and Clark 1992b; Hobson *et al.* 1993; Vanderklift and Ponsard 2003; Caut *et al.* 2009). Rather than as a result of amino acid hydrolysis it occurs as a result of proteins being catabolized into nitrogenous waste products, i.e. ammonia (NH_4^+). These waste products are often toxic and need to be excreted resulting in the loss of ^{14}N (Peterson and Fry 1987; Hobson *et al.* 1993; Vanderklift and Ponsard 2003; Caut *et al.* 2009). Different taxa excrete waste products in different forms, e.g. mammals excrete urea and birds uric acid. In arid environments or during water stress water is conserved; however, N is still required to be removed from the tissues and N-concentrated

urea or uric acid is excreted (Ambrose and DeNiro 1986; Hobson *et al.* 1993; Vanderklift and Ponsard 2003; Caut *et al.* 2009). The excretion of N-concentrated urea and uric acid depletes the tissues of N, resulting in the tissues being replenished by the remaining N_2 stores, being ^{15}N (Ambrose and DeNiro 1986). The replacement of ^{14}N by ^{15}N results in more positive $\delta\,^{15}N$ tissue values (Ambrose and DeNiro 1986; Peterson and Fry 1987; Hobson and Clark 1992b; Hobson *et al.* 1993; Vanderklift and Ponsard 2003; Caut *et al.* 2009). More positive $\delta\,^{15}N$ values of tissues can also occur outside of nutrient- and water-stressed situation, mainly when an organism's metabolic activity is increased (Bearhop *et al.* 2002; Vanderklift and Ponsard 2003). An increased metabolic rate may occur during migration, moult, reproduction, or bouts of poor health (Bearhop *et al.* 2002; Vanderklift and Ponsard 2003). Increased metabolic activity generally results in increased assimilation of biochemical components or altered assimilation so as to acquire more energy (Bearhop *et al.* 2002). Additionally organisms may source nutrients from reserves or pools in their bodies, which is beneficial during these bouts of increased metabolic activity but could alter isotopic values depending on the $\delta\,^{15}N$ values of the nutrient pools (Hobson and Clark 1992b; Mizutani *et al.* 1992; Bearhop *et al.* 2002).

Hydrogen/Deuterium

Hydrogen (H) in water is transferred into the tissues of plants through precipitation and ground water and further into the tissues of consumers after ingestion (Kennedy and Krouse 1989; Cormie *et al.* 1994; Hobson *et al.* 1999b; Hobson *et al.* 2012a). Analysis of hydrogen ($\delta\,^{2}H$) is used mainly in determining the geographic origins of organisms in relation to the global patterns of $\delta\,^{2}H$ in precipitation (Cormie *et al.* 1994; Chamberlain *et al.* 1997; Hobson and Wassenaar 1997; Wassenaar and Hobson 2000; Kelly *et al.* 2002; Hobson *et al.* 2004a; 2004b; Bowen *et al.* 2005; Hobson *et al.* 2012b).

Geographical influence on $\delta^{2}H$ values

The change in mean-annual air temperature across the latitudes; decreasing with distance from the equator or with increasing altitude, influences the amount, frequency, and type of precipitation that occur at different regions of the planet (Reich and Oleksyn 2004). With the latitudinal or altitudinal decline in temperature, $\delta^{2}H$ values also become more negative (Dansgaard 1964; Kennedy and Krouse 1989; Cormie *et al.* 1994; Hobson and Wassenaar 1997; Wassenaar and Hobson 2000; Kelly *et al.* 2002; Bowen *et al.* 2005; West *et al.* 2010; Hobson *et al.* 2012b). However, the African continent does not show as distinct changes in $\delta^{2}H$ with increasing latitude as North America and Europe, but there is an apparent east-west decrease in $\delta^{2}H$ values (Møller and Hobson 2004; Bowen *et al.* 2005; Hobson 2005; West *et al.* 2010; Wakelin *et al.* 2011; Bairlein *et al.* 2012; Reichlin *et al.* 2013). Usually cooler areas receive more precipitation and experience less evaporation than warmer areas, as a result of increased temperatures warmer areas tend to have more positive $\delta^{2}H$ values in precipitation (Dansgaard 1964; Wassenaar and Hobson 2000; McKechnie *et al.* 2004; Smith and Dufty 2005; Hobson 2007; Hobson *et al.* 2012a; 2012b). Enrichment occurs as the lighter ^{1}H molecules evaporate faster than the heavier ^{2}H molecules, leaving the heavier molecules behind (Dansgaard 1964; Lloyd 1966; McKechnie *et al.* 2004; Becker *et al.* 2007). Fractionation through evaporation also results in $\delta^{2}H$ values decreasing with distance from the sea (Lloyd 1966; Kennedy and Krouse 1989; Hobson 1999; Kelly *et al.* 2002; Hobson 2007; Ossa *et al.* 2012). $\delta^{2}H$ values of the ocean are relatively constant and remain around *c.* 0 ‰ (Lloyd 1966), after evaporation, fractionation occurs and the water removed from the ocean is more negative in its $\delta^{2}H$ values (Dansgaard 1964; Lloyd 1966; McKechnie *et al.* 2004). As this depleted water is deposited and re-evaporated further inland it becomes more negative in $\delta^{2}H$, resulting in the most depleted waters furthest inland.

Anthropogenic influences on $\delta^{2}H$ values

Anthropogenic affects often alter the immediate environment and thus can influence isotopic signatures. The introduction of water results in the presence of sources of water which may be isotopically different to that of the surrounding natural water sources and precipitation (Wolf *et al.* 2011). This could result in different $\delta^{2}H$ values being represented in the tissues of local organisms, if they are incorporating introduced water sources into their diet (Hobson *et al.* 1999b; Wassenaar and Hobson 2006; Wakelin *et al.* 2011; Wolf *et al.* 2011). The removal or introduction of vegetation can also alter $\delta^{2}H$ values of an environment (Hobson 2007). Vegetation removal can result in increased evaporation and $\delta^{2}H$ values become more positive compared to local precipitation (Hobson 2007). Whereas introduced vegetation not only reduces evaporation, but may also alter the vegetation type of the area (C3, C4, and CAM) resulting in different $\delta^{2}H$ values due to differences in stomatal activity (Fogel and Cifuentes 1993). C3 plants have the most negative $\delta^{2}H$ values and CAM plants the most positive (Kennedy and Krouse 1989; Yohannes *et al.* 2007).

Physiological influences on $\delta^{2}H$ by consumers

$\delta^{2}H$ fractionation occurs as a result of fractionation during photosynthesis within the plant. Whilst stomata are open water diffuses out of the plant, resulting in the water remaining within the plant having more positive $\delta^{2}H$ values, increasing the plant tissue $\delta^{2}H$ values above those of local precipitation (Fogel and Cifuentes 1993). There is no fractionation between trophic levels in terms of $\delta^{2}H$ (Kennedy and Krouse 1989; Hobson and Wassenaar 1997). This physiological influence on $\delta^{2}H$ values of plants can be observed in their consumers. Though this may not be a physiological process for the consumer it indirectly occurred as a result of the plants physiological process. However, after ingestion $\delta^{2}H$ can still experience physiological fractionation. $\delta^{2}H$ obtained through food sources or drinking

water can exchange with food molecules, usually in the form of proteins and carbohydrates in the stomach and other bodily fluids (Hobson *et al.* 1999b), resulting in the $\delta^{2}H$ values of consumer tissue differing to the environment. Though this has been observed in blood, muscle, and liver tissues it is suggested that the incorporation of food and drinking water is more pronounced in keratin based tissues (Hobson *et al.* 1999b). Even though feather $\delta^{2}H$ values are directly correlated to precipitation $\delta^{2}H$ values (Chamberlain *et al.* 1997; Hobson and Wassenaar 1997; Wassenaar and Hobson 2000; Kelly *et al.* 2002; Hobson *et al.* 2004a; Wolf *et al.* 2011), it is suggested that feather $\delta^{2}H$ values may be more negative than precipitation $\delta^{2}H$ values by 10–30 ‰ (Chamberlain *et al*. 1997; Hobson and Wassenaar 1997; Wassenaar and Hobson 2000; Hobson *et al.* 2004a; Wolf *et al.* 2011). However, Hobson and Wassenaar (1997) showed that Neotropical songbirds in North America had $\delta^{2}H$ feather values on average of 24 ‰ more positive than $\delta^{2}H$ precipitation values. Fractionation can occur via other means in organisms as well, however only with respect to $\delta^{2}H$ (Hobson *et al.* 2004a). One form of further fractionation occurs due to exposure of tissues to ambient water vapour (DeNiro and Epstein 1981; Hobson and Wassenaar 1997; Hobson 1999; Hobson *et al.* 1999b; McKechnie *et al.* 2004; Hobson 2007). Hydrogen is weakly bound to O_2 and N_2 and thus H_2 exchanges with available O_2 and N_2 (Hobson 1999; Hobson *et al.* 1999b), resulting in more positive $\delta^{2}H$ values in tissues exposed to water vapour. Fractionation though is relatively small due to the composition of feather keratin containing only 40 % exchangeable H (Hobson 1999). Exposure to heat stress can also cause $\delta^{2}H$ fractionation in organisms. Certain species have developed evaporative-cooling to cope with heat stress, though the loss of water vapour through the skin causes more positive $\delta^{2}H$ values in the tissues of the organism (McKechnie *et al.* 2004; Smith and Dufty 2005; Wassenaar and Hobson 2006). Within and between species, different $\delta^{2}H$ values are observed and some possible reasons could be due to physiological and ecological differences (Smith and Dufty

2005; Wassenaar and Hobson 2006; Smith *et al.* 2008; Oppel *et al.* 2011; Hobson *et al.* 2012a). Certain feathers may grow in different regions, depending on the moult pattern and growth rate. The new feathers will reflect isotopic signatures of the region of growth which may differ to the region where the sample was taken (Smith and Dufty 2005; Smith *et al.* 2008; Oppel *et al.* 2011). Diet may differ between different species or different age classes, ultimately altering $\delta\,^{2}H$ values (Smith and Dufty 2005; Hobson *et al.* 2012a).

Isotope variation

Isotope values ($\delta\,^{13}C$, $\delta\,^{15}N$, and $\delta\,^{2}H$) may also show differences in variability between different regions. Isotope variability of an area could be influenced by vegetation type and climate. Africa has areas with homogeneous vegetation cover (Still and Powell 2010; Pardo and Nadelhoffer 2010) or similar climates (Aggarwal *et al.* 2010; Pardo and Nadelhoffer 2010), resulting in areas with reduced isotope variability. Therefore, regions with heterogeneous vegetation cover and climatic differences could reflect increased isotope variability compared to homogeneous regions, e.g. forest birds inhabiting C3 forests and feeding entirely on C3 fruits would be expected to show reduced isotopic variability compared to species which opportunistically inhabit and feed between C3 forest and agricultural C4 land. This application for determining difference in isotope variability is dependent on the ecology of the species being studied.

Multi-isotope analysis

Each element and its respective isotopes occur in different abundances at different geographic locations (Ehleringer *et al.* 1999; Chamberlain *et al.* 2000). These variations in isotopic signatures are what help allocate tissue samples of organisms to their geographic locations (Ehleringer *et al.* 1999; Hobson 1999; Chamberlain *et al.* 2000; Bearhop *et al.* 2003; Møller and Hobson 2004; Ehleringer *et al.* 2007). In certain situations a single isotope

may not vary significantly between two study sites, thus it is suggested that a multi-isotope method be used (van der Merwe *et al.* 1990; Hall-Martin *et al.* 1993; Chamberlain *et al.* 2000; Hobson *et al.* 2004a; Hobson 2005; Hobson *et al.* 2012b; Ossa *et al.* 2012). It must be emphasised that each factor that could influence isotopic signatures, if possible, must be acknowledged and analysed (Hobson 2007).

Prior to analysis and determining the influences caused by environmental and physiological factors it is important that the appropriate tissue of the sample species be selected (DeNiro and Epstein 1978; 1981; Tieszen *et al.* 1983; Hobson and Clark 1992a; Gannes *et al.* 1997; Hobson 1999; 2007) and that there is an understanding of how the chosen tissue type assimilates, alters, and reflects isotopes obtained from dietary intake (Hobson and Clark 1992b; Mizutani *et al.* 1992; Gannes *et al.* 1997; Bearhop *et al.* 2002; 2003; Cerling *et al.* 2006; Wassenaar and Hobson 2006; Smith *et al.* 2008; Symes and Woodborne 2011).

Stable isotopes in feathers

Feathers are inert tissues which hold isotopic information of the region where they were grown (Hobson and Clark 1992a; 1992b; Mizutani *et al.* 1992; Bearhop *et al.* 2002; Pain *et al.* 2004; Bowen *et al.* 2005; Hobson 2005; Yohannes *et al.* 2007). It allows for the origin of feathers to be determined easier than metabolically active tissues, as the isotopic signature of a feather does not change after formation (Bowen *et al.* 2005; Hobson 2005). The occurrence of lipids in the diet also do not influences isotopic signatures of feathers as much as they do other tissues, as feathers are formed primarily of proteins (Hobson and Clark 1992b; Mizutani *et al.* 1992; Bearhop *et al.* 2002; Cherel *et al.* 2005; Hobson 2005; Ehleringer *et al.* 2007). This suggests that the δ^{13}C and δ^{15}N values of feathers are less likely to be influenced by lipids in the diet while δ^{2}H values of feathers can be influenced by ambient water vapour (DeNiro and Epstein 1981; Hobson and Wassenaar 1997; Hobson

1999; Hobson *et al.* 1999b; McKechnie *et al.* 2004; Hobson 2007). There is one major negative draw back to using feathers as the selected tissue and that is, different species assimilate, fractionate, and reflect isotopes differently (Mizutani *et al.* 1992; Gannes *et al.* 1997; Bearhop *et al.* 2003; Cherel *et al.* 2005). As certain isotopes are influenced as a result of other isotopes being enriched or depleted, a multi-isotope approach can provide possible interpretation when other analyses were less successful and also allows for inter-isotope correlation to be taken into account.

Background

The African grey parrot is a popular pet species that is heavily traded (Tamungang *et al.* 2013; Martin *et al.* 2014). Often trade quotas are ignored and birds are illegally caught and imported into different countries (Mulliken 1995). These shipments often come from regions throughout the native distribution of the species (Martin *et al.* 2014). In December 2010 a consignment of *c.* 700 African grey parrots, part of a larger consignment of *c.* 1 650 individual birds, from the Democratic Republic of Congo, died during transport (News 24 2011). The parrots were being transported on a 1Time flight from O.R. Tambo International Airport, Johannesburg, Gauteng, to King Shaka International Airport, Durban, KwaZulu-Natal (Mail and Guardian 2011; News 24 2011). Of the consignment, *c.* 250 African grey parrot carcasses were obtained by the University of KwaZulu-Natal for further studies and retained as evidence in the ongoing court case. For further information on this incident see the links in the references; Mail and Guardian 2010 and News 24 2011.

It is believed that the deceased African grey parrots were illegally caught from the wild and traded into South Africa from a country where they may not have originated, as it would be unlikely that such large consignments would be made up of captive parrots. The main outcome of this research is to determine whether the stable isotope analysis of African

grey parrot feathers can be applied to forensic ecology and the tracking and monitoring of the illegal trade of this species.

Aims and objectives

The aim of this study was to analyse stable light isotope ratios in feathers from known captive and wild African grey parrots to determine the provenance of specimens from undetermined geographical origin.

The research had the following specific objectives

1. To determine the stable light isotope values ($\delta\ ^{13}C$, $\delta\ ^{15}N$, $\delta\ ^{2}H$) of different feather types (primary, body, tail) within individual deceased African grey parrots (of unknown origin), to understand inter and intra-individual variation in isotopic signatures, and how this may be relevant for studies that sample individual feathers from birds (including forensic studies like this).
2. To compare the stable light isotopic values ($\delta\ ^{13}C$, $\delta\ ^{15}N$, $\delta\ ^{2}H$) of feathers from deceased African grey parrots (of unknown origin) with those of African grey parrot feathers of known (wild and captive) origin.
3. To determine the stable light isotope values ($\delta\ ^{13}C$, $\delta\ ^{15}N$, $\delta\ ^{2}H$) of primary wing feathers from deceased African grey parrots (of unknown origin) to determine if finer scale isotope analysis within individuals provides additional and relevant information for forensic studies.

Hypotheses and predictions

A. The isotope values of different feather types from individual African grey parrots differ significantly from one another, because of spatial and temporal differences during the growing phases of each feather type.

B. The isotopic signature of the feathers obtained from known wild African grey parrots differ significantly from that of known captive African grey parrots, because of the different environments to which they are exposed.

C. Isotope values among the primary feathers of African grey parrots will differ significantly, because of spatial and temporal differences during the growing phases of each primary feather.

It is predicted that, if the deceased African grey parrots of unknown origin were captured from the wild, they will have similar isotope signatures and reflect similar isotope variability to those of the African grey parrots of known wild origin.

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Chapter 2: Isotopic differences between feather type

"He who is cruel to animals becomes hard also in his dealings with men. We can judge the heart of a man by his treatment of animals." **- Immanuel Kant**



ISOTOPIC VARIATION ($\delta^{13}C$, $\delta^{15}N$, $\delta^{2}H$) AMONG FEATHER TYPES OF AFRICAN GREY PARROTS (*PSITTACUS ERITHACUS*): IMPLICATIONS FOR DIETARY AND FORENSIC STUDIES

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Abstract

Stable isotope analysis of feathers has become increasingly popular in avian ecological studies. While it is understood that the stable isotope signatures of feathers represent the period of feather growth, few studies have critically assessed the use of different feathers in dietary and migratory studies. Three feathers types; primary (P10), body (breast), and tail (outer right) of 32 African grey parrots (*Psittacus erithacus*), from a larger consignment of deceased parrots from unknown origins were analysed for $\delta^{13}C$, $\delta^{15}N$, and $\delta^{2}H$ and were all significantly different, except for primary and tail feathers for $\delta^{2}H$. The isotope differences between feather types were compared; 80 % of tail feathers had more positive $\delta^{13}C$ isotope values than both primary (2.7 ± 1.4 ‰) and body feathers (2.0 ± 3.1 ‰). For $\delta^{15}N$, 78 % of tail feathers were more negative than primary (1.8 ± 2.5 ‰) and body (1.0 ± 2.0 ‰) feathers. For $\delta^{2}H$, 71 % of tail feathers were more negative (9.8 ± 17.7 ‰) compared to body feathers, whilst primary feathers were negative (2.3 ± 16.1 ‰) compared to tail feathers. The variable use of the landscape, the different times at which different

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feathers grow, and the different isotopic routing and turnover rate result in the variable incorporation of isotopes into different feather types. Using these differences, we developed standardising factors that allow for the comparison of isotope values in different feathers of African grey parrots, specifically; primary, tail, and body feathers. By maintaining consistent sampling, isotope analysis could be incorporated into forensic ecology, so that comparisons between individuals using different feathers may be possible.

Keywords: *Feather isotope differentiation, isotope analyses, feather type, isotope standardising, feather standardising factor*.

Introduction

Isotope analysis has been used to determine diets (DeNiro and Epstein 1978; 1981; Tieszen *et al.* 1983), trophic positions (Peterson and Fry 1989; Hobson 1990), and geographic origins of animals (Peterson and Fry 1989; Hobson 1990; Chamberlain *et al.* 1997; Hobson and Wassenaar 1997). In birds, differences in isotopic turnover, period of tissue growth (feathers), isotopic routing, and the variable fractionation from diet to tissue results in isotopic values of tissues differing within and between individuals (Hobson and Clark 1992b; Gannes *et al.* 1997; Cherel *at al.* 2005; Wassenaar and Hobson 2006), e.g. different tissues of captive domestic chicken (*Gallus gallus*) and Japanese quail (*Coturnix japonica*) differed in $\delta^{13}C$ and $\delta^{15}N$ values (Hobson and Clark 1992b). These isotopic differences that occur between tissues types could also occur between different feather types within the same individual (Ramos *et al.* 2009; Symes and Woodborne 2011). Within individual variation as a result of physiological and geographical differences may therefore limit interpretations made using single feathers (Ramos *et al.* 2009), which in turn may have implications on

extrapolating to other species (Cherel *et al.* 2005; Greer *et al.* 2015), as isotope values may be incorrectly interpreted as a result of immediate differences between feather types.

Analysis of stable isotopes in different tissues provides information of diet and location of individuals from different time periods; from many years for bone and enamel, to a few days for blood and liver (Hobson and Clark 1992a). However, feathers are one of the most commonly sampled tissues for avian species because they are easily sampled with no ill-effect to the individual and provide interpretations for the period of feather growth (Hobson and Clark 1992a; Mizutani *et al.* 1992; Hobson *et al.* 1999; Bearhop *et al.* 2002). Many studies have been performed on avian species using blood and feathers (Hobson and Clark 1992b; Bearhop *et al.* 2002; Cherel *et al.* 2005; Greer *et al.* 2015), claws (Bearhop *et al.* 2003; Hobson and Clark 1992b), and liver and bone collagen (Hobson and Clark 1992b) and it is known that tissue choice is highly dependent on the nature of the study. For example, migration studies tend to use tissues which hold their isotopic signatures for longer periods, whereas studies on diet could use tissues which hold their signatures for mere hours to years depending on what needs to be inferred (Hobson and Clark 1992a; Thompson and Furness 1995). When considering the possible migratory origins of species it is suggested that feathers are sampled (Hobson and Clark 1992b). Even in this regard an understanding of when feathers are grown (moulted) is required (Hobson and Wassenaar 1997; Chamberlain *et al.* 2007). Wing feathers are usually only grown annually, and alternate in a breeding-moult cycle (Thompson and Furness 1995), whereas body or contour feathers could be grown year round or replaced when lost, this moult is generally reflected in smaller birds, parrots, falcons, and some sea-birds (Thompson and Furness 1995; Mallet-Rodrigues 2012; Pyle 2013).

Studies on both marine, e.g. penguin spp., Leach's storm-petrel (*Oceanodroma leucorhoa*), cormorant spp., and wandering albatross (*Diomedea exulans*) (Mizutani *et al.*

1992; Hedd and Montevecchi 2006, Becker *et al.* 2007; Jaeger *et al.* 2009) and terrestrial birds, e.g. ibis spp., willow warbler (*Phylloscopus trochilus*), golden plover (*Pluvialis dominica*), and American plover (*Pluvialis fulva*) (Mizutani *et al.* 1992; Chamberlain *et al.* 2000; Rocque *et al.* 2006; Evans *et al.* 2012) have suggested that there can be no difference in δ^{13}C or δ^{15}N values between feather types. However, this is not always the case as some species do show isotope differences between feather types (Mizutani *et al.* 1992; Hedd and Montevecchi 2006; Becker *et al.* 2007; Jaeger *et al.* 2009). δ^{2}H values of; black-throated blue warbler (*Dendroica caerulscens*), Wilson's warbler (*Wilsonia pusilla*), Baikal teal (*Anas formosa*), golden plover (*Pluvialis dominica*), and American plover (*Pluvialis fulva*) did not differ significantly between feather types (Chamberlain *et al.* 1997; Kelly *et al.* 2002; Rocque *et al.* 2006; Fox *et al.* 2007), whilst birds of prey; Cooper's hawk (*Accipiter cooperii*) and northern goshawk (*Accipiter gentilis*) have shown significant differences in δ^{2}H values between feather types (Meehan *et al.* 2003; Smith and Dufty 2005; Langin *et al.* 2007; Smith *et al.* 2008). It is thus important to account for feather type when considering isotope signatures and the interpretations they provide for diet and migratory origin (Smith and Dufty 2005).

While the potential reasons for isotope differences are not clear, a number of reasons have been suggested, such as physiological influences in tissues with different growth rates (Tieszen *et al.* 1983; Kennedy and Krouse 1989; Mizutani *et al.* 1992). Also, different feather types growing at different rates may result in differing isotope values between feather types (Smith *et al.* 2008). Certain feathers grow faster than others as a result of their final size and purpose, and experience variable diet-tissue fractionation (Bearhop *et al.* 2003; Smith *et al.* 2008). Fractionation may differ as larger feathers may be sourcing nutrients from body stores as opposed to immediate dietary nutrients (Bearhop *et al.* 2003), or faster growing feathers are incorporating nutrients at a quicker rate reducing fractionation (Smith *et al.* 2008). The

location and period of tissue growth can also influence tissue isotope values (Hobson and Clark 1992a; Mizutani *et al.* 1992; Bearhop *et al.* 2002; Symes and Woodborne 2011; Johnson 2014). Isotope values in primary producers differ through the season with respect to δ^{2}H (Yohannes *et al.* 2005; Terzer *et al.* 2013) and across geographic localities, with respect to δ^{13}C, δ^{15}N (Vogel *et al.* 1990; van der Merwe *et al.* 1990; Hall-Martin *et al.* 1993; Hobson 1999), and δ^{2}H (Cormie *et al.* 1994; Hobson 1999). These differences are generally reflected in the consumer's tissues. Feathers which are grown at different times reflect different δ^{2}H values, as a result of the annual changes in precipitation (Smith *et al.* 2008; Yohannes *et al.* 2005), whilst feathers grown in different locations or environments will show different δ^{13}C, δ^{15}N (Chamberlain *et al.* 1997; 2000; Kelly 2000; Evans *et al.* 2003; Møller and Hobson 2004), and δ^{2}H values (Chamberlain *et al.* 1997; Hobson and Wassenaar 1997; Kelly *et al.* 2002; Møller and Hobson 2004).

Understanding the potential isotopic relationships between feather types could aid in further interpreting the dietary and migratory habits of species, as a there would be a better understanding of diet-tissue fractionation and the potential sourcing of nutrients. This could also prove valuable in forensic ecology, as feather standardising factors suggested may be applied to future studies (Symes *et al.* In prep). These will allow for a broader sample base to be analysed. To date no research has been done to compare isotope signatures between feather types for psittacines, thus this study could be the first to determine whether feather types of parrots differ isotopically. This could be beneficial as certain feather samples, i.e. those from museum collections, only allow body feather sampling and often tail feathers are sampled from live birds (Cherel *et al*. 2000; Hobson 2001; Fair *et al*. 2013; Ogden *et al.* 2015). The research could also potentially establish a working protocol to allow for the comparison of different feather types between individuals and species.

The aim of the study was to analyse stable isotopes ($\delta^{13}C$, $\delta^{15}N$, $\delta^{2}H$) in different African grey parrot (*Psittacus erithacus*) feather types and determine whether there were any isotope differences between the feather types. African grey parrots would be moving between isotopically different regions through the growing season, sourcing food, and moulting. We therefore hypothesised that there may be a difference in $\delta^{13}C$, $\delta^{15}N$, and $\delta^{2}H$ isotope values between feather types as a result of physiological and geographical influences. If there are in fact differences between the feathers types and the differences are uni-directional these will be used to formulate standardising factors that will normalise all feather values to those of tail feathers.

Methods and materials

Study species

Thirty two African grey parrots were randomly selected from a sub-sample of *c.* 100 randomly selected deceased parrots, originally forming part of *c.* 250 randomly selected parrots which died during transit from Johannesburg to KwaZulu-Natal, South Africa. The 250 parrots were part of a larger consignment *c.* 700 suggested to be wild caught in the Democratic Republic of Congo, as such a large consignment would unlikely derive solely from captivity in that region. African grey parrots were believed to be a sufficient surrogate species to determine if there was inter-feather isotope variation as they are generally frugivorous birds, meaning their isotopic signatures should represent C3 values, these birds also rarely feed on C4 plants unless exposed to a C4 food source (Tamungang *et al.* 2013, 2014). African grey parrots also inhabit a broad area across the tropical forests of central

Africa reducing the isotopic variation that may occur through geographic changes (Mulliken 1995; Forshaw 2006).

From the deceased African grey parrot sub-sample a primary feather (P10), tail feather (outer right) and body feather (breast) were taken from each individual. The feathers were washed in a 2:1 chloroform:methanol mixture to remove any contaminating debris or oils and dried at 60˚C for a day in a drying oven (Wassenaar and Hobson 2000).

δ^{13}C and δ^{15}N isotope analysis

Samples weighing *c*. 0.5 mg were cut from the distal region (*c.* 20 mm beneath the tip of the feather) of the vane of each feather and placed into toluene-washed tin cups. Merck Gel (δ^{15}N = +6.8 ‰, δ^{13}C = -20.57 ‰) standards weighing 0.2, 0.4, and 0.6 mg were weighed out to allow sample peak values to fall within the range of peaks of δ^{13}C and δ^{15}N. A standard and a blank were sampled at the beginning, end, and after every 12 unknown samples to determine if there was any remaining residue from the previous sample as well as if corrections are needed if the standard values had deviated. The weighed samples and standards were analysed in a Thermo Fisher Scientific Inc. Flash EA1112 Elemental Analyser coupled to a DeltaV mass spectrometer via a Conflo IV interface. Combustion took place at 1020˚C and a copper reduction oven was used at 650˚C. Water was trapped in a perchlorate water trap. N_2 and CO_2 were separated in a Gas Chromatograph column at 45˚C.

δ^{2}H isotope analysis

Samples weighing 0.2–0.4 g were cut from beneath the δ^{13}C and δ^{15}N sample point on the distal region of the vane of each feather and placed in toluene-washed silver cups. Twelve (one per every seven samples) 0.2, 0.4, and 0.6 g standards comprising of 6 matrix-matched Caribou hoof (CBS; δ^{2}H = -197 ‰) and Kudu horn (KHS; δ^{2}H = -54.1 ‰) (Wassenaar and

Hobson 2010) were weighed out to correct for any sampling discrepancies (Meier-Augustine *et al.* 2013). The weighed samples and standards were left to equilibrate in the same ambient conditions for 10 days to allow for the free exchange of atmospheric $\delta^{2}H$ (Wassenaar and Hobson 2003; Meier-Augustine *et al.* 2013). Samples were analysed through pyrolysis using a Thermo DeltaV Advantage mass spectrometer coupled to a Flash HT Plus elemental analyser. Corrections were made to the sample values using a linear regression based on the actual values for the standards versus the measured value to account for non-exchangeable hydrogen (Wassenaar and Hobson 2003).

Statistical analysis

A Shapiro-Wilk test was used to test for normality (Shapiro and Wilk 1965). The mean (± SD) was calculated for each isotope and feather type. Differences between feather types were tested using ANOVA/Kruskal-Wallis tests and specific differences between feather types were confirmed using Paired *t*-Tests/Wilcoxon Signed-Rank tests, which were Bonferroni adjusted, results presented in Appendix 1. The isotope difference between primary and body feathers, body and tail feathers, and primary and tail feathers for each individual were calculated. A 95 % confidence interval of the differences was calculated for each difference between feather types for each isotope in an attempt to determine the possible range of differences in a larger population. Average differences were calculated to establish standardising factors. Percentages of the different directions of the differences between the feather types were calculated to determine whether or not the suggested standardising factors could be applied.

Results

Only δ^{15}N samples were normally distributed. There were significant differences between feather types for all three isotopes δ^{13}C (H = 39.01, df = 2, P < 0.0001), δ^{15}N (F = 10.5, df = 2, P < 0.0001), and δ^{2}H (H = 14.72, df = 2, P = 0.0006). The δ^{13}C and δ^{15}N isotope values for the primary African grey parrot feathers (δ^{13}C = -24.4 ± 1.3 ‰, δ^{15}N = +7.3 ± 1.6 ‰) were significantly different to both the body (δ^{13}C = -23.7 ± 0.6 ‰, δ^{15}N = +6.5 ± 1.4 ‰) and tail feathers (δ^{13}C = -21.7 ± 3.1 ‰, δ^{15}N = +5.5 ± 1.7 ‰) (Fig. 1A and 1B; Appendix 1). The δ^{2}H values for primary feathers (δ^{2}H = -85.0 ± 13.0 ‰) and tail feathers (δ^{2}H = -82.4 ± 11.0 ‰) were significantly different to the body feathers (δ^{2}H = -72.6 ± 11.4 ‰) (Fig. 1C; Appendix 1).

For δ^{13}C, primary feathers were more negative by 0.7 ± 1.4 ‰ than body feathers and 2.7 ± 3.2 ‰ more negative than tail feathers (Table 1). Body feathers were also 2.0 ± 3.1 ‰ more negative than tail feathers for δ^{13}C (Table 1). Of the 32 African grey parrots 80 % of tail feathers had more positive δ^{13}C values than both primary and body feathers, suggesting an overall enrichment from primary through to body and tail feathers (Fig. 1A). However, primary feathers were 0.8 ± 1.7 ‰ and 1.8 ± 2.5 ‰ more positive for δ^{15}N than body and tail feathers respectively (Table 1). Body feathers were also more positive by 1.0 ± 2.0 ‰ for δ^{15}N than tail feathers (Table 1). Of the 32 African grey parrots 78 % of primary feathers were more positive for δ^{15}N than both body and tail feathers suggesting an overall decrease from primary through to body and tail feathers (Fig. 1B). Primary feathers were 12.1 ± 16.7 ‰ and 2.3 ± 16.1 ‰ more negative than tail and body feathers respectively for δ^{2}H (Table 1). Tail feathers were also 9.8 ± 17.7 ‰ more positive than body feathers for δ^{2}H (Table 1). With 70 % of body feathers being more positive than both primary and tail feathers it is suggested that there is an increase from primary to tail feather and reduction from body to tail feather in isotope values (Fig. 1C). Overall >60 % of the 32 samples for each isotope follow the same

directional difference. The 95 % confidence intervals of the differences between each feather type for each isotope suggest that with a larger sample (n = 50) the range of differences between feather types would be less than observed in this relatively small sample group (Appendix 1).

Table 1: Matrix for $\delta^{13}C$, $\delta^{15}N$ and $\delta^{2}H$ of 32 African grey parrots, showing the percentage of feathers which are more positive or negative in a specific isotope than the other feather types, P = primary (P10), B = body (breast), and T = tail feather (outer right).

	$\delta^{13}C$			**$\delta^{15}N$**			**$\delta^{2}H$**		
%	**P**	**B**	**T**	**P**	**B**	**T**	**P**	**B**	**T**
P <		81	97		25	16		74	65
B <	19		63	75		28	26		29
T <	3	37		84	72		35	71	

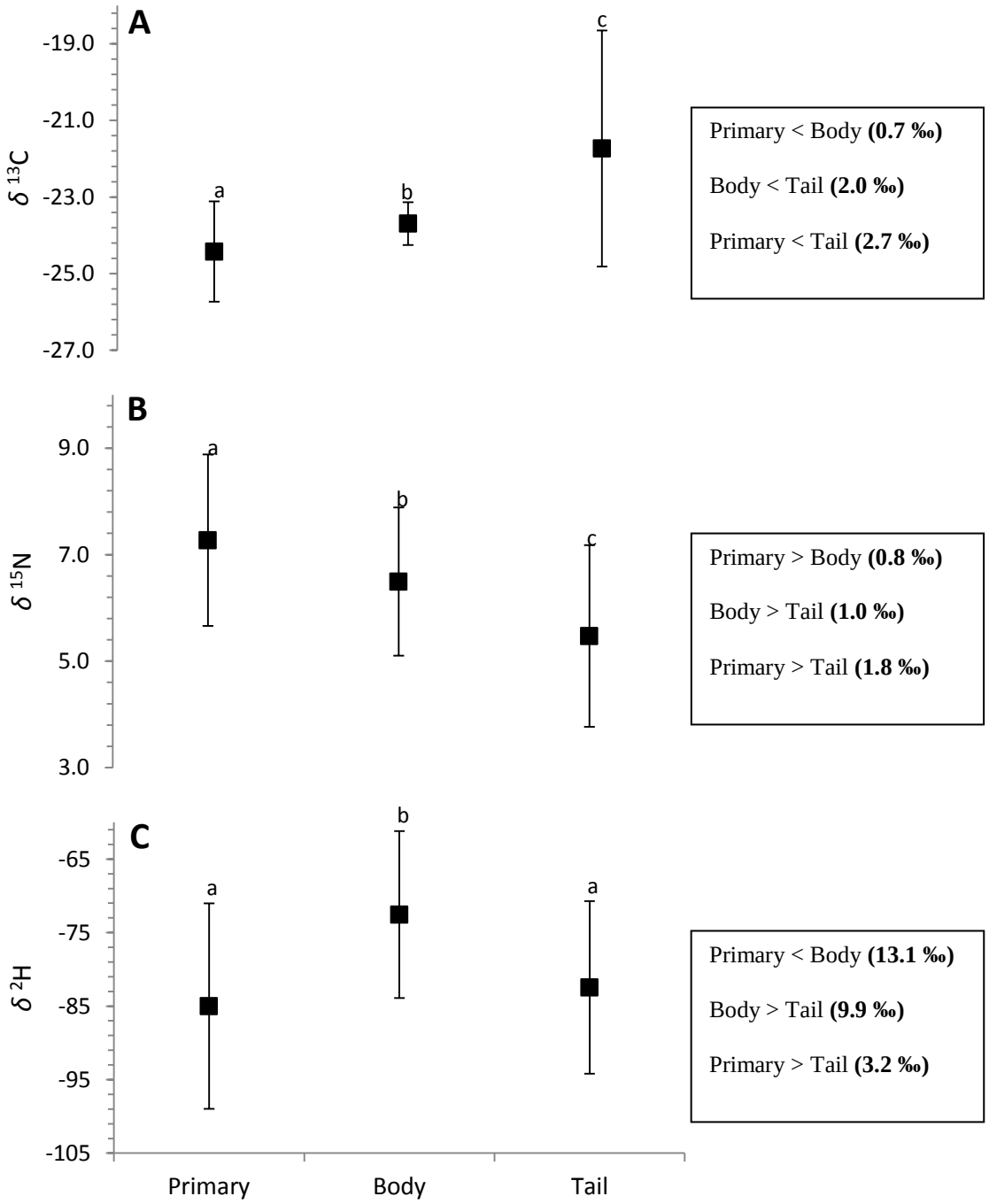


Figure 1: Mean (± SD) of, A) δ^{13}C, B) δ^{15}N, and C) δ^{2}H for primary (10), body (breast), and tail feathers (outer right) of 32 African grey parrots. Standards: Merck Gel (δ^{15}N = 6.8 ‰, δ^{13}C = -20.57 ‰), Caribou hoof (δ^{2}H = -197 ‰) and Kudu horn (δ^{2}H = -54.1 ‰). Difference in letter denotes significant difference between feather types (Paired *t*-Test/Wilcoxon Signed-Rank Test; $P < 0.0167$) (Appendix 1).

Discussion

The significant difference in δ^{13}C, δ^{15}N, and δ^{2}H values between feather types of African grey parrots may be different to the isotopic patterns observed between feather types of other species (Mizutani *et al.* 1992; Thompson and Furness 1995; Chamberlain *et al.* 1997; Kelly *et al.* 2002; Hedd and Montevecchi 2006; Rocque *et al.* 2006; Becker *et al.* 2007; Fox *et al.* 2007; Jaeger *et al.* 2009; Evans *et al.* 2012), but is understandable due to several factors.

Physiological influences on isotope values

The fractionation and assimilation rate of isotopes as a result of variable growth rate in certain feather types (Tieszen *et al.* 1983; Kennedy and Krouse 1989; Mizutani *et al.* 1992; Gannes *et al.* 1997; Bearhop *et al.* 2003; Smith *et al.* 2008) may explain the isotope difference between feathers types. It is suggested that primary feathers of bullfinches (*Pyrrhula pyrrhula*) and greenfinches (*Carduelis chloris*) grow slower than both body and tail feathers (Newton 1967) and thus assimilate nutrients at different rates; this may also apply to African grey parrots. This growth rate pattern could be suggested by the results of all the isotope values between feather types. Both δ^{13}C and δ^{15}N are routed into the tissues of an organism through diet (DeNiro and Epstein 1978; 1981) and δ^{2}H via dietary water and exchangeable hydrogen in precipitation (DeNiro and Epstein 1981; Cormie *et al.* 1984; Hobson and Wassenaar 1997; Hobson *et al.* 1999). However, isotopes may be routed differently or stored in body pools for different tissues/feather types, resulting in opposite trends being observed among the feathers for each isotope (Tieszen *et al.* 1983; Kennedy and Krouse 1989; Mizutani *et al.* 1992; Gannes *et al.* 1997; Bearhop *et al.* 2002; Ayliffe *et al.* 2004; Dalerum and Angerbjörn 2005; Wassenaar and Hobson 2006; Storm-Suke *et al.* 2012). It is known that isotopes fractionate differently between tissues (Sealy *et al.* 1987; Hobson

and Clark 1992b; Gannes *et al.* 1997; Cherel *et al.* 2005) resulting in different isotopic values being observed and fractionation may even differ within individual tissues (Sealy *et al.* 1987; Wassenaar and Hobson 2006). Ayliffe *et al.* (2004) suggest that the production of horse blood and growth of horse hair source nutrients from three different pools at three different rates, resulting in variations of δ^{13}C values between tissues and within tissues. δ^{2}H may also be sourced from different pools at different rates depending on the metabolic requirements (Wassenaar and Hobson 2006; Storm-Suke *et al.* 2012). Similar δ^{13}C and δ^{2}H sourcing could be occurring in African grey parrots. Feathers which are less likely to fall out and which are moulted annually (primary feathers) might be sourcing nutrients from different stores and at a slower rate than those of feathers which may be replaced more frequently (body feathers) and would require nutrients at a faster rate. Whether feather types differ as significantly as different tissues for fractionation is still questionable.

If the difference in feather growth rate and nutrient sourcing are not influencing the isotope values, there could be potential that physical feather size and its volume to surface area ratio could be influencing at least δ^{2}H values (Bearhop *et al.* 2003). Feathers with an increased surface area to volume ratio, such as primary and tail feathers compared to body feathers experience increased exchange of deuterium (Bearhop *et al.* 2003). This could be the reason that the δ^{2}H values of these two larger feather types have more negative values than body feathers, as these feathers exchanged with more negative δ^{2}H at some point in their growing phase (Chamberlain *et al*. 1997).

Geographical influences on isotope values

Like feather growth rate influencing isotope values, so too can the location in which the feather was grown (Hobson and Clark 1992a; Mizutani *et al.* 1992; Bearhop *et al.* 2002; Symes and Woodborne 2011; Johnson 2014). One possibility resulting in δ^{13}C values being

more negative and δ^{15}N values being more positive in primary feathers than in body and tail feathers is that primary feathers are moulted prior to tail and body feathers (R. Hines, pers. comm.). Primary feathers may reflect these positive and negative isotope values respectively as the parrots might have been feeding in isotopically different locations during the different moult phases (Dalerum and Angerbjörn 2005; Symes and Woodborne 2011) or moving between areas of different aridity (van der Merwe *et al.* 1990; Hall-Martin *et al.* 1993; Hobson 1999; Chamberlain *et al.* 2000; Hobson 2005; Hobson *et al.* 2012b; Larson *et al.* 2013). With respect to δ^{2}H values differing between feather types, feathers reflect the global δ^{2}H precipitation model. As it is suggested that primary feather are moulted before tail and body feathers respectively (R. Hines, pers. comm.) it is possible that the parrots were moving between regions of differing δ^{2}H isotope values during the moult phase. Body feathers having more positive δ^{2}H values compared to both primary and tail feathers further suggests that the parrots had moved from an isotopically negative or moist region (Neto *et al.* 2006) into a more positive δ^{2}H or drier region (Neto *et al.* 2006) between moulting their primary feathers and growing new body feathers. After which they then had moved back into the isotopically negative region prior to growing new tail feathers (Chamberlain *et al.* 1997; Hobson and Wassenaar 1997; Hobson 1999; Kelly *et al.* 2002; Hobson *et al.* 2012; Larson *et al*. 2013). The changes in isotope values between feather types suggests the possible movement and tracking of food sources throughout the moulting period. This would explain the possible differences in δ^{13}C and δ^{15}N of the primary feathers. Alternatively body feathers could have been grown during an isotopically more positive time of the season with respect to δ^{2}H (Yohannes *et al.* 2005; Smith *et al.* 2008; Terzer *et al.* 2013).

Standardising factors

The proposed standardising factors, derived from the predictable isotope differences between feather types were suggested as the majority of the differences were in the same direction and

this maintains consistency (Greer *et al.* 2015). An increase of 2.7 ‰ for $\delta^{13}C$ from primary and 2.0 ‰ from body is suggested to standardise to tail feather values. For $\delta^{15}N$ a decrease of 1.8 ‰ for primary and 1.0 ‰ for body feathers is suggested to standardise to tail feather values. Whilst for $\delta^{2}H$ an increase of 2.3 ‰ is suggested for primary to tail feather standardisation, though being not significantly different it may not be influential. Body feathers require a 9.8 ‰ decrease to standardise to tail feather values. The isotope differences between feather types suggest that it is important to select a specific tissue with respect to the intended research and to consistently maintain this choice. However, consistently selecting the same tissue type or feather type is not always possible. This study suggests that it may be possible to formulate factors which will standardise the isotope values of primary and body feathers to those of tail feathers, or additional combinations. The reason the standardising factors were calculated to convert primary and body feather values to tail feather values was that feathers sampled from living birds are usually those from the tail (Cherel *et al*. 2000; Hobson 2001; Fair *et al*. 2013; Ogden *et al.* 2015) as removal of primary (flight) feathers may negatively impact the mobility of the sampled individual. In addition, feathers sampled from museum specimens are usually body feathers, to preserve the integrity of the museum specimens. These standardising factors will further maintain consistent sampling and allow for comparison within and between studies resulting in isotope analysis becoming more applied in forensic ecology. These standardisation values may only be appropriate for use with parrot species and more specifically African grey parrots.

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Appendix

Appendix 1: Statistical differences between feather types of 32 African grey parrots for each isotope ($P < 0.0167$), * shows significant difference between feather types.

Feather comparison	95 % C.I. of differences	Statistic (Paired *t*-Test/Wilcoxon Signed-Rank test)
$\delta^{13}C$		
Primary > Body	1.2–0.2	W = -326, df = 31, P = 0.002*
Body > Tail	3.1–0.9	W = -317, df = 31, P = 0.001*
Primary > Tail	3.8–1.5	W = -446, df = 31, P < 0.001*
$\delta^{15}N$		
Primary < Body	0.2–1.4	t = 2.5759, df = 31, P = 0.015*
Body < Tail	1.8–0.3	t = 2.7970, df = 31, P = 0.009*
Primary < Tail	0.9–2.7	t = 4.1131, df = 31, P = 0.001*
$\delta^{2}H$		
Primary < Body	19.2–7.0	W = -371 df = 30, P = 0.001*
Body > Tail	3.4–16.4	W = 273, df = 30, P = 0.008*
Primary > Tail	9.1–2.7	W = -158, df = 30, P = 0.124

Chapter 3: Isotope analysis determining provenance

"Not responding is a response - we are equally responsible for what we don't do." **- Jonathan Safran Foer**



IDENTIFYING CAPTIVE AND WILD AFRICAN GREY PARROTS (*PSITTACUS ERITHACUS*) USING STABLE ISOTOPES

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Abstract

The global model of the distribution of hydrogen in precipitation, and how this is predictably assimilated into feathers, has proved useful in delineating the geographical origins of migrating birds. The relationship between the isotope signature of tissue and environment has also become a useful tool in forensic ecology. This study assessed stable isotopes in a suspected illegal shipment of African grey parrots (*Psittacus erithacus*) that died in transit during a domestic flight in South Africa, in an effort to determine their original provenance. Feather values were normalised using a tail feather standardisation factor to allow a comparison to be made across feather types and determine whether standardising feathers to a single feather type was appropriate. The mean (± SD‰) isotope values for the tail feather standardised and Suess effect corrected wild parrot feathers, from known origins ($\delta^{13}C$ = -22.1 ± 1.1 ‰; $\delta^{15}N$ = +5.6 ± 2.5 ‰; $\delta^{2}H$ = -86.4 ± 16.6 ‰) differed significantly from the values for the tail feather standardised parrot feathers from known captive locations ($\delta^{13}C$ = -19.1 ± 1.4 ‰; $\delta^{15}N$ = +5.4 ± 1.6 ‰; $\delta^{2}H$ = -58.6 ± 18.5 ‰) for $\delta^{13}C$ and $\delta^{2}H$. The unknown parrot feather values ($\delta^{13}C$ = -22.09 ± 0.9 ‰; $\delta^{15}N$ = +5.1 ± 1.7 ‰; $\delta^{2}H$ = -84.2 ±

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16.0 ‰) differed significantly from the captive parrot feathers, but did not differ significantly from wild parrot feathers for $\delta^{13}C$ and $\delta^{2}H$. This suggests that the unknown feathers had a wild provenance. It is clear that provenance can be determined using stable isotope analysis, at least with African grey parrots, and that standardising isotope values to those of a single feather type may improve comparisons between different feather types.

Keywords: *Forensic ecology, African deuterium, deuterium gradient, isotope variability, illegal trade, provenance, Suess effect.*

Introduction

Isotope analyses are a valuable tool in determining the migratory origins of animals (Peterson and Fry 1989; Vogel *et al.* 1990; van der Merwe *et al.* 1990; Cormie *et al.* 1994), specifically birds that move across continents (Hobson 1990; Chamberlain *et al.* 1997; Hobson and Wassenaar 1997; Wassenaar and Hobson 2000; Kelly *et al.* 2002). Similarly, stable isotopes have become an important method in forensic ecology for determining the provenance of illegally traded materials, such as ivory from African elephants (*Loxodonta africana*), horns of white (*Ceratotherium simum*) and black rhinoceros (*Diceros bicornis*) (Vogel *et al.* 1990; van der Merwe *et al.* 1990; Hall-Martin *et al.* 1993; Koch *et al.* 1995; Ehleringer *et al.* 2007), and plants such as the opium poppy (*Papaver somniferum*), coca (*Erythroxylum* spp.), cannabis (*Cannabis sativa*), and cycads (*Encephalartos* spp.) (Ehleringer *et al.* 1999; 2000; 2007; Retief 2013). Additional uses of stable isotopes in forensic ecology included determining the origin of deceased humans (Beard and Johnson 2000; Sharp *et al.* 2003; Fraser *et al.* 2006).

To date, to the best of our knowledge, no research, has been done on avian species with respect to isotope analysis and its uses in forensic ecology. The death of a consignment of *c.* 700 African grey parrots (*Psittacus erithacus*) in December 2010 presented an opportunity to use stable isotope analysis as a forensic technique, with the potential to determine the captive or wild origin of African grey parrots from an unknown location. Currently their provenance is unknown, but it is suggested that many of the traded African grey parrots that are claimed to be captive bred are in fact wild caught (Mulliken 1995). We used stable isotope analysis of $\delta^{13}C$, $\delta^{15}N$, and $\delta^{2}H$ in a forensic ecology approach to investigate the provenance of the deceased African grey parrots. Most studies have used $\delta^{2}H$ to understand the origins of migrating animals and/or illegally traded material. However, the main challenge in using $\delta^{2}H$ in Africa is, there is a weak $\delta^{2}H$ gradient and very little variation across the continent (Møller and Hobson 2004; Pain *et al.* 2004; Bowen *et al.* 2005; Wakelin *et al.* 2010). This approach should not be rejected though, and the potential for using multiple isotopes remains to be further explored (see Hobson *et al.* 2012). The use of multiple isotopes may be more informative, as a single isotope's values may not differ between sites whilst another isotope may, allowing for interpretation of the range of isotope values in a region (van der Merwe *et al.* 1990; Vogel *et al.* 1990; Hall-Martin *et al.* 1993; Ehleringer *et al.* 1999; 2000).

Factors influence captive and wild $\delta^{13}C$ values

African grey parrots inhabit the tropical forests of central and western Africa (Mulliken 1995; Forshaw 2006; Melo and O'Ryan 2007; Tamungang *et al.* 2013; Martin *et al.* 2014) suggesting they would possess $\delta^{13}C$ values aligned with the C3 plants on which they feed; between -37 ‰ to -24 ‰ (Peterson and Fry 1987; Kennedy and Krouse 1989; Marshall *et al.* 2007; Schoeninger 2010; Still and Powell 2010; Symes 2012; Tamungang *et al* 2013; 2014). However, research on primates by Schoeninger (2010) and forest birds by Scott (2012)

suggest that C3 forest δ^{13}C values may be more in the range of -30 ‰ to -24 ‰. Wild species of fish; Atlantic salmon (*Salmo salar*), gilthead sea bream (*Sparus aurata*), and bluefin tuna (*Thunnus thynnus*) have been distinguished from their captive bred counterparts as a result of differences in isotope values influenced by dietary differences (Dempson and Power 2004; Serrano *et al.* 2007; Vizzini *et al.* 2010). It therefore may not always be as important to suggest the exact origin of a sample, as it is to determine the samples provenance, especially in terms of trade. The difference in δ^{13}C values between wild and captive species may also be observed in terrestrial species, like African grey parrots. δ^{13}C values of captive African grey parrots (-20.1 ± 0.2 ‰ to -20.9 ± 0.2 ‰; Symes *et al.* In prep) were more positive than; frugivorous bird species from forests (-24.6 ± 0.3 ‰ to -25.6 ± 0.2 ‰; Scott 2012), Cape parrots (*Poicephalus robustus*) (-21.9 ± 0.8 ‰; Symes and Woodborne 2009), and red-crowned parakeets (*Cyanoramphus novaezelandiae*) (-24.0 ± 0.6 ‰; Hawke and Holdaway 2009). The respective studies included different species from different locations; however, it suggests that isotope values may differ between known wild and captive birds and parrots. Captive and wild δ^{13}C values may also differ (Kennedy and Krouse 1989; Hobson and Clark 1992a; 1992b; Mizutani *et al.* 1992; Bearhop *et al.* 2003) or show increased variability (Symes *et al*. In prep) as a result of wild stresses and anthropogenic influences (Rubenstein and Hobson 2004).

Factors influencing captive and wild δ^{15}N values

Factors which could influence δ^{15}N values between wild and captive species include differences in precipitation (van der Merwe *et al.* 1990; Amundson *et al*. 2003), soil and vegetation type (Vitousek *et al*. 1989, Schoeninger *et al.* 1997; Evans 2001), and ultimately dietary differences (DeNiro and Epstein 1981). All of which are interconnected. With increased precipitation; common to tropical forests there is a notable decrease in δ^{15}N values (van der Merwe *et al.* 1990; Amundson *et al*. 2003). These more negative δ^{15}N values are

reflected in the soil and vegetation of tropical forests (Vitousek *et al*. 1989; Schoeninger *et al.* 1997; Evans 2001) and further in the tissues of the animals of that region (DeNiro and Epstein 1981; Schoeninger *et al.* 1997). In these circumstances, determining the provenance of African grey parrots would focus mainly on diet with respect to δ^{15}N (DeNiro and Epstein 1981). As diet is the most important factor when using δ^{15}N as a forensic tool, it must be noted that many of these differences may only occur as the result of anthropogenic influences (Pardo and Nadelhoffer 2010). Anthropogenic influences may alter the δ^{15}N values of food sources, through irrigation or the introduction of exotic food sources (Evans 2001; Amundson *et al.* 2003). The difference in δ^{15}N values between wild and captive species potentially being under the influence of anthropogenic change should therefore be accepted with caution. δ^{15}N values may also be influenced by stresses; moult, nutrient stress, and reproduction in African grey parrots (Kennedy and Krouse 1989; Hobson and Clark 1992a; 1992b; Mizutani *et al.* 1992; Bearhop *et al.* 2003). Isotope values may maintain reduced variability with respect to captive parrots, as Symes *et al.* (In prep) showed reduced isotopic variability in captive African grey parrots. This could be an important factor to differentiate between captive and wild traded species.

Factors influencing captive and wild δ^{2}H values

The δ^{2}H gradient on the African continent is not as strong as in America and Europe and makes determining the geographical origins of species from Africa significantly more difficult (Møller and Hobson 2004; Pain *et al.* 2004; Bowen *et al.* 2005; Yohannes *et al.* 2005; 2007; Wakelin *et al.* 2010), but may still prove valuable to specific studies. Species originating in the west of Africa will have significantly different δ^{2}H values from those originating in central and eastern Africa (Bowen *et al.* 2005; Neto *et al.* 2006; Bairlein *et al.* 2012; Reichlin *et al.* 2013). The east-west δ^{2}H gradient of central Africa can prove useful when determining the provenance of species traded from this region; such as African grey

parrots. There is a possibility that $\delta^{2}H$ may differ between wild and captive species as a result of different water sources being incorporated; wild species incorporating dietary water through natural water sources and captive species through tap or borehole sources from regions with different precipitation $\delta^{2}H$ values. This may only prove beneficial if the comparison between wild and captive species is being drawn in the same geographical region, as $\delta^{2}H$ gradients and even tap water sources are similar across extensive regions of the African continent (West *et al.* 2011). Captive African grey parrot, $\delta^{2}H$ values also showed reduced variability (Symes *et al.* In prep). This could distinguish captive from wild bred African grey parrots as wild parrots are distributed across central Africa, with fluctuating $\delta^{2}H$ values, ranging from more negative values ($\delta^{2}H$ = -33.1 ± 1.7 ‰) in the west (Ivory Coast and Ghana) and positive values ($\delta^{2}H$ = -15.2 ± 9.6 ‰) in the east (Uganda and Kenya) (Bairlein *et al.* 2012; Gutiérrez-Expósito *et al.* 2015).

The aim of this study was to sample feathers from known captive and wild African grey parrots for $\delta^{13}C$, $\delta^{15}N$, and $\delta^{2}H$ and compare their isotope values with those of deceased African grey parrot feathers from unknown origins, here forth referred to as the unknown African grey parrots. The results may help determine whether using stable isotope analyses can infer African grey parrot provenance. This could create a method for monitoring and possibly controlling the illegal trade of African grey parrots and other illegally traded animals. Feather standardising factors (Chapter 2) were applied to standardise all sampled feathers to values equivalent of tail feathers.

We hypothesised that, i) the stable isotope ($\delta^{13}C$, $\delta^{15}N$, and $\delta^{2}H$) values of the African grey parrot feathers of known wild and captive origin would differ significantly, ii) the African grey parrot feathers of unknown origin (deceased birds) were from wild populations, iii) we also hypothesised that the $\delta^{2}H$ values of the wild African grey parrot feathers would match the east-west $\delta^{2}H$ gradient of central Africa (Bairlein *et al.* 2012). In

addition to using isotope values to differentiate between wild and captive African grey parrot feathers, iv) the wild African grey parrot feathers were also believed to show increased isotope variability compared to those of captive African grey parrots. The same hypotheses were tested for the tail feather standardised feather values, v) it was thought that the standardised values would show the same differences as the uncorrected values but with reduced isotope variability.

Methods and Materials

Study species

A total of *c.* 192 African grey parrot feathers were analysed for $\delta^{13}C$, $\delta^{15}N$, and $\delta^{2}H$ from three separate groups; unknown (n = 100), captive (n = 50), and wild (n = 42). The unknown African grey parrot feathers (primary feathers) were selected randomly from a subsample of *c.* 250 randomly selected parrots from a larger consignment of *c.* 700 parrots that died in transit from Johannesburg to KwaZulu-Natal, South Africa in 2010. The parrots were from unknown origins. Feathers of all types were collected from captive African grey parrots across South Africa, only including feathers from birds >1 year in captivity, ensuring the feathers were grown in captivity as African grey parrots generally moult annually (R. Hines pers. comm.). Body feathers from wild African grey parrots were obtained from museum collections, allowing preservation of the specimens (n = 37) and tail feathers from some wild sites in their native distribution (n = 5). Museum samples were obtained from the Ditsong National Museum of Natural History (DNMNH), Pretoria, South Africa (n = 1) and The American Museum of Natural History (AMNH), New York, USA (n = 37). The museum samples all originated from known wild origins (Appendix 1).

$\delta^{13}C$ and $\delta^{15}N$ isotope analysis

Samples weighing *c*. 0.5 mg were cut from the distal region of the vane of each feather and placed into toluene washed tin cups. Merck Gel ($\delta^{15}N$ = +6.8 ‰, $\delta^{13}C$ = -20.57 ‰) standards weighing 0.2, 0.4, and 0.6 mg respectively were weighed out to allow sample peak values to fall within the range of peaks of $\delta^{13}C$ and $\delta^{15}N$. A standard and a blank were sampled at the beginning, end, and after every 12 unknown samples to determine if there was any remaining residue from the previous sample as well as if corrections were needed if the standard values had deviated. The weighed samples and standards were analysed in a Flash EA1112 Elemental Analyser coupled to a DeltaV mass spectrometer via a Conflo IV interface. Combustion took place at 1020˚C and a copper reduction oven was used at 650˚C. Water was trapped in a perchlorate water trap. N_2 and CO_2 are separated in a Gas Chromatograph column at 45˚C.

$\delta^{2}H$ isotope analysis

Samples weighing 0.2–0.4 g were cut from beneath the $\delta^{13}C$ and $\delta^{15}N$ sample point on the distal region of the vane of each feather and placed in toluene washed silver cups. Twelve 0.2, 0.4, and 0.6 g standards comprising of 6 matrix-matched Caribou hoof (CBS; $\delta^{2}H$ = -197 ‰ and Kudu horn (KHS; $\delta^{2}H$ = -54.1 ‰) (Wassenaar and Hobson 2010) were weighed out to correct for any sampling discrepancies (Meier-Augenstein *et al.* 2013). The weighed samples and standards were left to equilibrate in the same ambient conditions for 10 days to allow for the free exchange of atmospheric deuterium to occur (Meier-Augenstein *et al.* 2013). Samples were analysed through pyrolysis using a Thermo DeltaV Advantage mass spectrometer coupled to a Flash HT Plus elemental analyser. Corrections were made to the sample values using a linear regression based on the actual values for the standards versus the measured value (Wassenaar and Hobson 2003).

Statistical analysis

A Shapiro-Wilk test was used to test for normality (Shapiro and Wilk 1965). To account for the Suess effect the $\delta^{13}C$ values of the museum obtained specimens were corrected to the 2010 predicted $\delta^{13}C$ values (Appendix 2) (Francey and Allison 1995; Francey *et al.* 1999). Differences between sample groups; unknown, captive, and wild were confirmed using ANOVA/Kruskal-Wallis tests coupled with Tukey HSD/Dunn's post hoc tests, results presented in Table 1. To determine the variability between sample groups the range was calculated. Primary (P10) and body feathers isotope values were normalised to the isotopic values of tail feathers using the feather type standardisation factors for African grey parrots suggested in Chapter 2, to maintain consistent sampling of feather material. These differences were compared to those of the uncorrected values to determine whether it is applicable to standardise to a specific feather type. The uncorrected and tail feather standardised wild feather values were plotted and correlated against the longitude from which the feathers were obtained. Their $\delta^{2}H$ values were plotted along with the predicted annual $\delta^{2}H$ in precipitation values of those regions; calculated using the Online Isotopes in Precipitation Calculator (OIPC) (Bowen and Revenaugh 2003; Bowen 2015). A one-way ANCOVA was used to determine if $\delta^{2}H$ values differed significantly between the different African countries where feathers were obtained from, with longitude as the covariate.

Results

The museum samples varied in collection date from 1899–1944, which required correction of $\delta^{13}C$ values for the Suess effect of between 1.26–1.48 ‰ (Appendix 2). A Shapiro-Wilk test confirmed that the $\delta^{15}N$ and $\delta^{2}H$ samples were normally distributed,

whilst the $\delta^{13}C$ samples were not. The original uncorrected African grey parrot feathers from all three sample groups were significantly different for $\delta^{13}C$ and $\delta^{2}H$, but not $\delta^{15}N$ (Table 1; Fig. 1a). As there was no significant difference between any of the sample groups for $\delta^{15}N$, we felt it would be appropriate presenting a bi-plot of $\delta^{13}C$ and $\delta^{2}H$ values to graphically describe our comparisons. For the feather standardised African grey parrot feathers a Shapiro-Wilk test confirmed that the $\delta^{15}N$ and $\delta^{2}H$ samples were normally distributed, whilst the $\delta^{13}C$ samples were not. Analysis of the tail feather standardised African grey parrot feathers revealed that there were significant differences in $\delta^{13}C$ and $\delta^{2}H$ values between the three sample groups, but not for $\delta^{15}N$. The unknown and wild feathers did not differ from one another, but were both significantly different to the captive feathers for $\delta^{13}C$ and $\delta^{2}H$ (Table 1; Fig. 1b). As again there was no significant difference between any of the sample groups for $\delta^{15}N$, we felt it would be appropriate presenting a bi-plot of only $\delta^{13}C$ and $\delta^{2}H$.

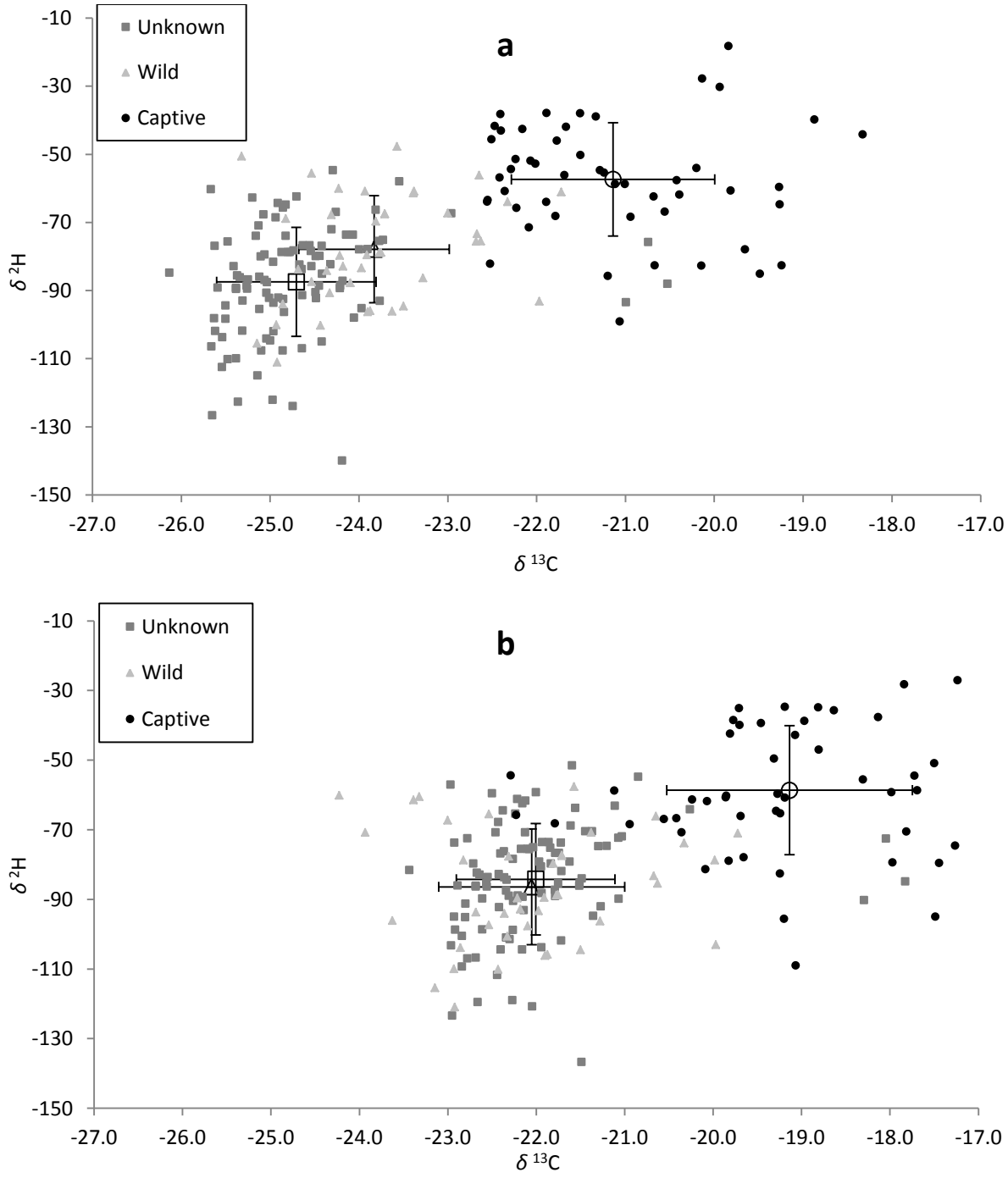


Figure 1: Stable $\delta^{13}C$ and $\delta^{2}H$ ‰ values of unknown, wild, and captive African grey parrot feathers corrected for the Suess effect (Francey and Allison 1995; Francey *et al.* 1999), showing, a) original uncorrected values of feathers, and b) feather values standardised for feather type (tail feather). Standards: Merck Gel ($\delta^{13}C$ = -20.57 ‰), Caribou hoof ($\delta^{2}H$ = -197 ‰) and Kudu horn ($\delta^{2}H$ = -54.1 ‰). Mean (± SD ‰) values of unknown, wild, and captive feathers are shown.

Table 1: Mean (± SD ‰) values and range (in parentheses) of $\delta^{13}C$, $\delta^{15}N$, and $\delta^{2}H$ of original uncorrected and tail feather standardised unknown, captive, and wild African grey parrot feathers corrected for the Suess effect, different lettering showing significant difference between sample groups (Tukey HSD/Dunn's $P < 0.01$).

Isotope	Unknown (‰) (range)	Captive (‰) (range)	Wild (‰) (range)	Statistics
Original uncorrected feathers				
$\delta^{13}C$	-24.7 ± 0.9 [a] (-20.5 to -26.1)	-21.1 ± 1.1 [b] (-18.3 to -22.6)	-23.6 ± 0.8 [c] (-21.5 to -25.0)	Kruskal-Wallis: $H = 121.03$; df = 2; $P < 0.001$
$\delta^{15}N$	+6.9 ± 1.7 [a] (3.8 to 11.5)	+6.6 ± 1.6 [a] (3.0 to 10.8)	+6.5 ± 2.3 [a] (0.4 to 14.1)	ANOVA: $F = 0.72$; df = 2; $P = 0.488$
$\delta^{2}H$	-87.4 ± 16.0 [a] (-54.7 to -139.9)	-57.3 ± 16.6 [b] (-18.3 to -99.1)	-77.9.3 ± 15.7 [c] (-47.7 to -111.0)	ANOVA: $F = 58.11$; df = 2; $P < 0.001$
Standardised to tail feather				
$\delta^{13}C$	-22.0 ± 0.9 [a] (-17.8 to -23.4)	-19.0 ± 1.4 [b] (-15.6 to -22.3)	-22.1 ± 1.1 [a] (-19.5 to -24.6)	Kruskal-Wallis: $H = 89.44$; df = 2; $P < 0.001$
$\delta^{15}N$	+5.1 ± 1.7 [a] (2.0 to 9.7)	+5.4 ± 1.6 [a] (2.0 to 9.0)	+5.6 ± 2.5 [a] (-0.6 to 14.1)	ANOVA: $F = 1.35$; df = 2; $P = 0.262$
$\delta^{2}H$	-84.2 ± 16.0 [a] (-51.5 to -136.7)	-58.6 ± 18.5 [b] (-27.0 to -109.0)	-86.2 ± 16.6 [a] (-57.6 to -120.9)	ANOVA: $F = 45.18$; df = 2; $P < 0.001$

The original uncorrected unknown feathers had the greatest $\delta^{13}C$ variability and the wild feathers the smallest (Table 1). For $\delta^{15}N$ the wild feathers had the greatest variability and the unknown the smallest (Table 1). For $\delta^{2}H$ the greatest variability was that of the unknown feathers and the smallest occurred in the wild feathers (Table 1). The tail standardised feathers showed a similar trend for $\delta^{2}H$, with the unknown feathers having the greatest variability and the unknown the smallest (Table 1). For $\delta^{15}N$ and, the wild feathers had the greatest variability and the captive the smallest (Table 1). However, for $\delta^{13}C$ the trend was opposite to that of $\delta^{15}N$, with the captive feathers having the greatest variability and the wild feathers the smallest range (Table 1).

The $\delta^{2}H$ values of the uncorrected and tail feather standardised feathers showed a similar increase with increased longitude to the predicted $\delta^{2}H$ in precipitation values based

on the OIPC, despite the average difference between the predicted and uncorrected feather value being $\delta^{2}H$ = 67.7 ‰ ± 16.3 (Fig. 2). The trend showed an increase in $\delta^{2}H$ feather values from the west to east coast of Africa (Fig. 2) for uncorrected (Pearson; R^2 = 0.18; N = 42; *P* = 0.005) and tail feather standardised (R^2 = 0.24; N = 42; *P* = 0.001) African grey parrot feathers.

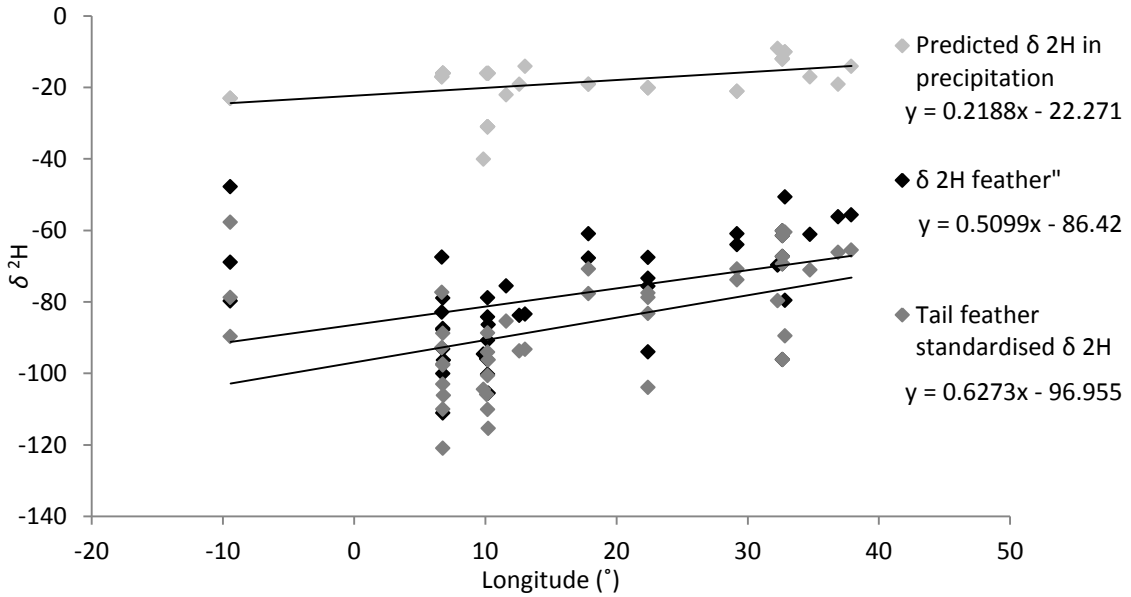


Figure 2: Predicted annual $\delta^{2}H$ in precipitation (OIPC; Bowen and Revenaugh 2003; Bowen 2015) and $\delta^{2}H_{feather}$ values of uncorrected and tail feather standardised wild African grey parrot feathers (n = 42) obtained from known origins (Ditsong National Museum of Natural History, South Africa, and American Museum of Natural History, USA) against longitude. Standards: Caribou hoof ($\delta^{2}H$ = -197 ‰) and Kudu horn ($\delta^{2}H$ = -54.1 ‰).

Table 2: Observed mean(±SD) and adjusted mean (covariate = longitude) δ^{2}H (‰) values of original uncorrected and tail feather standardised wild African grey parrot feathers obtain from museum specimens for African countries (ANCOVA $P = 0.05$). Democratic republic of Congo abbreviated to DRC

		Original uncorrected δ^{2}H (‰)		Tail feather standardised δ^{2}H (‰)	
ANCOVA		$F = 3.92$; df = 7; $P = 0.004$		$F = 5.0$; df = 7; $P < 0.001$	
Country	**Sample size (n)**	**Observed (x̄)**	**Adjusted (x̄)**	**Observed (x̄)**	**Adjusted (x̄)**
Liberia	3	-65.4 ± 16.3	-42.2	-75.3 ± 16.3	-51.7
Príncipe	2	-75.1 ± 10.9	-66.9	-85.0 ± 10.9	-76.7
Nigeria	7	-90.5 ± 12.3	-82.3	-103.4 ± 10.3	-95.1
Cameroon	5	-89.7 ± 8.4	-84.6	-99.6 ± 8.4	-94.4
Gabon	5	-89.4 ± 11.6	-85.1	-99.3 ± 11.6	-94.9
DRC	9	-71. 9 ± 11.6	-77.7	-81.1 ± 11.6	-87.0
Uganda	5	-70.8 ± 14.6	-86.7	-70.8 ± 14.6	-86.9
Kenya	3	-57.6 ± 3.0	-77.1	-67.5 ±3.0	-87.2

δ^{2}H values differed significantly between the different longitudes (respective countries) for the original uncorrected ($F = 3.92$; df = 7; $P = 0.004$) and tail feather standardised ($F = 5.0$; df = 7; $P < 0.001$) wild African grey parrot feathers (Table 2). The mean observed δ^{2}H values were lowest in east Africa and highest along the west coast of central Africa. δ^{2}H values adjusted to the influences of longitude (covariate) were the lowest in the west of Africa and highest along the west coast of central Africa

Discussion

The stable δ^{13}C and δ^{2}H values of the original uncorrected wild and unknown African grey parrot feathers were significantly different from the original uncorrected captive African grey parrot feathers and suggest that stable isotopes can be used to differentiate the provenance of African grey parrots as captive bred or wild caught. The δ^{13}C and δ^{2}H values of the tail feather standardised wild and unknown feathers did not differ significantly, but both differed significantly from the tail standardised captive feathers, which suggests that the

feathers from the African grey parrots of unknown origin were not from a captive provenance. This emphasises that stable isotope analysis can be used to differentiate the provenance of African grey parrots, but it is suggested that tail feather correction factor be applied. There were several reasons why the isotopic values of the wild and unknown feathers differed from the captive feathers.

Isotopic signatures to determine the provenance of organisms

African grey parrots or organisms that are given C3 diets would have significantly different $\delta^{13}C$ values to those of species that have utilised primarily C4 diets such as commercial parrot feed (Symes *et al.* In prep). The wild and unknown feathers reflected $\delta^{13}C$ values in alignment with feeding on C3 plants (Peterson and Fry 1987; Kennedy and Krouse 1989; Marshall *et al.* 2007; Still and Powell 2010; Symes 2012), whilst the feathers from known captive African grey parrots reflected a diet of C4 origin (Kennedy and Krouse 1989; Marshall *et al.* 2007; Still and Powell 2010; Symes 2012; Symes *et al.* In prep). These differences in $\delta^{13}C$ values between C3 and C4 are naturally occurring but an additional factor could be attributing to the isotope difference. The canopy affect is the notable decrease in $\delta^{13}C$ values between areas of increased canopy cover and areas exposed to the atmosphere (van der Merwe and Medina 1991; Schoeninger *et al.* 1997; Cerling *et al.* 2007). This depletion occurs as a result of the continual cycling of ^{13}C depleted CO_2 released by the decomposition of forest floor leaf litter in the zone between the floor and canopy layer (Schleser and Jayasekera 1985; van der Merwe and Medina 1991) and the low light conditions resulting in the decrease of $\delta^{13}C$ during photosynthesis (Farquhar *et al.* 1982). The canopy affect and its influence on $\delta^{13}C$ values could be a fundamental factor in forensic isotope ecology as tropical-forest-inhabiting species should reflect significantly different isotope values to organism that do not inhabit areas with increased tree cover; such as African grey parrots.

Over time the increase in pollution, mainly in the form of CO_2 through the burning of fossil fuels has caused a marked depletion in atmospheric ^{13}C, resulting in more negative $\delta^{13}C$ values over time (Keeling 1979; Friedli *et al.* 1986; Hilton *et al.* 2006; Verburg 2007); this is known as the Suess effect. It is suggested that correcting for the Suess effect is fundamentally important when using archived museum samples as failing to correct for changes in $\delta^{13}C$ values over time could result in inaccurate interpretation of museum sample $\delta^{13}C$ values, reflecting the incorrect provenance (Barquete 2012).

We expected that the $\delta^{2}H$ values would differ significantly between the wild and captive feathers, as the feathers come from completely different regions of Africa, though it is promising to see that there was no difference in values between the tail standardised unknown and wild feathers. This suggests that the unknown feathers were not from a captive provenance, and more likely from a wild provenance.

Isotopic variability to determine the provenance of organisms

Though the isotopic variability appeared similar between the three sample groups with respect to $\delta^{13}C$, the unknown and captive feathers in the original uncorrected and tail feather standardised samples respectively had the greatest variability. The variance may have been higher in the known captive African grey parrots than in those from the wild as a result of the captive samples coming from a broad range of locations and possibly receiving a variety of commercial parrot feeds. The wild African grey parrots, would also have been feeding on a varied diet, however possibly more consistent than captive feeds. For $\delta^{15}N$ the wild feathers in both the original uncorrected and tail feather standardised samples had the greatest variability. This increased variability could be as a result of the variable wild diets that the different individuals were exposed to, as well as the variable source locations across Africa. Wild feathers were 30 % less variable in $\delta^{2}H$ compared to both the unknown and captive

feathers from both the original uncorrected and tail feather standardised samples. This could be a result of the increased stresses and dietary variability that may occur as a result of the captive parrots being selected from a variety of locations (Kennedy and Krouse 1989; Hobson and Clark 1992a; 1992b; Mizutani *et al.* 1992; Bearhop *et al.* 2003; Symes *et al.* In prep). Symes *et al.* (In prep) show reduced variability in the $\delta^{13}C$ and $\delta^{15}N$ values of captive African grey parrots which could be a result of controlled living conditions and consistent diet; however, this study obtained feather samples from a single sample group in the same location. Studies on captive domestic chicken (*Gallus gallus*), Japanese quail (*Coturnix japonica*), ring-billed gulls (*Larus delawarensis*) (Hobson and Clark 1992b), and great skua (*Catharacta skua*) (Bearhop *et al.* 2002) suggest that nutritional stress can result in different fractionation rates of isotopes, resulting in tissues reflecting isotopic values different to when not under nutritional stress. Not only can nutritional stress result in isotopic differences through increased turnover rate, but so can water stress or dehydration (McKechnie *et al.* 2004; Smith and Dufty 2005; Symes 2012). Captive rock doves (*Columba livia*) which were restricted drinking water exhibited increased water loss through evaporative cooling and subsequently their tissue reflected isotopically more positive $\delta^{2}H$ values, as the lighter ^{1}H molecules are evaporated leaving the heavier ^{2}H molecules behind (McKechnie *et al.* 2004). It is also suggested that northern goshawk (*Accipiter gentilis*) $\delta^{2}H$ values differ as a result of the same exposure to water stresses (Smith and Dufty 2005). The wild feathers may have less variability as these parrots were free roaming and capable of not only sourcing locations to avoid the heat but also sourcing fresh and non-evaporated water sources. Captive birds may have been exposed to different levels of heat stress being kept in cages and their water sources were also exposed to different levels of evaporation. The unknown feathers had similar variability to the captive feathers, both being higher than the variability observed in the wild feathers. It can be suggested that these parrot feathers were also obtained from a

variety of locations, with small numbers of individuals representing different locations. This trend follows the patterns of illegal trade, as many birds are caught from a variety of locations to make the harvesting of these parrots profitable and because many locations are over harvested (Mulliken 1995; Eniang *et al.* 2008).

Isotopic correlation with the $\delta^{2}H$ gradient of Africa.

The $\delta^{2}H$ gradient of Africa is suggested to show a weak increase from the west coast to the east coast of central Africa (Hobson 2005; Bairlein *et al.* 2012; Hobson *et al.* 2012; Reichlin *et al.* 2013; Gutiérrez-Expósito *et al.* 2015). The predicted $\delta^{2}H$ values of precipitation for longitudinal points across central Africa did, as expected show a west to east increase in $\delta^{2}H$. The $\delta^{2}H$ values of the uncorrected and tail standardised wild feathers also followed the west to east increase of $\delta^{2}H$. The observed and adjusted mean $\delta^{2}H$ values suggested for the sampled countries in Africa provided a more robust means of determining where in the African continent the unknown African grey parrots could have come from. Parrots with $\delta^{2}H$ values in the -70 ‰ range suggested a more eastern to central African provenance, whereas $\delta^{2}H$ values <-90 ‰ suggested a more central and west-central coast provenance. The more positive $\delta^{2}H$ values of West Africa were different to what has previously been suggested (Hobson 2005; Bairlein *et al.* 2012; Hobson *et al.* 2012; Reichlin *et al.* 2013; Gutiérrez-Expósito *et al.* 2015). The samples from Liberia, which was the only far western country sampled, could have been from African grey parrots sourcing water from agricultural regions. This allows for suggestions to be made about whether confiscated African grey parrots and other organisms originated from the west or east coast of central Africa, creating opportunity for modelling and more accurate defining of origins if the $\delta^{2}H_{precipitation}$ and $\delta^{2}H_{feather}$ relationship is understood.

Using $\delta^{13}C$ and $\delta^{2}H$ isotope analysis showed promising results for forensic ecology and determining the difference between wild and captive of African grey parrots. Standardising multiple feathers to a single feather type enhances the interpretation of provenance when compared to feathers that were not standardised. Furthermore, considering that standardising the feathers maintains consistent sampling (Greer *et al.* 2015) and allows for intra- and inter-study comparisons, it is suggested that this method could be incorporated as well as enhanced over additional avian species. Using the west to east $\delta^{2}H$ gradient of central Africa could also suggest finer scale origins of African grey parrots. The ability to determine the provenance of an African grey parrot as well as the potential to suggest an exact origin could help with monitoring the illegal trade and tracking which countries or areas are involved (Martin *et al* 2014). Species which are illegally caught and traded from the wild may reflect wild isotope signatures, allowing for suggested identification of provenance to be made on confiscated or deceased traded parrots. By further coupling the west-east $\delta^{2}H$ gradient with multi-isotope analysis could help define more precise locations of illegally traded parrots and this will help in determining the countries or areas most affected and involved in the illegal trade of African grey parrots. Further research involving modelling of African grey parrots $\delta^{2}H$ values and the west-east gradient observed would enhance the ability to monitor and track the illegal trade of this species. These analyses could also potentially be replicated on organisms other than African grey parrots, also affected by the illegal trade.

Acknowledgments

Captive African grey parrot feathers were collected from many locations in South Africa. Wild feathers were supplied by The Ditsong National Museum of Natural History and

The American Museum of Natural History (CITES import permit No. 141367; for the import of feathers). The African grey parrot specimens were obtained from the University of KwaZulu-Natal, Pietermaritzburg. Isotope analyses were performed at The Mammal Research Institute Stable Light Isotope Laboratory, University of Pretoria, Pretoria (δ^{13}C and δ^{15}N) and iThemba LABS, Johannesburg (δ^{2}H).

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Appendices

Appendix 1: List of museum obtained African grey parrot feathers, displaying respective catalogue numbers, collection date, coordinates (decimal; negative values reflect south and west), and origins. Democratic Republic of Congo abbreviated to DRC.

Code	Museum	Catalogue Number	Feather type	Collection date	Coordinates		Location
					Latitude	Longitude	
WAG1	DNMNH (SA)	TM25343	Primary	1939	-5.8922	22.4027	DRC
WAG2	ANMH (USA)	257950	Body	1925	-5.8922	22.4027	DRC
WAG3	ANMH (USA)	703410	Body	1946	-5.85	13.05	DRC
WAG4	ANMH (USA)	620238	Body	1901	0.3645	6.6874	Príncipe
WAG5	ANMH (USA)	620237	Body	1901	0.3645	6.6874	Príncipe
WAG6	ANMH (USA)	344665	Body	1944	6.4281	-9.4295	Liberia
WAG7	ANMH (USA)	620217	Body	1902	4.7695	6.7681	Nigeria
WAG8	ANMH (USA)	208009	Body	1924	0.2827	34.7519	Kenya
WAG9	ANMH (USA)	620218	Body	1902	4.7695	6.7681	Nigeria
WAG10	ANMH (USA)	620226	Body	1908	-6.2755	29.1973	DRC
WAG11	ANMH (USA)	620214	Body	1902	4.7695	6.7681	Nigeria
WAG12	ANMH (USA)	620216	Body	1902	4.7695	6.7681	Nigeria
WAG14	ANMH (USA)	344666	Body	1944	6.4281	-9.4295	Liberia
WAG15	ANMH (USA)	620215	Body	1902	4.7695	6.7681	Nigeria
WAG16	ANMH (USA)	620221	Body	1907	-0.7015	10.2403	Gabon
WAG17	ANMH (USA)	620222	Body	1907	-0.7015	10.2403	Gabon
WAG18	ANMH (USA)	408936	Body	1929	-3.8957	17.8732	DRC
WAG19	ANMH (USA)	415159	Body	1930	4.9914	9.8618	Cameroon
WAG20	ANMH (USA)	415161	Body	1930	5.15	10.1833	Cameroon
WAG21	ANMH (USA)	415160	Body	1930	5.15	10.1833	Cameroon
WAG22	ANMH (USA)	408935	Body	1929	-3.8957	17.8732	DRC
WAG23	ANMH (USA)	415157	Body	1930	5.15	10.1833	Cameroon
WAG24	ANMH (USA)	344667	Body	1944	6.4281	-9.4295	Liberia
WAG25	ANMH (USA)	620219	Body	1902	4.7695	6.7681	Nigeria
WAG26	ANMH (USA)	620231	Body	-	1.3733	32.2903	Uganda
WAG27	ANMH (USA)	620227	Body	1908	-1.2973	36.9114	Kenya
WAG28	ANMH (USA)	620234	Body	1917	-0.0236	37.9062	Kenya
WAG29	ANMH (USA)	257949	Body	1925	-5.8922	22.4027	DRC
WAG30	ANMH (USA)	620232	Body	1899	0.986	32.8536	Uganda
WAG31	ANMH (USA)	344861	Body	1943	0.1702	10.1087	Gabon
WAG32	ANMH (USA)	415158	Body	1930	5.15	10.1833	Cameroon
WAG33	ANMH (USA)	257951	Body	1925	-5.8922	22.4027	DRC
WAG34	ANMH (USA)	620233	Body	1899	0.986	32.8536	Uganda
WAG35	ANMH (USA)	620223	Body	1907	-0.8365	12.6074	Gabon
WAG36	ANMH (USA)	620220	Body	1907	-0.8037	11.6094	Gabon
WAG37	ANMH (USA)	620213	Body	-	5.7117	6.8094	Nigeria
WAG38	ANMH (USA)	620225	Body	1908	-6.2755	29.1973	DRC

Appendix 2: The Suess effect correction factors based on Francey and Allison (1995) and Francey *et al.* (1999) used in $\delta^{13}C$ measured from African grey parrot feathers between 1899 and 1946. Corrections factors used to correct the historical $\delta^{13}C$ values of African grey parrot feathers to $\delta^{13}C$ values of 2010.

Decade	Correction factor
1890–1899	-1.728
1900–1909	-1.745
1910–1919	-1.615
1920–1929	-1.580
1930–1939	-1.531
1940–1940	-1.481

Chapter 4: Isotope variation in wing tracts

"Often, the greater our ignorance about something, the greater our resistance to change." **- Marc Bekoff**



STABLE ISOTOPE ANALYSIS OF PRIMARY FEATHERS OF AFRICAN GREY PARROTS (*PSITTACUS ERITHACUS*)

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Abstract

Stable isotope analyses of individual tissues and feathers have been used to infer geographic origins and at a finer scale (along wing tracts of individual birds) have been used to infer geographic origin and dietary shifts spatially and temporally. However, no research has been done using isotope analysis of primary feathers to infer the provenance of birds, such as African grey parrots. The primary feathers from the wings of deceased African grey parrots (*Psittacus erithacus*) (n = 6), thought to be illegally wild caught, were analysed for $\delta^{13}C$, $\delta^{15}N$, and $\delta^{2}H$. We hypothesised that birds undergoing active moult would reflect changes in isotope signatures consistent with a change from a wild to captive environment. We therefore sampled all primary feathers from the wings of parrots in active primary moult (n = 4) and wings of parrots not displaying moult (n = 2). Feathers which had not undergone or started moult reflected isotopic values representative of known wild African grey parrot isotope values, and showed little isotope variation among primary feathers. Certain feathers which were known to have moulted or started moult reflected values representative of known captive parrots and showed increased variability among the primary feathers. Suggesting a

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change from wild to captive locations during the moulting process and prior to death. After being caught from the wild, African grey parrots are believed to be exposed to stressful conditions during transportation. We present potential evidence that the African grey parrots were exposed to stresses while in captivity as their isotope values reverted from already captive values back to those of wild isotope values. Possibly as a result of stress induced increases in δ^{15}N and δ^{2}H values, or from sourcing nutrients from capital body stores acquired in wild conditions.

Keywords: *Isotopic variation, moult, provenance, nutritional stress, water stress, body stores, nutrient pools*

Introduction

Stable isotopes are naturally occurring forms of elements with different nuclear masses. The technique of isotope analysis compares the ratio of these different massed molecules that occur within different tissues (Rubenstein and Hobson 2004). The ratios of stable isotopes in tissues differ as a result of physiological factors; fractionation, assimilation rates, and stresses (Tieszen *et al.* 1983; Hobson and Clark 1992b; Bearhop *et al.* 2002) and source values. The isotope values of sources are influence by geographical factors; longitude, latitude, altitude, and aridity (Kennedy and Krouse 1989; Hobson 1999; 2005). Differences in isotopic values can vary as a result of incorporating food (van der Merwe *et al.* 1990; Hall-Martin *et al.* 1993; Hobson 1999a; Chamberlain *et al.* 2000; Hobson 2005; Hobson *et al.* 2012b; Larson *et al.* 2013) or water sources (Cormie *et al.* 1994; Chamberlain *et al.* 1997; Hobson and Wassenaar 1997; Hobson 1999a; Kelly *et al.* 2002; Hobson *et al.* 2012; Larson *et al*. 2013) from isotopically different locations or habitats. Isotopes obtained through diet or

water are assimilated into different tissues (DeNiro and Epstein 1978; 1981; Kennedy and Krouse 1989; Hobson *et al.* 1999b) at different rates as a result of the time tissues take to grow (turnover time) (Tieszen *et al.* 1983; Kennedy and Krouse 1989; Hobson and Clarke 1992b; Mizutani *et al.* 1992; Gannes *et al.* 1997; Bearhop *et al.* 2002; Ayliffe *et al.* 2004; Dalerum and Angerbjörn 2005). These source values are discriminated through fractionation into the tissues and result in differing isotope values. Isotopically different regions can be a result of differences in aridity, with xeric regions being isotopically more negative for $\delta^{13}C$ and $\delta^{15}N$ compared to mesic regions (van der Merwe *et al.* 1990; Hall-Martin *et al.* 1993; Ehleringer *et al.* 1999; Hobson 1999; 2005; Yohannes *et al.* 2007; Hobson *et al.* 2012b). Differences in stable isotopes have been used to infer dietary differences in organisms (DeNiro and Epstein 1978; 1981; Schoeninger *et al.* 1997; Sponheimer *et al.* 2003), delineate trophic levels (DeNiro and Epstein 1978; 1981; Hobson and Welch 1995; Herrera *et al.* 2003; Symes and Woodborne 2009), and determine the origins of migrating animals (van der Merwe *et al.* 1990; Cormie *et al.* 1994; Chamberlain *et al.* 1997; 2000; Yohannes *et al.* 2007; Ramos *et al.* 2009; Hobson *et al.* 2012b; Reichlin *et al.* 2013). However, few studies have been done on determining the provenance of illegally traded material (Vogel *et al.* 1990; van der Merwe *et al.* 1990; Hall-Martin *et al.* 1993; Koch *et al.* 1995; Ehleringer *et al.* 1999, 2000; 2007; Retief 2013), with no known studies focusing on avian material.

Isotopic differences have been observed in tissues which follow a time series in their growth, both in captive (Hobson *et al.* 1996; Ayliffe *et al.* 2004; Symes *et al.* In prep) and wild environments (Bearhop *et al.* 2003; Cerling *et al.* 2006; Ramos *et al.* 2009; Symes and Woodborne 2011). A change in isotope values along the time series, brought about by a change in diet, can be used to infer movement over temporal and spatial scales (Cerling *et al.* 2006; Ramos *et al.* 2009). Feathers are suggested to be a reliable tissue for inferring geographical origin or provenances, as feathers lock in the isotope values obtained through

dietary intake ($\delta^{13}C$ and $\delta^{15}N$) and $\delta^{2}H$ in precipitation values during the growing phase from the locations they were grown (Hobson 1999a). Feathers grown on opposite ends of the migratory route or at different times in the season may reflect isotopically different values, because either the two locations differ isotopically, e.g. they are dominated by C3 or C4 vegetation (Marra *et al.* 1998), or because the species migrating feeds on different food sources in the two locations (Dalerum and Angerbjörn 2005; Symes and Woodborne 2011).

Feathers grow at different rates (Smith *et al.* 2008) as well as moult at different times through the season, following predictable moult patterns (Inger and Bearhop 2008). These differential moult patterns and feather growth rates coupled with the movement between isotopically different regions aid in delineating origins, migratory routes, and dietary shifts of animals, mainly birds (Smith and Dufty 2005; Yohannes *et al.* 2007; Ramos *et al.* 2009). However, this is not isolated to migratory species but could also be observed if moult occurred between different isotope regions along trade routes or if there were a shift in diet and water consumption. Wild and captive Atlantic salmon (*Salmo salar*), gilthead sea bream (*Sparus aurata*) and bluefin tuna (*Thunnus thynnus*) provenance has been distinguished from one another as a result of differences in diet between locations i.e. fish farms and the open ocean (Dempson and Power 2004; Serrano *et al.* 2007; Vizzini *et al.* 2010). The potential isotopic difference between wild and captive diets could be applied to determining the provenance of illegally traded material, such as birds even if they are not a migratory species. If moult has occurred during the shift of location it is expected that the isotope values of primary feathers will differ in relation to the isotopic changes between the different locations. It is hypothesised that this same change in isotope values of primary feathers may be observed in birds that have been taken from wild environments into captivity.

Utilising African grey parrots (*Psittacus erithacus*) was beneficial in testing this hypothesis as they are a heavily traded species of African bird often caught illegally from

wild locations (Mulliken 1995; Melo and O'Ryan 2007; Tamungang *et al.* 2013; Martin *et al.* 2014). African grey parrots only move between different locations within their central African distribution in search of fruiting trees (Amuno *et al.* 2007; Tamungang *et al.* 2014) and rely almost entirely on C3 trees for food (Chapman *et al.* 1993; Juste 1995; Amuno *et al.* 2007; Tamungang *et al.* 2013; 2014). Lastly African grey parrots have a predictable annual moult pattern, which follows continuous divergent moult, starting at the inner primaries (P5 or P6) and first proceeding inwards towards P1 and then outwards to P10 (Howell 2010; Mallet-Rodrigues 2012; Pyle 2013). The moult process takes about *c.* seven weeks with large (primary) feathers growing at up to 8 mm/day (Mallet-Rodrigues 2012). Species caught from wild environments and transferred into captive environments could show isotopic differences if moult were to occur during this process.

The aim of this study was to determine if isotope values ($\delta^{13}C$, $\delta^{15}N$, and $\delta^{2}H$) of primary feathers along a moult sequence reflect temporal or spatial variation linked with possible source shifts in illegally traded African grey parrots. If there is isotopic differentiation observed along the moult sequence, what does this infer and which isotopes best reflect this isotopic change?

Methods and materials

Study species

Six African grey parrots were randomly selected from a sub-sample of *c.* 100 deceased parrots, originally randomly selected from a further sub-sample of *c.* 250 African grey parrots forming part of *c.* 700 parrots which died during transit from Johannesburg to KwaZulu-Natal, South Africa. From the *c.* 100 deceased African grey parrot sub-sample three left and

three right wings were selected, each from different parrots. Wings with varying degrees of moult were selected to determine whether isotope values changed among feathers at different stages of moult. Each of the primary feathers (P1–P10) were taken from each wing. Feathers undergoing moult were compared to fully grown feathers to determine how far into growth they were and were classed as, ">⅓ grown" and "<⅓ grown". Of the 6 wings, two were missing P10 and one was missing P6. Two of the wings had no moulting feathers and were selected as controls and four wings had feathers growing at the time of death (one with a feather <⅓ grown, two with feathers >⅓ grown, and one had both <⅓ grown and >⅓ grown feathers). The feathers were washed in 2:1 chloroform:methanol mixture to remove any contaminating debris or oils and dried at 60˚C for a day in a drying oven (Wassenaar and Hobson 2000).

$\delta^{13}C$ and $\delta^{15}N$ isotope analysis

Samples weighing *c*. 0.5 mg were cut from the distal region (*c.* 20 mm beneath the tip of the feather) of the vane of fully grown and <⅓ grown feathers. Samples weighing *c*. 0.5 mg were cut from the distal and proximal region of >⅓ grown feathers. All samples were placed into toluene-washed tin cups. Merck Gel ($\delta^{15}N$ = +6.8 ‰, $\delta^{13}C$ = -20.57 ‰) standards weighing 0.2, 0.4, and 0.6 mg were weighed out to allow sample peak values to fall within the range of peaks of $\delta^{13}C$ and $\delta^{15}N$. A standard and a blank were sampled at the beginning, end, and after every 12 unknown samples to determine if there was any remaining residue from the previous sample as well as if corrections were needed if the standard values had deviated. The weighed samples and standards were analysed in a Thermo Fisher Scientific Inc. Flash EA1112 Elemental Analyser coupled to a DeltaV mass spectrometer via a Conflo IV interface. Combustion took place at 1020˚C and a copper reduction oven was used at 650˚C. Water was trapped in a perchlorate water trap. N_2 and CO_2 were separated in a Gas Chromatograph column at 45˚C.

$\delta^{2}H$ isotope analysis

Samples weighing 0.2–0.4 g were cut from beneath the $\delta^{13}C$ and $\delta^{15}N$ sample point on the distal region of the vane of each fully grown and <⅓ grown feather, and from the distal and proximal region of the >⅓ grown feathers, and placed in toluene-washed silver cups. Twelve (one per every seven samples) 0.2, 0.4, and 0.6 g standards comprising of 6 matrix-matched Caribou hoof (CBS; $\delta^{2}H$ = -197 ‰) and Kudu horn (KHS; $\delta^{2}H$ = -54.1 ‰) (Wassenaar and Hobson 2010) were weighed out to correct for any sampling discrepancies (Meier-Augustine *et al.* 2013). The weighed samples and standards were left to equilibrate in the same ambient conditions for 10 days to allow for the free exchange of atmospheric deuterium (Wassenaar and Hobson 2003; Meier-Augustine *et al.* 2013). Samples were analysed through pyrolysis using a Thermo DeltaV Advantage mass spectrometer coupled to a Flash HT Plus elemental analyser. Corrections were made to the sample values using a linear regression based on the actual values for the standards versus the measured value to account for non-exchangeable hydrogen (Wassenaar and Hobson 2003).

Statistical analysis

A Shapiro-Wilk test was used to test for normality (Shapiro and Wilk 1965). Kruskal-Wallis tests coupled with Dunn's post hoc tests determined if there was any significant difference between the isotope values of the wings. Wilcoxon Signed-Rank Tests were used to determine if there were any differences between isotope values of primary feather tracts and average known wild and captive African grey parrot isotope values (Chapter 3). Non-parametric testing was used as assumptions could not be effectively evaluated by parametric *t*-Tests as a result of small sample sizes of primary feathers on each wing (n = 9/10). Statistic results were presented in Appendix 1 to maintain the flow of the results. The known wild African grey parrot isotope values were obtained from wild African Grey Parrot body

feathers (n = 42), obtained from museum collections (n = 37) and tail feathers from some wild sites in their native distribution (n = 5). Museum samples were obtained from the Ditsong National Museum of Natural History (DNMNH), Pretoria, South Africa (n = 1) and The American Museum of Natural History (AMNH), New York, USA (n = 37). The museum samples all originated from known wild origins (Appendix 1). The known captive African grey parrot isotope values were obtained from different captive African grey parrot feathers (n = 52). Both the wild and captive average isotope values were obtained from chapter 3. All non-primary feathers were standardised to the isotope values of primary feathers using feather type standardising factors for African grey parrots (Chapter 2). This was to maintain consistent sampling of feather material (Greer *et al.* 2015). It is understood that these standardising factors normalise isotope values to those of P10 feathers. It was decided that maintaining isotope values of a primary feather was better for suggesting wild and captive signatures than using average isotope values from a variety of feather types.

Results

For δ^{13}C there was no significant difference between the wings (H = 8,684; df = 5; P = 0.122). Wings did differ significantly for both δ^{15}N (H = 15,528; df = 5; P = 0.008) and δ^{2}H (H = 18, 478; df = 5; P = 0.002). Wing (b) differed significantly to wings (a, c, and f; P < 0.01) for δ^{15}N. Whereas for δ^{2}H wings (b) and (c), (c) and (f), and (d) and (f) differed significantly (P <0.01).

Control wings

Wing (a) (Fig. 1a) reflected δ^{13}C values significantly different to the captive average (Appendix 1). Wing (b) (Fig. 1b) reflected δ^{13}C values significantly different to both the wild and captive averages (Appendix 1), however the primary feather δ^{13}C values were more

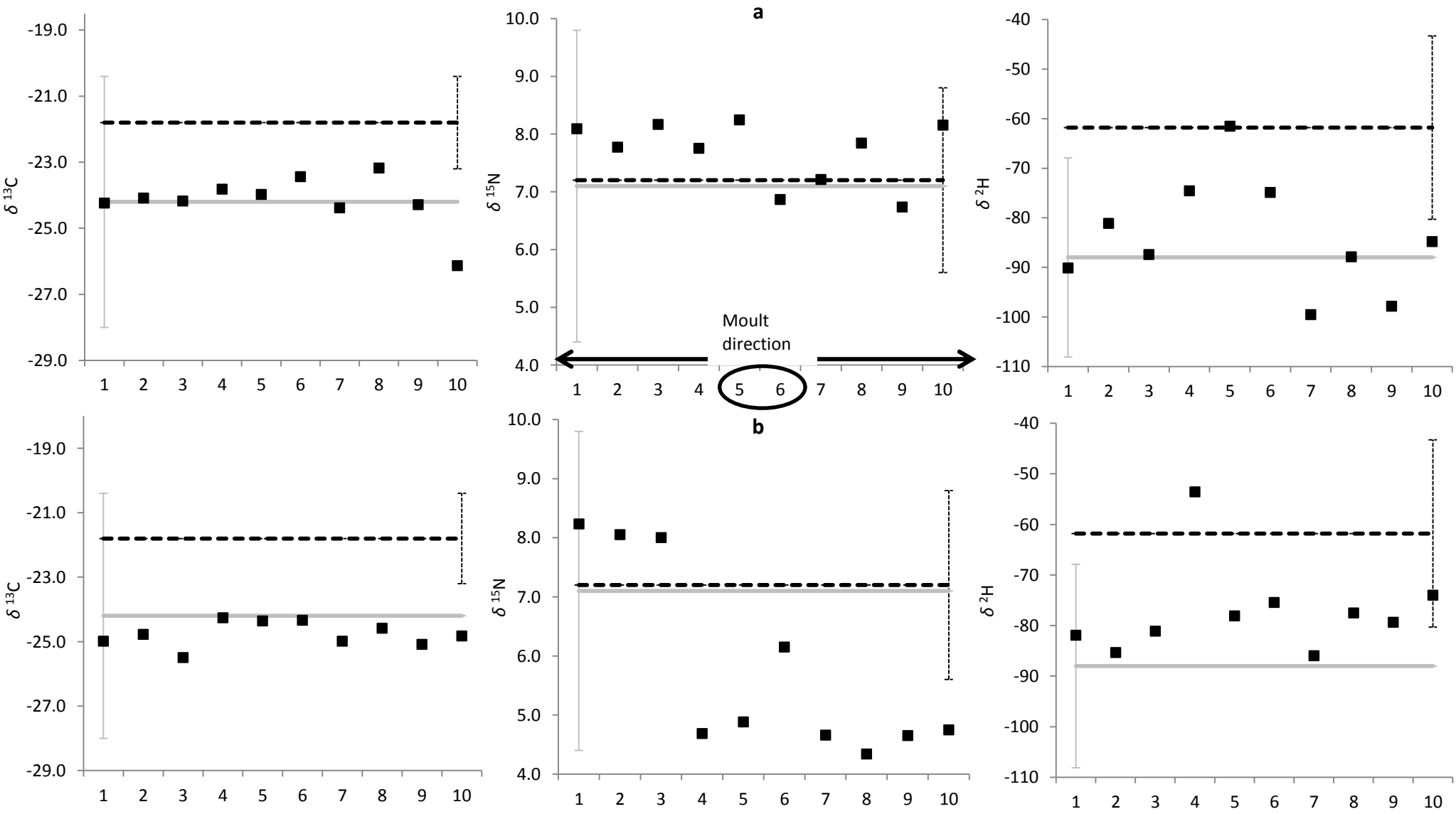
negative than the wild average. Both wings showed little variation (<1.5 ‰) in δ^{13}C values among the primary feathers. The δ^{15}N values of wings (a) and (b) were both significantly different to the wild and captive averages (Fig. 1a–b, Appendix 1). There was little δ^{15}N variation (<1.5 ‰) among the primary feathers of wing (a) (Fig. 1a), whereas wing (b) (Fig. 1b) showed more positive δ^{15}N values in primaries (P1–3). The δ^{2}H values of wing (a) (Fig. 1a) were not significantly different to the wild average, but were significantly different to the captive values (Appendix 1). However, wing (b) (Fig. 1b) was significantly different to both the wild and captive δ^{2}H averages (Appendix 1), with primary feather values being more negative than the captive average. Both wing tracts (a) and (b) showed isotopic variation (<30 ‰) among the primary feathers (Fig. 1a–b).

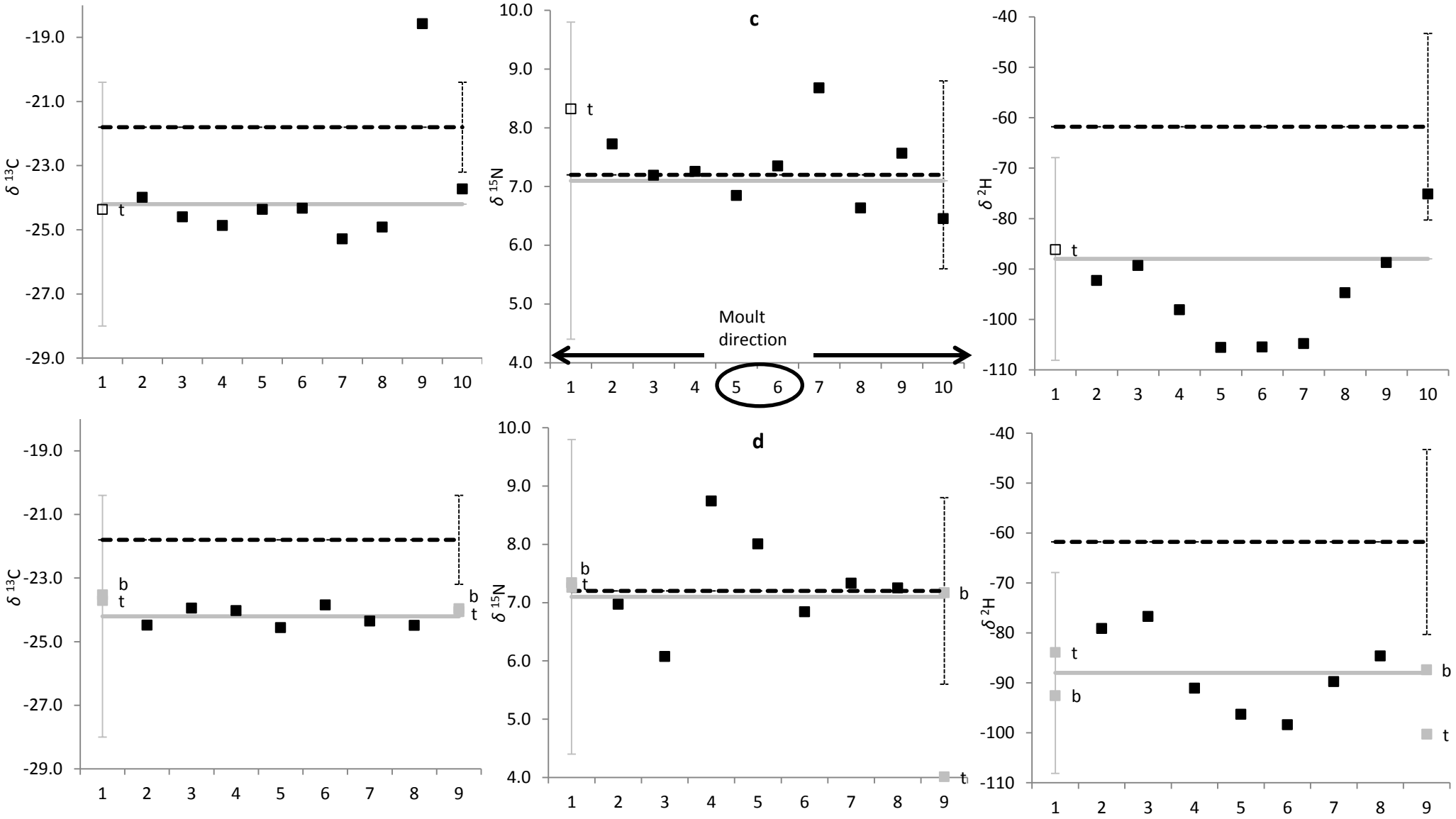
Wings with moulting/growing primaries

Wing (c) (Fig. 1c) δ^{13}C values were not significantly different to the wild and captive averages (Appendix 1). Wing (d) (Fig. 1d) δ^{13}C values were significantly different to the captive average but not the wild average (Appendix 1). Wings (c and d) both had δ^{13}C isotope value more negative than the wild average (Fig. 1c–d), with the feathers undergoing moult (P1; Fig. 1c) and (P1 and P9; Fig. 1d) also more negative than the wild average. Wing (e) (Fig. 1e) had δ^{13}C values significantly different to the captive average (Appendix 1), with only the moulting primary (P2) having a captive isotope value (Fig. 1e). The δ^{15}N values of wings (c) and (d) were not significantly different to both the wild and captive average values (Appendix 1), with the moulting feather of (P1; Fig. 1c) being more negative than the captive average and the moulting feathers of (P1 and P9; Fig. 1d) being similar to wild and captive averages. There was no significant difference to both the wild and captive δ^{15}N averages (Appendix 1) for wing (e). The moulting primary (P2; Fig. 1e) reflected δ^{15}N values similar to the wild and captive averages. The δ^{2}H values of wings c) and (d) are both not significantly different to the wild average but are significantly different to the captive average

(Appendix 1). Wings c) and (d) both show an increase from the inner primaries towards the outer, moulting primaries (Fig. 1c–d). Wing (e) primary values were significantly different to both the wild and captive $\delta\ ^{2}H$ averages (Appendix 1), with the moulting primary (P2) reflecting more negative values than the captive average (Fig. 1e).

Wing (f) (Fig. 1f) had values significantly different to the captive $\delta\ ^{13}C$ average (Appendix 1), with the primary feathers values becoming more negative from the inner to the outer primaries (Fig. 1f). The $\delta\ ^{15}N$ values are also significantly different to both the wild and captive averages (Appendix 1), with the values being more positive than the captive average and becoming more positive from the inner to the outer primaries (Fig. 1f). For $\delta\ ^{2}H$ the primary feather values of wing (f) are significantly different to both the wild and captive averages (Appendix 1), with the primary feather values becoming more positive from the inner to the outer primaries (Fig.1f).


a
b
Moult direction
δ 13C
δ 15N
δ 2H
-19.0
-21.0
-23.0
-25.0
-27.0
-29.0
10.0
9.0
8.0
7.0
6.0
5.0
4.0
-40
-50
-60
-70
-80
-90
-100
-110
1 2 3 4 5 6 7 8 9 10


c
d
δ 13C
δ 15N
δ 2H
t
b
Moult
direction

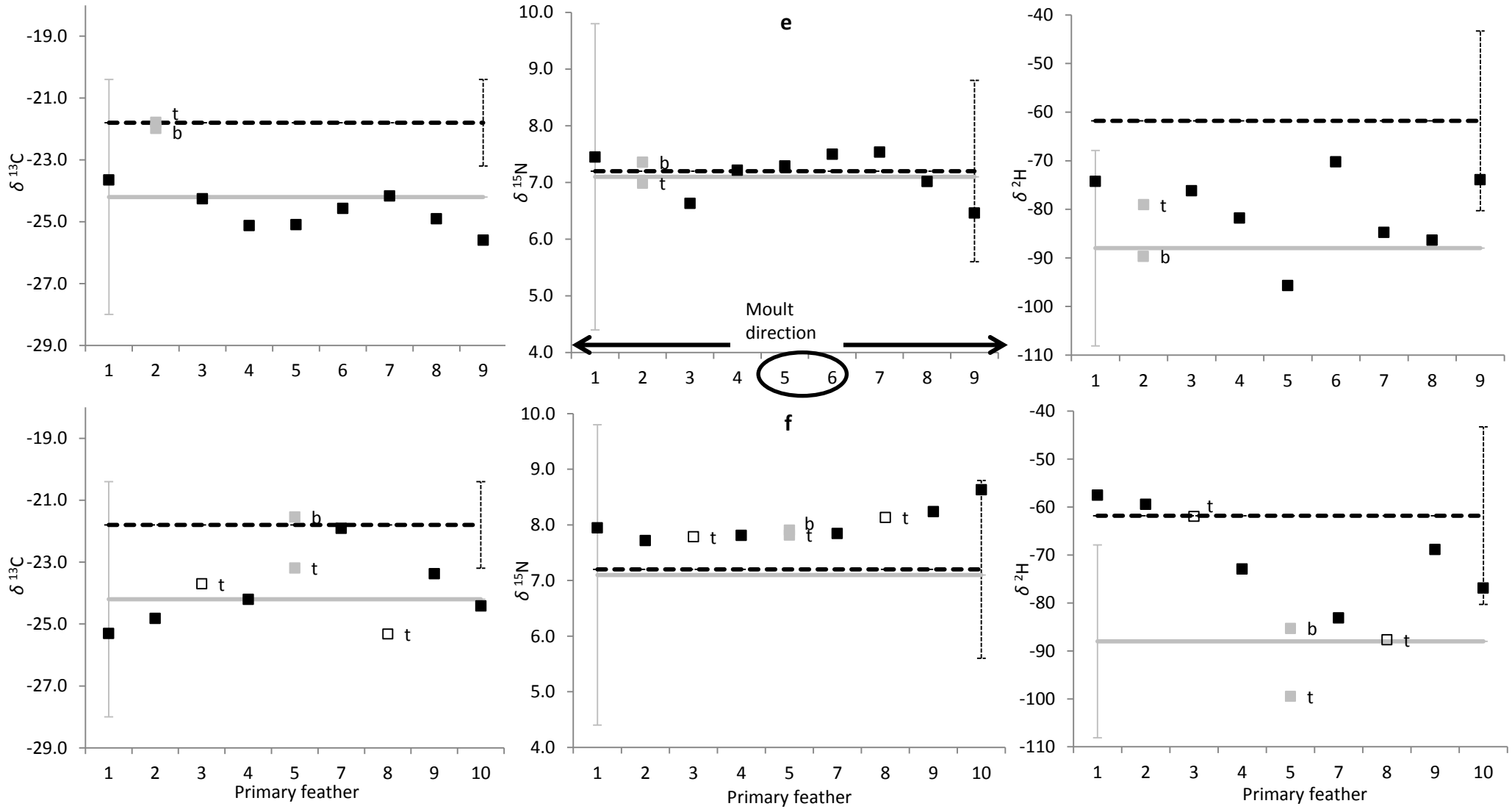


Figure 1: $\delta^{13}C$, $\delta^{15}N$, and $\delta^{2}H$ values of primary feathers along individual wings of six (a–f) African grey parrots. Mean (± SD) ‰ known wild and captive $\delta^{13}C$, $\delta^{15}N$, and $\delta^{2}H$ values represented by the grey and dashed lines respectively. Black boxes = fully grown feathers, grey boxes = > ⅓ grown and open boxes = < ⅓ grown. t = tip and b = base of > ⅓ grown feathers. Moult starts at either P5 or P6 (black ring) and progresses to P1 first and then to P10, as indicated by the black arrows. Standards: Merck Gel ($\delta^{15}N$ = +6.8 ‰, $\delta^{13}C$ = -20.57 ‰), Caribou hoof ($\delta^{2}H$ = -197 ‰) and Kudu horn ($\delta^{2}H$ = -54.1 ‰).

Discussion

Isotope variation among primary feathers

All the primary feathers from the control wings and wings with moulting/growing feathers showed isotopic variation, which was expected as each primary feather along the wing moults at a different time and possibly in a different location, reflecting dietary variation at different spatial and temporal scales or changes in the δ^{2}H precipitation values along the deuterium gradient (Marra *et al.* 1998; Bearhop *et al.* 2003; Ayliffe *et al.* 2004; Dalerum and Angerbjörn 2005; Cerling *et al.* 2006; Ramos *et al.* 2009; Symes and Woodborne 2011). However, the isotopic differences among the primary feathers of the control wings (Fig. 1a and b) were <1.5 ‰ for δ^{13}C and δ^{15}N, which are less than the suggested trophic level fractionations (DeNiro and Epstein 1978; 1981), suggesting that the African grey parrots could have been maintaining a relatively consistent diet. Not only was there isotopic variation within each wing, but isotope values of the control wings differed significantly to some of the wings which had begun moulting. This difference between the control and moulting wings could suggest that the increased variation in the moulting wings was a result of the shift from wild to captive values between the feathers. Inferring a shift from a wild location to a captive location using isotope analysis proved more difficult. Certain isotopes reflected a suggested wild to captive shift in certain wings, but the same isotopes did not reveal any apparent shifts for other wings, which emphasises the importance of the timing of moult to observe a shift in location, especially for implementing a forensic ecology approach. If moult did not occur at the time of a wild to captive shift, it is unlikely that it would be reflected in the isotope values of the primary feathers. It is also suggested that using δ^{15}N to determine a location shift is not accurate as the difference between wild and captive nitrogen averages is so similar (<0.1 ‰). Of the six wings and three different isotopes analysed, four wings (Fig. 1c, d, e, and f)

suggest possible isotope values from wild values (in old feathers) to captive values (in new or growing feathers).

Inferring a shift from the wild to captivity using isotope differences

The potential shifts from wild environments to captivity observed in the four wings were not observed for each of the isotopes analysed but rather certain isotopes for certain wings. Feathers which were growing that had isotope values different (reflecting captive values) to the values of grown feathers (reflecting wild values) were suggested to have been grown in captive environments. This would suggest that the feathers moulted and began to grow after the African grey parrots were removed from the wild and whilst they were in captive environments, and before they died. These changes were expected as feathers would reflect the diet ($\delta\ ^{13}C$ and $\delta\ ^{15}N$) and $\delta\ ^{2}H$ in precipitation of the location where the feather was grown or began growing prior to death (Hobson 1999a), especially if there were isotopically distinct values between the two locations (Marra *et al.* 1998; Dalerum and Angerbjörn 2005; Ramos *et al.* 2009; Symes and Woodborne 2011). The ability to infer a shift from a wild to captive environment based on isotope analysis depends on the timing of capture, the time spent in captivity, the time of death as well as the time of moult. If moult has not occurred during the shift of environment (or movement of the bird) there is little that can be inferred from stable isotope analysis using feathers. The diet of the African grey parrots in captivity will also influence the ability to infer a shift from a wild environment to captivity. If the African grey parrots are being fed a diet similar to that of their natural diet whilst in captivity the newly grown feathers will reflect isotope signatures similar to wild environments.

Influence of stress on isotope variation

Organisms have been known to source nutrients from body stores and pools acquired at different location or from different sources during the egg production and laying phase

(O'Brien *et al.* 2000; Gauthier *et al.* 2003). During egg production and laying more nutrients are required and the organisms are experiencing a form of nutritional stress. It is believed that once African grey parrots have been caught in wild location they are placed into nutrient and water deficient cramped cages or boxes for extended periods of transportation, creating stressful environments (May 2001; Fahlman 2002). Wings (c–e) might be showing the use of body stores and pools to source nutrients whilst in a nutritionally deficient captivity (O'Brien *et al.* 2000; Bearhop *et al.* 2002; Gauthier *et al.* 2003; Carleton and Martínez del Rio 2005). The use of body stores is suggested as the values of moulted feathers were not reflecting isotope values different to the feathers which were potentially grown in wild conditions. This makes defining captive from wild African grey parrots difficult when comparing the isotope values among the primary feathers along the wing tract. However, it does suggest a shift from a wild environment into a stressful captive environment.

Wing (f) might be the only wing which shows the influence of stressful environments in more detail. For δ^{13}C the primary (P5), which is suggested to moult first (Howell 2010; Mallet-Rodrigues 2012; Pyle 2013) reflects captive isotope values and as moult progress through to primaries (P1 and later P9) the isotope values begin to reflect wild values. Though it is the opposite trend compared to the five prior wings, it could be reflecting the influences of stress on the isotopic signatures of tissues. Stress, in this case presumed nutrient stress can result in increased metabolic activity (Bearhop *et al.* 2002; Carleton and Martínez del Rio 2005), which would result in the uptake of nutrient stores and possible fat reserves (Bearhop *et al.* 2000; 2002; Carleton and Martínez del Rio 2005; Kempster 2007). Therefore the decrease in δ^{13}C values in the primaries as moult progresses could be a result of the parrots exposed to stress sourcing nutrients from isotopically negative stores obtained from wild environments (O'Brien *et al.* 2000; Bearhop *et al.* 2002; Gauthier *et al.* 2003; Carleton and Martínez del Rio 2005; Cherel 2005). Uptake of δ^{13}C negative lipids through the breakdown

of fat stores could also possibly alter the isotope values of the newly formed feather tissues (Bearhop *et al.* 2000). For δ^{15}N there was an apparent increase in δ^{15}N values along the moult pattern. This again can be a result of nutrient and water stress (Hobson and Clark *et al*. 1992b; Hobson *et al*. 1993; Bearhop *et al.* 2000). Japanese quail (*Coturnix japonica*), Ross's goose (*Chen rossi*), and American crow (*Corvus brachyrhynchos*) under nutritional stress showed increased δ^{15}N values in their tissues, as well as reduced muscle mass. It is suggested that muscle is broken down as a source of amino acids, resulting in an increase δ^{15}N values in tissues (Hobson *et al*. 1993). Additionally, under water stressed situations organisms excrete more negative δ^{15}N concentrated urine to retain water, the tissues therefore become isotopically more positive for δ^{15}N (Steele and Daniel 1978; Hobson and Clark 1992; Hobson *et al.* 1993). Amino acids could be sourced from now more negative δ^{15}N stores for the production of new tissues under water and nutrient stressed situations (Steele and Daniel 1978). African grey parrots could also be sourcing δ^{15}N from more positive body stores which had been built up during periods of time in wild (Gauthier *et al.* 2003). The increase of δ^{15}N in the primary feathers may only be by 0.7 ‰, but the increase is uniform from the inner to outer primaries. δ^{2}H values also became increasingly more positive (20–30 ‰) as moult progressed. In the cramped cages or boxes it would be expected that the parrots would be panting as a result of increased temperature through body heat as well as the potential lack of water. Panting would result in evaporative cooling and this is known to cause an increase of δ^{2}H values (Wolf and Martínez del Rio 2000; McKechnie *et al.* 2004; Smith and Dufty 2005). As water is lost through the body to the environment, isotopically positive δ^{2}H would remain within the body and tissues of the organism (McKechnie *et al.* 2004). The decrease and increase of the respective isotopes are therefore suggested to be a result of stress and not a return from captive to wild situations as the birds died in a captive environment. It must be noted that because these African grey parrots were obtained with no known origin or

provenance there is possibility that there could be other factors, unbeknown to us, influencing the shifts in isotope values along the moult sequences of the African grey parrot wings.

It is possible to determine the provenance of African grey parrots with the fine scale isotope analysis of wing tracts, and the changes from wild to captive isotope values between old and new feathers. A captive provenance could also be inferred using the assumption that wild African grey parrots are stressed in captive situations. African grey parrots are often transported under poor conditions once they have been caught from wild locations and this resulting stress could influence the isotope values. However, the timing of moult is fundamentally important. If moult had not begun or occurred between the shift in location from the wild to captivity it would not be possible to infer provenance. It is suggested that utilising δ^{13}C and δ^{2}H isotope analysis will more accurately infer provenance than when using δ^{15}N isotope analysis, mainly as a result of the reduced difference between the wild and captive δ^{15}N average values. This may differ between species and location; however, it is believed that this fine scale method for determining provenance will be beneficial for forensic ecology as the sequential growth of certain tissues at different spatial and temporal scales can reflect isotopic shifts.

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The Mammal Research Institute Stable Light Isotope Laboratory, University of Pretoria, Pretoria (δ^{13}C and δ^{15}N) and iThemba LABS, Johannesburg (δ^{2}H).

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Appendix

Appendix 1: Wilcoxon-Signed Rank test results for wing tracts compared to known wild and known captive African grey parrot $\delta^{13}C$, $\delta^{15}N$, and $\delta^{2}H$ values. Bold text highlights comparisons of significant difference ($P < 0.05$).

	$\delta^{13}C$		**$\delta^{15}N$**		**$\delta^{2}H$ values**	
Wing tract	**Wild $\bar{x}$**	**Captive $\bar{x}$**	**Wild $\bar{x}$**	**Captive $\bar{x}$**	**Wild $\bar{x}$**	**Captive $\bar{x}$**
a	*W* = 13.5, df = 9, *P* > 0.05	***W* = 0, df = 9, *P* = 0.005**	***W* = 5, df = 9, *P* = 0.022**	***W* = 3, df = 9, *P* < 0.05**	*W* = 16, df = 9, *P* > 0.05	***W* = 1, df = 9, *P* = 0.007**
b	***W* = 0, df = 9, *P* = 0.005**	***W* = 0, df = 9, *P* = 0.005**	***W* = 7.5, df = 9, *P* = 0.041**	***W* = 6, df = 9, *P* = 0.029**	***W* = 0, df = 9, *P* = 0.005**	***W* = 1, df = 9, *P* = 0.007**
c	*W* = 19, df = 9, *P* = 0.384	*W* = 9, df = 9, *P* = 0.059	*W* = 16.5, df = 9, *P* = 0.263	*W* = 17, df = 9, *P* > 0.05	*W* = 10, df = 9, *P* = 0.075	***W* = 0, df = 9, *P* = 0.005**
d	*W* = 21, df = 8, *P* > 0.05	***W* = 0, df = 8, *P* < 0.05**	*W* = 21, df = 8, *P* > 0.05	*W* = 20, df = 8, *P* > 0.05	*W* = 20, df = 8, *P* > 0.05	***W* = 0, df = 8, *P* = 0.005**
e	*W* = 11, df = 8, *P* > 0.05	***W* = 0, df = 8, *P* < 0.05**	*W* = 18, df = 8, *P* > 0.05	*W* = 12.5, df = 8, *P* > 0.05	***W* = 5, df = 8, *P* < 0.05**	***W* = 0, df = 8, *P* < 0.05**
f	*W* = 15, df = 8, *P* > 0.05	***W* = 0, df = 8, *P* < 0.05**	***W* = 0, df = 8, *P* < 0.05**	***W* = 0, df = 8, *P* < 0.05**	***W* = 2, df = 8, *P* < 0.05**	***W* = 3, df = 8, *P* < 0.05**

Chapter 5: Conclusion: isotope analyses determining provenance

"People need to be educated so that they can make intelligent moral choices." **- Gary L. Francione**



This is one of few studies on the stable isotope analysis of parrot species (Hawke and Holdaway 2009; Somerville *et al.* 2010; Greer *et al.* 2015, Symes *et al.* In prep), and one of two studies focusing specifically on African grey parrots *Psittacus erithacus* (Symes *et al.* In prep). To my knowledge this study is also the first to incorporate stable isotope analysis in a forensic approach on avian material. The results from this study have been applied to suggest a protocol for determining wild from captive African grey parrots as well as suggest areas where additional research is required.

We hypothesised that the isotope values (δ^{13}C, δ^{15}N, and δ^{2}H) of wild African grey parrot feathers would differ significantly to those of captive African grey parrot feathers and that the isotope values of wild African grey parrots would show increased variability compared to captive African grey parrots. These differences were believed to occur as a result of both environmental and physiological (stress, malnutrition, and dehydration) factors. To account for physiological factors influencing the isotope values of African grey parrot feathers, isotope values of different African grey parrot feather types were compared.

Feather differentiation

We hypothesised that the isotope values of different feather types of African grey parrots would differ significantly. Isotope values between feather types differed in other avian species (Mizutani *et al.* 1992; Hedd and Montevecchi 2006; Becker *et al.* 2007; Jaeger *et al.* 2009; Ramos *et al.* 2009). These differences could be a result of different feathers growing at different times, therefore potentially in geographically different areas (Marra *et al.* 1998; Ramos *et al.* 2009). The isotope values (δ^{13}C, δ^{15}N, and δ^{2}H) did differ between feather types of African grey parrots and that the differences were not completely uni-directional. However >60 % of the isotope differences between feather types were uni-directional. Primary feathers were the most negative for δ^{13}C and δ^{2}H and positive for δ^{15}N, tail feathers

were the most positive for $\delta^{13}C$ and negative for $\delta^{15}N$ and body feathers were the most positive for $\delta^{2}H$. These isotope differences between feather types creates inaccuracy when comparing isotope values between similar species, between different species, and museum, wild, and captive specimens, as well as when comparing different studies (Cherel *et al.* 2005; Greer *et al.* 2015). Although different studies require specific tissues and feather types it is suggested that when possible the same feather types be selected for isotope analysis. Understandably this is not always possible, as a result of feather availability and sampling ethics. Therefore the use of feather standardising factors creates an additional means for more consistent sampling and could make intra- and inter-study comparisons more appropriate (Greer *et al.* 2015). The feather standardising factors for African grey parrots established in chapter 2 could provide an additional means to analyse multiple feather types (in the event that the same feather cannot be sampled, i.e. museum samples often being body feathers). However, the standardising factors suggested are African grey parrot specific, though the protocol for obtaining these feather correction factors should be considered for other species. These standardising factors were used to normalise all isotope values of African grey parrots feathers to a single feather type isotope value when determining provenance. Along with standardising the isotope values to a single feather type isotope changes in $\delta^{13}C$ over time were also corrected.

Suess effect

Ice core samples show that atmospheric CO_2 values have increased as a result of burning fossil fuels and nuclear activity, resulting in the decrease of atmospheric $\delta^{13}C$, known as the Suess effect (Keeling 1979; Friedli *et al.* 1986). Samples from 1890–2010 were markedly more positive for $\delta^{13}C$, by up to *c.* 1.3 ‰/century (Hilton *et al.* 2010; Barquete 2012) and could not be directly compared to contemporary samples as a result of this change. Museum samples were dated back in comparison to contemporary samples, often by more than half a

century and it is suggested that when sampling museum tissues and feathers, corrections for the Suess effect should be applied (Barquete 2012; Chapter 3). The corrections can either be applied to the museum samples reducing their values to the values of contemporary samples or increasing the values of contemporary samples to the values of the museum samples (some prescribed date of comparison), depending on the objectives of the study.

Inferring provenance

When applying stable isotope analysis to forensic ecology, inferring the provenance of a sample can be as important as inferring the exact geographic origin of the sample, as it is not always possible to suggest exact origins. Exact origins may be difficult to determine across the African continent as a result of weak $\delta^{2}H$ gradients and very little $\delta^{2}H$ variation across the continent (Møller and Hobson 2004; Bowen *et al.* 2005; Wakelin *et al.* 2010). It is therefore suggested that when applicable, multiple isotopes be analysed (van der Merwe *et al.* 1990; Vogel *et al.* 1990; Hall-Martin *et al.* 1993; Ehleringer *et al.* 1999; 2000; Hobson *et al.* 2012). As previously mentioned, if applicable, standardising for feather type differences (Chapter 2–4) and the Suess effect (Chapter 3 and 4) should be applied to maintain consistent sampling and to allow for intra- and inter-study comparisons (Greer *et al.* 2015).

We hypothesised that isotope values of African grey parrots from wild origins would differ significantly from captive African grey parrots and additionally the $\delta^{2}H$ values of wild African grey parrots would follow the predicted east-west $\delta^{2}H$ gradient of Africa. The hypothesis was supported as the isotope values of wild African grey parrots differed significantly from captive African grey parrots for $\delta^{13}C$ and $\delta^{2}H$ but not for $\delta^{15}N$. African grey parrots naturally occur in a distribution across central Africa, dominated by C3 vegetation (Forshaw 2006) and their isotope signatures (-22.1 ± 1.1 ‰) reflect the isotope values of their C3 diet (Chapter 3; DeNiro and Epstein 1978; Schoeninger 2010; Scott 2012).

These values differed from the signatures of captive African grey parrots (-19.0 ± 1.4 ‰), which were representative of captive diets supplemented with C4 plants (Chapter 3; Kennedy and Krouse 1989; Marshall *et al.* 2007). This $\delta^{13}C$ isotopic difference between wild C3 diets and captive C4 diets was suggested to be the main factor, coupled with $\delta^{2}H$ in determining the provenance of African grey parrots. With respect to $\delta^{15}N$ there was large isotopic variation in both the wild and captive samples and there was no significant difference. It was suggested that the provenance of samples other than African grey parrots could be determined if the species analysed occurs in a regions with distinct $\delta^{13}C$ values i.e. tropical $\delta^{13}C$ negative forests (Schoeninger *et al.* 1995) or arid $\delta^{13}C$ positive regions (van der Merwe *et al.* 1990; Vogel *et al.* 1990; Hall-Martin *et al.* 1995). The isotope variations of wild African grey parrots were greater than that of captive African grey parrots, possibly as a result of the spatial and temporal changes that occur in wild environments. Parrots in wild environments feed on a variety of naturally occurring food sources (Forshaw 2006) which they track through different seasons (Chapman *et al.* 1993; Tamungang *et al.* 2014). Whereas captive African grey parrots would be exposed to a more homogenous environment with similar food sources throughout seasons and little to no spatial variation. When inferring the provenance of African grey parrot samples through the comparison of wild and captive African grey parrots it is suggested that isotope values be standardised to a single feather type as well as $\delta^{13}C$ values being corrected to a common date using Suess correction factors.

Wing tract analysis

When analysing entire primary feather tracts of African grey parrots for isotope differences, the above mentioned factors must be applied to maintain consistent sampling (Greer *et al.* 2015). The same interpretations applied in chapter 3 were applied when analysing wing tracts, but the changes in isotope values along the primary feather tract, through temporal and spatial shifts become more apparent (Marra *et al.* 1998; Ramos *et al.* 2009; Symes and

Woodborne 2009). Changes in isotope values as a result of stresses (nutrient, water, and heat) were observed and suggest that wild African grey parrots were kept in poor captive conditions. However, these changes in isotope values of the primary feathers along wings were only observed if moult had occurred or was occurring along or between spatial and temporal shifts.

Through this study it is apparent that the stable isotope ($\delta\ ^{13}C$, $\delta\ ^{15}N$, and $\delta\ ^{2}H$) analyses of African grey parrot feathers can be used to infer the provenance of these heavily traded birds. This forensic approach and the methods and protocol suggested may prove crucial in identifying African grey parrots that have been illegally caught from wild locations in central Africa. These methods may only prove valuable if the African grey parrots have been caught and transported within a short time frame and have not been in captivity for extended periods. If the African grey parrots have completed moult the new feathers will incorporate the isotopic signatures of the new captive environment and there will be no proof of wild signatures remaining in feathers. However, the use of tissues with slower turn over times like claw, beak and bone material may still hold isotope values of wild environments. Tissues such as beak and claw may also reflect shifts from wild to captive environments in a time series as with the primary wing tracts. The base of the beak and claws would reflect isotope values incorporated more recently than the tips of these tissues which are older (Bearhop *et al.* 2003). These methods should be elaborated on and additional research conducted on more parrot species and other traded avifauna in an attempt to track and monitor the illegal trade of threatened species.

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