A Potential Enforcement Tool for Regulating Trade in Tortoises: Stable Isotope Analysis

Submitted by Emma Mary Wood to the University of Exeter
as a thesis for the degree of
Masters by Research in Biosciences (Cornwall)
In October 2012

This thesis is available for Library use on the understanding that it is copyright material and that no quotation from the thesis may be published without proper acknowledgement.

I certify that all material in this thesis which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University.

Signature: ………………………………………………………………..

ABSTRACT

A POTENTIAL ENFORCEMENT TOOL FOR REGULATING TRADE IN TORTOISES: STABLE ISOTOPE ANALYSIS

Tortoises of the genus Testudo are imported to the UK in large numbers. It is currently legal to trade both wild-caught and ranched *Testudo horsfieldii*, however there are incentives to fraudulently claim wild-caught specimens as ranched. In addition, only captive-bred *Testudo graeca* and *Testudo hermanni* specimens can be traded legally. Currently, it is difficult to ascertain the breeding source of tortoises in trade and a forensic tool that distinguishes between them is called for. I verified that tortoise scute keratin can be non-invasively longitudinally sampled for stable isotope analysis. I sampled 30 *T. horsfieldii* specimens that died on import to the UK and found that whilst they were from a single batch they clustered into two groups. I associated these groups with a known morphological measure of breeding origin (degree of scute pyramiding) and compared the two nominal groups using general linear mixed models (GLMMs). I found differences in $\delta^{15}N$ at all ages of growth, and in $\delta^{13}C$ and δD in old growth. I also longitudinally sampled 8 mature *T. graeca* and 4 mature *T. hermanni* specimens of unknown origin, and 1 mature *T. graeca* and 2 juvenile *T. hermanni* known to be imported. GLMMs revealed a shift in δD, $\delta^{13}C$ and $\delta^{15}N$ between old and recent growth of *T. graeca* but not *T. hermanni* specimens. δD of the *T. hermanni* specimen known to be imported did however appear to differ between old and recent growth. The greatest potential for stable isotope analysis as a regulatory tool for the tortoise trade appears to be in determining 1) if batches of captive specimens also include wild-caught individuals, and 2) if wild-caught specimens have been fraudulently claimed as UK captive-bred. However, the majority of specimens available for this thesis were of unknown origin so the conclusions I have drawn are based on assumptions and conjecture and will need further testing. Stable isotope analysis can be used to distinguish between individuals that have experienced different conditions, and it has potential to facilitate understanding of the scale of the illegal tortoise trade and identify areas to which further resources should be directed.

ACKNOWLEDGEMENTS

I could not have completed this Masters without the advice, guidance and encouragement of my supervisors: I am extremely grateful to Professor Brendan Godley for giving me the freedom to discover things for myself but always being there to steer me in the right direction and give me moral support when I needed it; to Professor Stuart Bearhop for getting me interested in stable isotope analysis in the first place and for welcoming me into his research group who broadened my understanding and suggested ways to analyse my data; and to Professor Robbie McDonald for his early involvement, enthusiasm, and input into the project. I also thank my supervisor at Fera, Dr. Simon Kelly, who guided me through the methods of stable isotope analysis and who, along with his colleagues, made me feel so welcome.

I certainly could not have done this research without samples to analyse so I am thankful to those who gave me access to their specimens: to Andrew Kitchener at the National Museum of Scotland (NMS), Rob Quest at Heathrow Animal Reception Centre, Amanda Yeomans and Rebecca Parry at Colchester Zoo, Mike Jessop who also gave me veterinary and sampling advice, to Eleanor Chubb who went out of her way to help me, Dillon Prest who brightened my day by introducing me to his herd of giant tortoises, Rick Wilton who gave up many hours to discuss the tortoise trade and to show me round his facilities, to Rachel Mowbray who also collected answers to the dietary questionnaire, and to Rachael Krueger, who gave me insights into on-the-ground law enforcement. I am also grateful to Alan Lothian and Phil Howard for finding specimens for me at NMS and showing me some tricks of the taxidermy trade.

A number of people helped me in the lab, particularly Gareth Rees, to whom I am very grateful for all the help he gave in the practical aspects of stable isotope analysis and for running several plates of samples for me. Also to Andy Robertson and Rich Inger who gave up their time to explain techniques and analysis. I also owe thanks to those who provided advice, guidance and equipment: Joe Faulks, Chris Mitchell, Anna Leonard, Corrina Lowry, and Malcolm Baxter.

For valuable insight into the wildlife trade and its regulation, for introductions to numerous people, and for heavy involvement at the beginning of the project I am grateful to Nevin Hunter at AHVLA. For advice and information I am also grateful to Evgeniy Peregontsev, Don Freeman, Claire Guy, Helen Pisarska, and Jan Stipala.

To my family for their constant support of all my decisions, whether they agree or not, and to my friends whose emotional support I could not have done without this year I couldn’t be more grateful.

And lastly, I also thank Defra Seedcorn for funding this research.

TABLE OF CONTENTS

Page

ABSTRACT……………………………………………………………………………………….. 2

ACKNOWLEDGEMENTS…………………………………………………………………….. 3

TABLE OF CONTENTS……………………………………………………………………… 5

LIST OF FIGURES AND TABLES………………………………………………………….. 6

CHAPTER:

1. General introduction: Use of stable isotope analysis to assess the origin of biological samples, and its potential as a regulatory tool for the wildlife trade……………………..... 7
2. Introduction to tortoises: trade, ecology and husbandry…………………………………….. 17
3. Resolving temporal patterns in isotope signature: a preliminary longitudinal study of tissues of the tortoise *T. horsfieldi*……………………………………………………………….. 24
4. Stable isotope analysis as a potential method to distinguish between ranched and wild *T. horsfieldii*……………………………………………………………………………….. 30
5. Longitudinal isotope record in carapacial scutes: a potential method for determining the source of tortoises?... 36
6. General discussion……………………………………………………………………… 40

FIGURES………………………………………………………………………………………. 43

TABLES………………………………………………………………………………………… 56

APPENDICES:

Appendix A…………………………………………………………………………………….. 58

Appendix B…………………………………………………………………………………….. 60

Appendix C…………………………………………………………………………………….. 62

REFERENCES………………………………………………………………………………… 63

LIST OF FIGURES AND TABLES

FIGURE: Page

1. Numbers of imported Testudo specimens 43
2. Range maps for focal species 44
3. Proportion of diet consisting of UK-sourced food 45
4. Scute growth pattern and sampling methods 46
5. $\delta^{13}C$, $\delta^{15}N$, and δD of *T. horsfieldii* of juvenile, old and recent growth 47
6. $\delta^{15}N$, and δD of *T. horsfieldii* front and back claws with days spent in the UK 48
7. Method for photographing specimens for measurement of pyramiding 49
8. Lines of measurement for determining degree of pyramiding 50
9. Clustering of *T. horsfieldii* in isotopic space 51
10. Distribution of measurements of scute pyramiding 52
11. $\delta^{13}C$, $\delta^{15}N$, and δD of *T. horsfieldii* of juvenile, old and recent growth 53
12. Change in $\delta^{13}C$, $\delta^{15}N$, and δD between old and recent growth in *T. graeca* and *T. hermanni* 54
13. Change in $\delta^{13}C$, $\delta^{15}N$, and δD between old and recent growth for known origin specimens of *T. graeca* and *T. hermanni* 55

TABLE:

1. Country of export of *T. graeca* and *T. hermanni* specimens between 2001 and 2011 56
2. Number of tortoise breeders in the UK and species bred 56
3. Dietary items given to tortoises in the UK 56
4. Results of cluster analysis for *T. horsfieldii* batch 57

Chapter 1

General Introduction

Use of stable isotope analysis to assess the origin of biological samples, and its potential as a regulatory tool for the wildlife trade

Over the last two decades stable isotope analysis has increasingly been used in ecological studies to investigate the diet and movement of animals (Crawford et al 2008, Inger & Bearhop 2008). However, its potential for assessing the origin of animals and animal products in the wildlife trade has only been superficially explored (Vogel et al 1990, van der Merwe et al 1990, Ishibashi et al 2000, Kelly et al 2007, Ziegler et al 2012). Here I give a brief overview of the wildlife trade, and of stable isotope analysis as a means to determine the provenance of samples of animal origin, with particular focus on terrestrial systems. I conclude with a discussion on the potential of stable isotope analysis as a regulatory tool for the wildlife trade. This is not intended as a comprehensive review of the now vast literature on the uses and caveats of stable isotopes in tracking animals but is meant as an outline of the technique with a view to identifying its potential as a regulatory tool for the wildlife trade.

The international trade in wildlife and its derivatives has revenues that run into billions of dollars per annum (IUCN 2000). Because of its often secretive nature it is not possible to accurately assess the extent of wildlife trafficking but high profits, low risk of detection and, in many cases, relatively low penalties for those prosecuted for illegal actions make it a major problem and a challenge for wildlife law enforcers (TRAFFIC 2011, Theile et al 2004). In cases where specimens are legal from one source but not another, legal trade can act as a cover for illegal trade: a protected species may be claimed to be a taxonomically similar but unprotected species, individuals from a protected location may be claimed to be from a non-protected location, wild-caught specimens may enter the trade under the guise of captive-bred specimens, and a legal one-off sale may provide cover for illegal specimens (Engler & Parry-Jones 2007, Lyons & Natusch 2011, Nijman & Shepherd 2009, Theile et al 2004). Total trade bans are a potential solution to these problems but the desirability and efficacy of such

measures have been called into question (Cooney & Jepson 2006). Improving trade regulation is likely to reduce threats to wildlife both by directly removing the threat and by increasing the perceived cost of trafficking.

There are several potential ways to ensure that traded wildlife is legally sourced, and methods used will depend on the species and the nature of the illegal trade. For example Lyons and Natusch (2011) suggest that laundering of wild-caught green pythons (*Morelia viridis*) through breeding farms could be controlled by requirement of proof of hatching (eggshell) with each exported individual. For many species, either extrinsic or intrinsic markers can be used to identify animals in trade. Traded specimens of Annex A species (EU Wildlife Trade Regulations) are required to carry a unique mark such as a microchip or, in the case of birds, a metal ring with a unique identification number. This tag then matches to paperwork that remains with the animal throughout the trade process (Theile et al 2004). Whilst these extrinsic marks are useful, they can be fraudulently obtained, or be inappropriate for small specimens (e.g. tortoises under 10cm; Theile et al 2004). They are also rarely appropriate for animal derivatives. In such cases, forensic analysis of intrinsic markers such as DNA (see Ogden et al 2009 for a review) or stable isotopes (Ishibashi et al 2000, Kelly et al 2005) may be used to confirm legality. Besides taxonomic classification, which is beyond the scope of this thesis, there are two broad categories of identification that can be addressed by forensic analysis and are required for regulation of the wildlife trade: 1) discrimination between individuals of captive and wild origin, and 2) determination of site or population of origin.

Genetic analysis has become an accepted forensic method in investigating wildlife crime, enabling enforcement- and investigative-officers to address such questions. It has been used to validate claims of captive breeding, check questionable material against databases of legal supplies, and link suspect animals and derivatives to specific wild populations (Manel et al 2002, Palsbøll et al 2005, Ogden et al 2009, White et al 2011). Yet despite the success of genetic techniques, they are not always suited to wildlife crime issues for either economic or practical reasons. This is the case, for instance, where the species is both traded and bred in high volume such as small passerines (Kelly et al 2007), pythons (Lyons & Natusch 2011), and tortoises of the genus *Testudo* (Vinke & Vinke 2010). In this case alternative methods must be employed to determine validity of claims of captive-bred origin. Often, the only method available is expert opinion; using telling signs such as degree of wear and parasite load

(Kelly et al 2007, Pietzsch et al 2006). In such circumstances stable isotope analysis may be useful as an additional and more objective approach.

General considerations when using stable isotopes as markers

When considering whether stable isotope analysis will be a valuable method to answer a particular question it is important to take several factors into consideration, including which isotope ratios to measure, which tissues to sample, and potential sources of error. Isotope ratios vary in time and space in a predictable manner according to a number of physiological and environmental processes. They can therefore be employed as both spatial and dietary markers, both of which are useful in discovering the provenance of biological material. The most informative isotope ratios will be those with the greatest variation among the populations to be differentiated. Below I discuss isotope ratios that are frequently used in ecological studies and the processes through which they are affected.

The most commonly used geographic marker in terrestrial stable isotope studies is hydrogen (δ^2H or δD; the ratio of Deuterium to Protium expressed relative to a standard) for which there are well studied continental-scale gradients (Hobson et al 2004a, Meehan et al 2004, Bowen et al 2005). These gradients have been found to correlate strongly with signature in a range of species (Hobson & Wassenaar 1997, Hobson et al 2004a, Cryan et al 2004, Ehleringer et al 2008). The ratio of hydrogen isotopes varies in large part due to fractionation during the water cycle, in which a greater proportion of the light isotope is left behind during phase changes (e.g. condensation). δD therefore tends to decrease with increasing altitude, precipitation, and distance from the sea (Bhattacharya et al 1985, Dansgaard 1964). In addition, cooler conditions enhance the effect of fractionation, leading to more negative δD at higher latitudes (Bowen & Wilkinson 2002). Measurement of hydrogen signature is complicated by the uncontrolled exchange of oxygen-bound hydrogen molecules with ambient moisture in the laboratory (Wassenaar and Hobson 2003). As the isotopic ratio of ambient moisture varies among laboratories at different geographic locations and over time within laboratories, such exchange leads to error in measurement among laboratories and over time. However, this can be addressed through equilibration with laboratory conditions and equal treatment of standards and samples (Wassenaar & Hobson 2003).

Carbon signature (δ^{13} C; C^{13}/C^{12}) can also be useful in geographic assignment (Hobson et al 1999a) as $\delta^{13}C$ of C-3 plants decreases with increasing latitude, altitude and

humidity (Körner et al 1991, Stuiver & Braziunas 1987, Graves et al 2002). Fractionation of carbon differs between photosynthetic pathways so $\delta^{13}C$ can be used to differentiate between C-3, C-4 and CAM plants (O'Leary 1981). This distinction between plant types can be used to differentiate between populations subsisting in landscapes of differing agricultural practices. For example, American and European people are isotopically distinct due to the greater dependence on corn (a C-4 plant) in America, and greater dependence on wheat (a C-3 plant) in Europe (Fraser et al 2006). Measurement of $\delta^{13}C$ can also be employed to differentiate between xeric habitats, which are dominated by CAM and C-4 plants, and mesic habitats dominated by C-3 plants (Marra et al 1998). In addition to its uses as a terrestrial dietary and geographic marker, carbon signature can be used to distinguish among marine, freshwater and terrestrial systems (Hobson 1990, Hobson et al 1999b, Bearhop et al 1999, Bodey et al 2010), and among regions within marine and freshwater systems (Hobson et al 1994, France 1995a, France 1995b).

Nitrogen signatures ($\delta^{15}N$; N^{15}/N^{14}) vary due to a number of geochemical, biological and anthropogenic factors. In ecological studies, $\delta^{15}N$ is most frequently used as an indicator of trophic level, with a typical increase of between 2‰ and 4‰ per trophic level (Post 2002; see Vanderklift & Ponsard 2003 for a meta-analysis of causes of variation in $\delta^{15}N$ enrichment). This is due to the loss of light nitrogen to nitrogenous waste products during the assimilation of dietary protein (Michener & Kaufman 2007). Amongst other uses it can also be employed to differentiate between different soils e.g. ridge tops and valley bottoms (Garten 1993), between areas of differing salinity and aridity (Heaton 1987), and between non-agricultural landscapes and agricultural where there is significant input of chemical fertiliser (Hebert & Wassenaar 2001).

Less commonly employed but also useful in determining geographic origin are sulphur ($\delta^{34}S$), strontium ($\delta^{87}Sr$), and oxygen ($\delta^{18}O$). $\delta^{18}O$ correlates well with δD in accordance with the meteoric water line(Craig 1961), $\delta^{34}S$ can be used to infer distance from the marine environment (Lott et al 2003) and volcanic inputs, and $\delta^{87}Sr$ varies depending on rock type and rock age and may be particularly useful when working at fine scales (Beard & Johnson 2000).

The above elements can provide useful information when used in isolation but analysis of multiple elements can increase resolution (Peterson et al 1985, Hobson et al 1998, Wassenaar & Hobson 2000, Lott et al 2003, Wunder et al 2005, Kelly et al 2005). Analysis of multiple elements is therefore likely to be necessary for wildlife trade

regulation purposes as high degree of certainty of assignment is paramount if, for example, the technique is to be used as evidence in legal proceedings.

Isotopic variation is reflected in the tissues of consumers as animals synthesise new tissue from the nutrients in their diet (Gannes et al 1998; Hobson et al 1999c). Cells in most tissues have a lifespan shorter than that of the organism and are constantly being replaced. The rate at which this replacement occurs (turnover rate) varies depending on a number of factors including tissue type, body size, growth, and temperature (reviewed in del Rio & Carleton 2012). Due to differences in turnover rates among tissues multiple time periods can be sampled in one sampling event. Thus, the tissue that is to be sampled must be carefully selected for best reflection of the period(s) of interest. For example, quail blood and quail bone collagen have different turnover rates. Analysis of blood will give information on diet or habitat from a period of a few days prior to sampling, whereas bone collagen will reflect a period of several months prior to sampling (Hobson and Clarke 1992). Other tissues such as keratin are metabolically inert once grown, so signatures are preserved indefinitely; avian feathers feature prominently in the literature but claws, mammal whiskers, hair, and turtle carapace have also been used in isotope studies (Bearhop et al 2003, Cherel et al 2009, Hobson et al 2004a, Ehleringer et al 2008, Cerling et al 2006, Reich et al 2007, Bodey et al 2010, Vander-Zanden et al 2010). Such tissues may be grown at particular times of year, and so reflect different stages of the annual cycle. Therefore, as with analysis of soft tissues, it is important to understand timing of keratin growth as misunderstandings of moult strategy or growth rate can lead to misinterpretation of the data (see Britzke et al 2009).

Assignment of animals to a geographic origin based on stable isotope measurements relies on knowledge of how isotope ratios vary spatially and temporally. Isotope ratios of biological samples are linked through diet to underlying patterns in geological or hydrological isotope ratios. Such patterns can be mapped as isoscapes and used to infer the origins of samples when there is a strong relationship between the isotope ratios of the two. The ideal situation is to use an isoscape built from samples that are as similar as possible to the samples of unknown origin; preferably the same tissue, age, species, and sex and over the same temporal period. Hobson & Wassenaar (1998) reared monarch butterflies on their sole larval plant across their Eastern breeding range, and from these individuals built an isoscape of $\delta^{13}C$ and δD of wing chitin. By comparing measurements of wintering individuals in the same year to these isoscapes the authors elucidated the migratory patterns of monarch butterflies in North

America. Creating an isoscape for the time period of interest and across the entire range is not possible for most applications. However, studies that base assignments on isotope ratios of known samples from the focal species from different temporal periods, or on a smaller scale are not uncommon in the literature (Kelly et al 2002, Hobson et al 2009b, Bearhop et al 2006, Lott & Smith 2006). In other studies, precipitation δD isoscapes are utilised by calibration with a smaller number of known origin samples of the study species (Bowen et al 2005, Hobson et al 2007, Hobson et al 2009c).

Accuracy of assignment is dependent on a number of factors. Multiple locations have the same isotopic signature so incorrect assignments may be made, or certainty of assignment may be extremely low if not all possible locations are correctly included (Fox et al 2010). Also, all isotopic measurements are prone to analytical error, so if the isotopic difference between potential populations is small, certainty of assignment will be low and results may be misleading (Wunder et al 2005, Wunder & Norris 2008). Consideration of isotopic variance within and between populations of interest is therefore essential. In cases where precipitation isoscapes are used incorrect assignment of discrimination factors (between tissue δD and precipitation δD) will reduce accuracy (see Hobson et al 2009a) and further reduction in accuracy may be incurred through errors in interpolation of measurements from survey stations in the construction of the isoscapes (Wunder & Norris 2008).

Earlier I described predictable sources of variation in isotope signatures that enable geographic or dietary assignment. However there are further sources of variation that may introduce assignment error if they are not considered, such as the physiological condition of individuals. For example heat stress may cause variation in δD (Meehan et al 2003, McKechnie et al 2004, Powell and Hobson 2006, Betini et al 2009), as may age (Meehan et al 2003, Smith & Dufty 2005, Langin et al 2007), body size (Betini et al 2009), and sex (Hobson et al 2009b). Nutritional stress can affect $\delta^{15}N$ and $\delta^{13}C$ (Hobson et 1993, Polischuk et al 2001, Williams et al 2007, though see Kempster et al 2007). Temporal variation in $\delta^{13}C$, $\delta^{15}N$ and δD should also be considered (Meehan et al 2003, Wunder et al 2005, Langin et al 2007).

Recently, describing and accounting for sources of error in models has become more common, as has improved statistical assignment techniques for example the inclusion of other sources of information alongside isotope data in a Bayesian framework is increasingly used (Langin et al 2007, Wunder & Norris 2008, Hobson et al 2009a).

Stable isotopes and the wildlife trade

If assignment of origin based on precipitation isoscapes is to be used as legal evidence, large isotopic gradients, extensive knowledge of the biology of the species (including discrimination factors and turnover rate or tissue growth rate), and knowledge of all potential origins are highly desirable; without these, assignment of origin is likely to be called into question (e.g. Rocque et al 2006). Although these are barriers to this technique being used as definitive legal evidence, isoscapes could be used as a tool in identifying heavily exploited populations to which greater protection can then be directed (see Wasser et al 2007, Wasser et al 2008). Vogel and colleagues (1990) and van der Merwe and colleagues (1990) conducted seminal pilot studies for the potential application of stable isotope analysis to determine the origin of elephant ivory. The authors used a trivariate approach using carbon, nitrogen and strontium signatures, which revealed distinct clusters for geographically and ecologically distinct populations. Following this work, Ziegler et al (2012) have developed isoscapes of elephant ivory to assist in the determination of ivory origin, which may facilitate identification of poaching hotspots so policing can be targeted (see Wasser et al 2007, Wasser et al 2008). However, it should be noted that genetic analysis is already being used successfully to this end in informing the wildlife trade (Wasser et al 2008, White et al 2011).

Stable isotope analysis has successfully been used in prosecutions relating to food fraud (Balling & Roßmann 2004), but thus far to my knowledge not in wildlife crime. The technique has however been applied in a few studies to determine whether individuals are of captive or wild origin, and its novelty as a regulatory tool in the wildlife trade lies in its potential to distinguish between such sources. Kelly et al (2007) compared signatures of captive bred passerines and individuals of two subspecies of goldfinch (*Carduelis carduelis britannica* and *C. c. major*) that were suspected to be of wild origin. The two subspecies were distinguishable by morphological differences, and were from isotopically distinct regions (the UK and Siberia). Isotopic signatures correlated with isoscapes based on precipitation, with the Siberian subspecies being more depleted than the UK subspecies. In addition, both differed significantly from captive bred canaries. However, there was not enough evidence to suggest that wild and captive specimens from isotopically similar geographic regions can be differentiated and the authors caution against extrapolation from one species to another. Whilst this study shows promise for stable isotope analysis to be used to

ascertain status of specimens as captive bred or wild caught, isotopic signature of the natural range and the captive location must differ to a high degree, and tissue must remain from the period in which the bird was taken from the wild.

Individuals that are isotopically distinct can be concluded to differ somehow in behaviour, physiology, or habitat. However, it cannot be concluded that individuals that have similar isotopic signatures are from the same location, only that they are from isotopically similar habitats. Fox et al (2010) used hydrogen values of feathers to determine whether a marbled duck (*Marmaronetta angustirostris*) shot in Essex (UK) was a natural vagrant or an escaped captive bird. Hydrogen signature of the shot bird was significantly different from that of birds originating from the closest wild source (Spain), however no other wild populations were tested. As the signature was similar to that of mallard (*Anas platyrhynchos*) and Baikal teal (*Anas formosa*) in Northern Europe the authors suggested that the specimen may have originated from a captive population in Northern Europe. However, the results were not conclusive: whilst it could be concluded that the specimen was unlikely to be from Spain, the similarity to ducks from Northern Europe only confirms that the two are from isotopically similar habitats. It could equally have been concluded that this duck was a true vagrant but from a population that the authors had not tested.

In a similar study, Fox and colleagues (2007) used hydrogen and oxygen isotopic signatures to investigate the origins of a single *A. formosa* specimen shot in Denmark, well outside its natural range of Siberia. Both hydrogen and oxygen are significantly depleted in Siberia compared to Denmark and the bird retained both juvenile and first-winter feathers. The authors therefore tested whether feathers grown at the breeding grounds showed significantly lower signature than feathers grown in Denmark. The authors found that the isotopic signature of the juvenile feathers complied with expected values for a vagrant, given known isoscapes. However, even without comparison with known hydrogen isoscapes, the extreme difference in values within tissues of the same individual suggested large scale movement, or a highly unlikely change in diet and drinking water, making the results of this study fairly conclusive.

Isotopic signature of a single period of growth could potentially be used to differentiate between specimens of wild and captive origin when isoscapes of the location of captivity and the natural range do not overlap (Kelly et al 2007). However, captive animals will often experience a ‘global supermarket diet’ that does not reflect the local isoscape, and may lead to overlap with the signature of wild populations (see Nardoto

et al 2006, Bol & Pflieger 2002). This may lead to an inability to definitively classify a captive individual as wild-sourced, although a specimen with a signature outside that found in wild populations could still be assumed to be captive. In this situation, isotope evidence could be used to clear a suspect but not to prosecute.

Keratinous tissues that are laid down sequentially over time have been successfully used in longitudinal studies of animal behaviour (Cherel et al 2009, Vander Zanden et al 2010). The ability to take samples that reflect multiple points in time opens the possibility to look at temporal patterns of change in isotope signature, which may reflect changes in behaviour or location (Bodey et al 2010, Fox et al 2007). It may also be useful in distinguishing captive bred from wild caught specimens. A single dramatic shift in signature or temporal pattern of signature (for example highly variable over time, to highly consistent) may be indicative of movement from a wild state to a captive state. Change in signature implies a change in diet, location, or physiology, all of which may differ between captive and wild individuals. A single jump from one signature to another is likely to be most useful in species that are expected to have a relatively constant signature throughout their life, such as sedentary dietary specialists. However, care should be taken in interpreting such shifts in signature as movement out of the wild and into captivity, as a jump is also likely to occur with change in ownership and consequent alteration to food-source.

It may be that longitudinal patterns in signature could be used as an indicator of captive or wild origin. Captive animals may be subject to erratic and opportunistic changes in food bought by owners and supplied by manufacturers. In contrast, wild animals, particularly those that are sedentary, are likely to experience predictable seasonal fluctuations that correspond with seasonal variation in natural food availability (Roth 2002). In such a scenario, it follows that wild specimens will display a less erratic temporal signature than those that are captive. However, it is possible that the opposite may be found, with wild animals having a temporally more variable signature due to dispersal, migration, and periods of nutritional or environmental stress. Several studies have shown that nutritional stress, which is more likely to be experienced by wild individuals, influences the carbon and nitrogen signature in multiple species and tissues (McCue & Pollock, 2008). Heat stress may be reflected in hydrogen and oxygen signature (McKechnie et al 2004), though this could be experienced by both wild and captive individuals, particularly in species that require an artificial heat source. It can then be seen that untangling the causes of temporal patterns could be very

difficult, and without extensive knowledge of the species biology and captive husbandry, misinterpretation of temporal patterns is possible.

Conclusions

Stable isotope analysis shows some promise as a tool for regulation of the wildlife trade, both as a method to trace geographical origins and to differentiate between captive bred and wild sourced specimens. However, it must be stressed that this approach is not a panacea that will be applicable to all species or situations. Much groundwork must be done before the approach can be validated and this process must occur for each new application of the technique in wildlife trade. Stable isotope analysis is most likely to be beneficial where there are large isotopic gradients, where the biology of the study species is well understood both in the wild and in captivity, where all potential isoscapes are known and where there is additional information that can be used alongside isotopic data. It is unlikely that it could be used as sole evidence for illegal trading, however it may have great value in provision of intelligence and adding to a body of evidence that may be required in a decision to prosecute.

Whilst stable isotope analysis may be useful in tracing geographical origins of wild specimens, genetic analysis is already being used to these ends when the DNA is intact and there is sufficient genetic variability between geographically distinct populations. The novelty of stable isotopes in the wildlife trade lies in its potential to differentiate between captive and wild specimens, for which there are three potential routes: 1) use of distinct values to compare between individuals 2) use of longitudinal data to compare within individuals (looking for marked shifts in signature) and 3) use of longitudinal data to compare between individuals. Each approach has its drawbacks, but used in combination they may prove to be a powerful tool. If using stable isototope analysis to distinguish between captive and wild specimens stable isotope analysis is likely to be most useful for species with isotopically restricted natural range, and from which tissue from multiple temporal periods can be measured.

CHAPTER 2

Introduction to tortoises: trade, ecology, and husbandry

In this thesis I consider the potential applications of stable isotope analysis to regulation of the tortoise trade. Understanding the ecology and captive husbandry practices of the species under consideration is necessary for the interpretation of isotope signatures. In this section I will therefore summarise the recent history of high volume tortoise trade in the UK, the ecology of the predominant species, and tortoise husbandry practices in the UK and in tortoise ranches and farms in exporting countries.

Trade history

Globally, tortoises (Testudinidae) were the third most heavily traded reptile family between 2004 and 2008, and species of the genus Testudo comprise the vast majority of the tortoise trade both globally and in the UK (UNEP-WCMC). There are five Testudo species, known as Mediterranean or garden tortoises: marginated (*Testudo marginata*), Kleinmann's (*Testudo kleinmanni*), Hermann's (*Testudo hermanni*), spur-thighed (*Testudo graeca*), and the Russian tortoise (*Testudo hordfieldii*), of which the latter three are the most heavily traded. Other species commonly found in the UK tortoise trade at lower volumes include leopard (*Geochelone pardalis*), Indian star (*Geochelone elegans*), African spurred (*Centrochelys sulcata*), redfoot (*Chelonoidis carbonaria*), yellowfoot (*Chelonoidis denticulata*), pancake (*Malochochersus tornieri*), and hingeback tortoises (*Kinixys spp.*).

High demand for the pet trade combined with habitat loss has led to the dramatic decline of many Testudo populations, particularly *T. graeca*, *T. hermanni*, and *T. kleinmanni* (van Dijk et al 2004, Perälä 2003, Van der Kuyl et al 2005). In 1975, the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) listed all Testudo species under Appendix II, making it necessary for import/export permits to be issued before trade is permitted. Despite these restrictions declines continued and in 1984 all Testudo species, with the exception of *T. horsfieldii*, were listed as Annex A species (EU Wildlife Trade Regulations): international trade in all wild caught specimens became illegal. It is required that with each change of ownership specimens of Annex A species are accompanied by an Article 10 Certificate, which states the name of the vendor, geographical origin and breeding source. There are four possible breeding sources listed by CITES: 'wild-caught' refers to individuals collected from the wild; 'farmed' specimens are those that have been sired in the

controlled environment; ‘ranched’ specimens must be raised in captivity but need not be either sired or born into it; ‘captive-bred’ specimens must satisfy several stipulations, but in basic terms are sired and born into populations that are maintained without the introduction of wild specimens.

In 1994, due to high threat of extinction, all commercial trade in *T. kleinmanni* became illegal. With such increases in protection for *T. hermanni*, *T.graeca* and *T. kleinmanni*, and with the lifting of the Iron Curtain, trade has recently focussed on *T. horsfieldii* (figure 1). Because of high mortality during transport and acclimation of specimens, a ban on imports of wild *T. horsfieldii* was imposed in 1999 (September 1999, European Union (EU) wildlife trade regulation 338/97). However, this ban was lifted in 2006 (UNEP-WCMC 2010). *T. horsfieldii* is now, globally, the most heavily traded tortoise species and the third most commonly imported reptile species, with a large proportion of specimens being of wild origin (UNEP-WCMC). As an Annex B species, it is legal to trade *T. horsfieldii* specimens of wild, ranched, farmed and captive-bred origin. However, despite relatively high import numbers for *T. horsfieldii*, trade has not risen to the level of that seen in the 1970s and 1980s for *T. hermanni* and *T. graeca* (figure 1). Because of their importance in the pet trade, in this thesis I will restrict my analyses to *T. hermanni*, *T. graeca* and *T. horsfieldii.*

Current trade: quantities and routes

I used data for the past ten years (2001-2011) from the CITES trade database (accessible at www.unep-wcmc-apps.org/citestrade/) to examine the source and origin of *T. horsfieldii, T. hermanni* and *T. graeca* entering the UK. It should however be noted that the last complete year of data for the database is 2005 as some countries report their statistics independently. I have used only import quantities, as export quantities from some countries, such as Uzbekistan, reflect the number of permits applied for rather than the number of specimens actually exported. I have not included any information on imports from within the EU as these quantities are not reported to CITES. Whilst approximate quantities of EU imports could, in theory, be obtained from collating information on Article 10 Certificates, it was not possible for this project.

T.horsfieldii specimens imported to the UK in the past ten years were reported as ranched (n=32,401), farmed (n=7,122), or wild (n=2,525). All but two specimens (re-exported from the USA) were exported from Uzbekistan (n=34,901) or exported/re-exported from Ukraine (n=8,582). For this period, re-exports of *T. horsfieldii* from Ukraine originated in Uzbekistan (n=160). Although it is legal to export wild specimens,

they fetch lower prices and may be less likely to survive in captivity (UNEP-WCMC, 2010). There are concerns that some wild specimens are laundered into the market as ranched specimens, in particular as re-exports from the Ukraine (*pers. comm.* Rick Wilton, company director, Zoological International Ltd.).

Despite trade restrictions, *T. graeca* and *T. hermanni* remain popular in the tortoise trade. In the last ten years 8,829 *T. graeca*, and 9,937 *T. hermanni* were imported into the UK from outside the EU (figure 1); the vast majority were from large scale breeding farms in Slovenia, Turkey and Macedonia. A breakdown of imports from non- EU exporting countries is shown in table 1. Tortoises from captive breeding farms are considered to be of questionable source by some, with fears that wild-caught specimens are being laundered into the market through these farms (Vinke & Vinke 2010).

A small proportion of the tortoise trade is supplied by enthusiast small-scale breeders in the UK. I collated information on 63 tortoise breeders listed on a number of advisory sites on the internet (www.tortoise-protection-group.org, www.tinytortoises.co.uk, www.tortsmad.com, www.tortoise-world.com, www.shelledwarriors.co.uk, www.tortoisemad.co.uk, www.thetortoisehouse.com). This information is summarised in table 2. The most commonly bred species were *T. hermanni* (n=35), followed by *T. graeca* (n=30) and *T. horsfieldii* (n=16). This list is not, however, exhaustive.

Wild-caught specimens of *T. graeca* and *T. hermanni* are also readily available on tourist markets in their natural ranges. False information is often given by vendors about the legality of such transactions (RSPCA 2001). It is possible that such specimens are laundered into the UK tortoise trade with fraudulently obtained Article 10 certificates.

Tortoises in the wild: range, habitat, diet and behaviour

Two of the three study species of this thesis are quite similar: *T. graeca* and *T. hermanni*, whose range is restricted to isolated patches around the Mediterranean basin. The other, *T. horsfieldii* has a range that extends from the Caspian Sea to China and from Kazhakstan to Iran (figure 2). *T. graeca* and *T. hermanni* occupy mosaic habitats that comprise both densely vegetated and open areas, though the habitat of *T. hermanni* tends to be less open than that of *T. graeca* (Anadon et al 2006, Wright et al 1988). Mediterranean forest and farmland are suitable habitat for both and *T. hermanni* are also found in dune scrub and maritime grassland (Stubbs 1989a). In

addition, some small, isolated populations of *T. hermanni* exist in urban parks, for example in Rome (Rugiero & Luiselli 2006). Regulation of body temperature is accomplished by shuttling between sunny and shady patches (Meek & Innskeep 1980).

In contrast to the above species, *T. horsfieldii* tends to inhabit arid environments: desert, semi-desert and steppe, though it can also be found in foothills, mountains, marshes, valleys, and floodplains (Stubbs 1989b, Theile 2000). Due to unfavourable conditions for most of the year, individual tortoises are active only in spring for approximately two months, during which time temperatures at night can drop to -10 °C and daytime temperatures can reach 45°C (Lagarde et al 2002). To cope with fluctuations in temperature this species is semi-fossorial, and spends the majority of time in burrows up to 2m long that facilitate moisture retention (Highfield 1992). It has been reported that individual Horsfield's tortoises are active for just 3.5% of the year and may spend less than twenty hours a year foraging (Lagarde et al 2002, Lagarde et al 2003).

In general all three species have a sedentary lifestyle and males are more active than females as they search and/ or fight for mates (Chelazzi & Calzolai 1986, Stubbs 1989a, Stubbs 1989c). *T. hermanni* that inhabit densely wooded areas are an exception as females migrate to specific isolated clearings from their usual home-range in order to lay eggs (Stubbs 1989a).

Testudo tortoises drink from puddles and streams when water is available although they are capable of tolerating water deprivation for substantial periods. During dry periods activity levels are reduced and tortoises aestivate in their relatively humid burrows in order to reduce fluid loss. Testudo tortoises are vegetarian, although there are anecdotal accounts of diet including molluscs, earthworms and insects. For example El Mouden et al (2006) found invertebrate fragments in 21% of *T. graeca* faeces analysed, however, to my knowledge, active selection of invertebrates has not been documented, and where invertebrates form part of the diet it is likely to be of negligible proportion. Diet of the three species is similar though differences exist among populations, for example plants in the carnation family were eaten by one population of *T. graeca* (Cobo & Andreu 1988) but actively avoided by another (El Mouden et al 2006). Flowering plants such as legumes (Fabaceae), buttercups (Ranunculaceae) and asters (Asteraceae) feature heavily in the diets of all three species (El Mouden et al 2006, Cobo & Andreu 1988, Meek & Inskeep 1980, Lagarde et al 2003). Crucifers (Brassicaceae), and poppies (Paperveraceae) have also been

reported to contribute significantly to the diet of *T. horsfieldii* (Lagarde et al 2003). In addition, grasses (Poaceae), and to a lesser extent sedges (Cyperaceae) and rushes (Juncaceae) form part of the diet of many populations of *T. graeca* and *T. hermanni* (Cobo & Andreu 1988, del Vecchio et al 2011). At least one population of *T. horsfieldii* has, however, been found to actively avoid grasses (Lagarde et al 2003). It is difficult to assess the contribution of C-3 and C-4 plants to the diets of these species. It is likely that the majority of diet consists of C-3 plants, however there is certainly some contribution of C-4 plants, for example Cobo & Andreu (1988) found evidence of consumption by *T. gracea* of *Cynodon dactylon* and *Panicum repens*, both C-4 grasses (Pyankov et al 2010).

Timing of reproduction is variable among species and populations due to the large differences in climate experienced. All three species mate soon after emergence from hibernation and in at least some populations of *T. gracea* and *T. hermanni* a second period of mating occurs in late summer, with eggs hatching in mid to late summer (Stubbs 1989a, Stubbs 1989c, Henen 2000). In *T. horsfieldii* eggs are produced from both stored resources (capital) and those acquired in the current season (income) (Henen 2004). As a consequence, although females spend a greater amount of time foraging than males their body condition may decrease during the active period (Henen et al 2000). In contrast, despite intensive activity in searching for mates, males increase in weight during the mating season (Henen et al 2000). Length of hibernation differs among species and populations but is shorter for *T. gracea* and *T. hermanni* than *T. horsfieldii*. Occasional and brief emergences during winter have been reported for *T. hermanni* (Stubbs 1989a).

Tortoises in captivity: diet and husbandry

The potential to distinguish between captive and wild tortoises using stable isotope analysis is dependent on there being differences between the diet, or the source of the diet, between the two groups. In order to gain an understanding of UK captive tortoise diet I created an online survey with kwiksurveys.com and sent it to breeders and owners, and posted links on facebook and on a website for the project (survey 1; see Appendix A). Owners attending post hibernation veterinary care sessions in Bristol also completed a questionnaire on diet (survey 2; see Appendix A). In addition to these surveys I searched websites for advice on tortoise diet.

a) Tortoises in the UK

The majority of online tortoise husbandry sites currently advise a varied diet of weeds and leafy vegetables with very occasional fruit. Root vegetables and high protein vegetables such as peas are advised against (BCG 2012, Highfield 2002, thetortoisetable.org.uk, tinytortoises.co.uk, tortoise.org.uk). Until recently textbooks advised a diet that included animal protein though this is now actively discouraged on specialist websites. Pre-packaged tortoise foods are also available and are occasionally advised as part of a balanced diet. Brands include Pre-Alpin, from Germany (agrobs.de), and Repcal tortoise food from California (repcal.com).

Survey 1 had 25 respondents, 18 of whom breed tortoises, and 23 tortoise owners completed survey 2. Data on whether the latter were tortoise breeders were not collected, but it is likely that the majority were not.

The number of respondents that fed their tortoises each of the dietary items is given in table 3. At least half the diet of tortoises of 75% of respondents (n=36) comprised of food definitely grown in the UK (figure 3). In most cases (n=30, 63% of respondents) garden weeds contributed to more than 50% of diet. Only 6% of respondents (n=3) did not feed their tortoises garden weeds. Other dietary items that contributed to more than 50% of any diet were supermarket greens (n=4), home-grown greens (n=2), and supermarket fruit (n=1).
No respondents fed their tortoises meat, although 2 noted that they had previously used cat or dog food.

In addition to questions on diet, in survey 1 I asked what source of water was available to tortoises. Of the 25 respondents, 11 gave only tap-water, 4 gave only rain-water, 9 gave both tap- and rain-water and 1 gave tap-, rain- and bottled-water.

Whilst these results reflect the advice found on websites, the sample is likely to be biased towards owners who use such sites. This is particularly likely to be the case for the online survey. I tested whether there was a difference in response to the two surveys using the Mann-Whitney-Wilcoxon test. There was a significant difference in percentage of diet sourced from the UK, with a greater proportion of the diet from the UK in the online survey; the median proportions of diet sourced in the UK for survey 1 and 2 respectively were 0.83 (n=25) and 0.6 (n=23) ($W=422$, $p<0.01$). Using data from survey 1, there was no difference in proportion of diet from the UK between owners who did and did not breed tortoises; median values for breeders (n=18) and non-

breeders (n=7) respectively were 0.77 and 0.83 (Mann-Whitney-Wilcoxon, W=64.5, p=0.95).

b) Tortoises in breeding facilities

Large-scale tortoise breeding facilities occur in Slovenia, Macedonia, Turkey (*T. graeca* and *T. hermanni)*, and Uzbekistan (*T. horsfieldii*). I could find no reference in the literature to the husbandry of these tortoises; however it is likely that they are force-grown on a more protein rich diet than wild individuals in order to increase productivity. The only legal tortoise breeding operation in Uzbekistan is the Uz ZooComplex. Tortoises in this facility are fed mainly on vegetables, salad leaves, pumpkins, wheat and protein supplement. Depending on the season apples, apricots and other fruits form part of the diet. Very young tortoises are also given yoghurt (*Pers. comm.* Evgeniy Peregontsev, Uz ZooKomplex).

Summary

As a method of trade regulation, stable isotope analysis is likely to be of greatest utility for *T. horsfieldii, T. hermanni* and *T. graeca* as they are imported in large numbers and are commonly bred in the UK. There are concerns that this high volume legal trade acts as a cover for the illegal trade; primarily as 1) wild *T. horsfieldii* falsely declared as ranched specimens (*pers. comm.* Rick Wilton, company director, Zoological International Ltd.); 2) wild *T. graeca* and *T. hermanni* from tourist markets claimed as UK-bred (RSPCA 2001); and 3) wild *T. graeca* and *T. hermanni* claimed as captive bred in large-scale farms (Vinke & Vinke 2010).

All three species typically hibernate for a substantial period and are vegetarian (though they may consume small quantities of invertebrates), and specialist websites advise that tortoises in captivity are given a similar diet to that of wild tortoises. Owners that completed diet surveys predominantly fed their tortoises garden weeds, but supermarket greens were also common dietary components: tortoise diet varies between owners and not all UK-kept tortoises will have a UK hydrogen signature. The diet of tortoises bred in large scale farms is unknown and may or may not differ to that of UK or wild tortoises.

CHAPTER 3

Resolving temporal patterns in isotope signature: a preliminary longitudinal study of tissues of the tortoise *T. horsfieldii*

Introduction

Longitudinal analysis of isotope signature may be useful for identifying the source of animal and plant specimens in trade (see chapter 1). There are three main approaches that can be used to resolve temporal patterns; firstly, sampling the same tissue on multiple occasions; secondly, sampling multiple tissues with different turnover rates and thirdly, serially sampling a single tissue (Dalerum & Angerbjörn 2005). From a wildlife trade regulation perspective, the latter two are of particular interest as these require only a single sampling event.

Temporal patterns can be resolved through measurement of tissues with different metabolic turnover rates. For example Hobson (1993) measured carbon and nitrogen stable isotope ratios of blood, liver and bone collagen of seven species of seabird to obtain short-, intermediate- and long-term dietary information. However research is required into fractionation factors, turnover rates and the effects of factors such as age, growth and metabolic routing for the taxa of interest, before this method can be confidently used (e.g. Bearhop et al 2002, Reich et al 2008).

Serial sampling of metabolically inert tissue is the method most likely to be of use in wildlife trade regulation as such tissue can incorporate information over long periods (Cherel et al 2009, Bodey et al 2010, Vander Zanden 2010). Tortoises of the genus Testudo are a good model for testing the potential of serial analysis for trade regulation purposes: they continue to grow past the age of sexual maturity, have a carapace formed of sequentially grown keratin, which is not shed (Wilson et al 2003), and they are traded extensively. Newly grown scute keratin forms a layer both below and around old growth causing the appearance of growth rings (Alibardi 2005, Seltzer & Berry 2005, Murray & Wolf 2012) (figure 4), which can be non-invasively sampled. Tortoise carapace therefore provides the opportunity to sample material from the entire lifespan of specimens entering the trade.

There have been very few stable isotope studies on tortoises. However, a recent diet switch experiment conducted by Murray & Wolf (2012) examined $\delta^{13}C$ of carapace keratin, red blood cells and plasma of desert tortoises (*Gopherus agassizii*). They sampled keratin before and after a diet switch. A single scute was sampled from each tortoise, by cutting and lifting a strip of keratin bisecting the growth rings. The carbon signature of growth rings grown before the diet switch was different between the two sampling events: carbon from the second diet was incorporated into growth rings that were present before the diet switch. This is consistent with the scute-growth pattern shown in figure 4a. Sampling in this way is therefore not ideal for resolving temporal patterns as weighted averages of several growth periods are obtained rather than values of individual growth periods. In addition, the sampling method used in the Murray & Wolf (2012) study may increase risk of infection as it exposes live tissues under the keratin. A preferable sampling method may be to remove a shallow layer of keratin from the surface, thus reducing the time period covered by material in the sample. The keratin covering the bony carapace is very thin, particularly in young tortoises, but pooling material from multiple scutes would enable the collection of larger samples. In order sample in this way it would be useful to know whether isotope ratios are conserved among scutes. To my knowledge there have, to date, been no such studies. It would also be of interest to know whether plastral and carapacial scutes differ, as sampling damage is less visible on the plastral scutes.

Claws are also formed of keratin and may therefore present an additional opportunity for collecting short to mid-term temporal data. For example Bearhop et al (2006) used δD of claw tips from blackcaps (Sylvia atricapilla) that had recently returned to their breeding grounds to infer wintering latitudes of individuals, which could not be achieved through sampling feathers (as they were grown on the breeding grounds). Use of claws in isotope studies is however limited by lack of knowledge of growth patterns so although claws are continuously grown they should not currently be used for time series (Bearhop et al 2003).

In this chapter I report the carbon, nitrogen and hydrogen values of claw, carapacial and plastral keratin of a batch of imported *T. horsfieldii*. I examine whether signatures vary among scutes in order to determine whether material from multiple scutes can be pooled, and look at whether there are differences between front and back claws to evaluate the potential of claw material in longitudinal studies. I also took the opportunity to sample blood, liver and muscle for future reference and have reported these results

in Appendix B. I discuss my findings in relation to stable isotope analysis as an approach for regulation of the tortoise trade.

Methods

Scute keratin standard

I prepared an in-house keratin standard from a single dead adult *T. graeca* specimen, the history of which is unknown. The keratin of two scutes was used. This material was cleaned with ethanol, homogenized using a high velocity ball mill, and dried at 60°C for 72 hours. I analyzed ten samples of the keratin standard at 0.7mg and 1mg to ascertain mean and standard deviation $\delta^{13}C$ and $\delta^{15}N$, respectively (values ± 1 sd: $\delta^{13}C$ = -25.88 ± 0.14; $\delta^{15}N$ = 5.01 ± 0.28).

For hydrogen analysis it is preferable to run samples in triplicate, due to a memory effect (Olsen et al 2006). In order to determine how best to divide small samples I therefore analyzed samples at two weights in both duplicate and triplicate as follows: I analyzed 8 triplicates at 1mg and 0.5 mg spaced by blanks or standards, removed the first of each triplicate from analysis and took an average of the final two. I also analyzed 8 duplicates at 1mg and 0.5mg spaced by blanks or standards and removed the first of each duplicate from analysis. The results are shown in Appendix C. As the standard deviation for hydrogen was smaller for triplicates at 0.5mg than for duplicates at 1mg, where sample weights obtained were small I divided them into triplicates of a lower weight rather than duplicates of a higher weight.

T. horsfieldii samples

I analysed 15 *T. horsfieldii* from a single batch re-exported from Ukraine. The specimens died soon after import (9-48 days) and had between 5 and 9 growth rings. Country of origin and source (wild/farmed/ranched) are unknown. I kept all specimens in a freezer at -18°C prior to sampling.

I cleaned scutes and claws to be sampled with water and scraped keratin samples from the carapace and plastron using a dental scaler. Growth stages sampled were the juvenile scute, which is grown before hatching, the growth bars adjacent to the juvenile scute (approximately 2; henceforth referred to as old growth), and the most recent growth bars (approximately 2) (figure 4c). These were taken from four carapacial and two plastral scutes (figure 4d). Due to low sample weight not all scutes could be

sampled for both carbon and nitrogen, and hydrogen. I therefore grouped the specimens as shown in figure 4d. I weighed material obtained for each sample. In addition to scute keratin I clipped all claws with intact tips, and used a high velocity ball mill to homogenise samples. I dried all keratin samples in an oven at 60°C for a minimum of 72 hours.

I loaded samples into tin capsules (approximate weights: C/N 0.5mg or 0.7mg, H 0.5mg or 1mg). Where sample weight was high enough I ran samples in triplicate or duplicate. Carbon ($\delta^{13}C$), nitrogen ($\delta^{15}N$), and hydrogen (δD) isotope ratios were determined using a Fisons 1108 Elemental Analyser coupled to a GV Instruments Isoprime in continuous flow mode. I included regularly spaced laboratory standards in all sample runs (see Appendix C for standards and values). These were calibrated against international standards. I also included the in-house scute keratin standard described above in all sample runs. All values are reported in delta notation (δ): δ(‰) = (R_{sample} / $R_{standard}$ – 1) x 1000, where R_{sample} and $R_{standard}$ are the ratio of heavy to light isotopes of the sample and standard respectively. Long term in-house repeatability for each element is as follows: 0.2‰ ($\delta^{13}C$), 0.3‰ ($\delta^{15}N$), 3‰ (δD). The standard deviation of scute keratin standard across all plates was 0.17‰ ($\delta^{13}C$), 0.36‰ ($\delta^{15}N$) and 4.6‰ (δD).

Statistical analysis

I used general linear mixed models (GLMMs) in R (R2.12.1, www.r-project.org), using the package lme4 to test differences among and within tissues. I specified identity of tortoise and sample number (whether sample was first, second or third in a triplicate/duplicate) as random factors in all models.

To test whether sample weight affected carbon or nitrogen signature I included an interaction between keratin age and original sample weight. I did not collect data on weight of hydrogen samples. To test whether signature was affected by number of days spent in the UK before death I included interactions between the number of UK days and recent scute growth, and also between number of UK days and position of claw (front / back). In all cases I checked the residual plots of minimum adequate models.

Results

Scute keratin

There was no difference among carapacial and plastral scutes in $\delta^{13}C$ (lmer: $\chi^2_{4,9}=0.57$, $p=0.97$), $\delta^{15}N$ (lmer, $\chi^2_{4,9}=3.40$, $p=0.49$), or δD (lmer, $\chi^2_{4,6}=8.65$, $p=0.07$). $\delta^{13}C$ did however differ among ages of keratin growth (lmer: $\chi^2_{2,5}=29.93$, $p<0.001$), as did $\delta^{15}N$ (lmer: $\chi^2_{2,5}=194.69$, $p<0.001$), and δD (lmer, $\chi^2_{2,4}=19.48$, $p<0.001$; figure 5). Dry weight of samples obtained for carbon and nitrogen analysis ranged from 0.35mg to 2.69mg (mean ± 1SD: 1.24 ± 0.48), yet despite differences among ages, original sample weight did not affect $\delta^{13}C$ (lmer: $\chi^2_{2,11}=1.74$, $p=0.42$), or $\delta^{15}N$ (lmer: $\chi^2_{2,11}=0.026$, $p=0.99$).

There was no effect of number of days alive in the UK on signature (lmer, $\delta^{13}C$: $\chi^2_{1,4}=2.16$, $p=0.14$; $\delta^{15}N$: $\chi^2_{1,4}=0.02$, $p=0.89$; δD: $\chi^2_{1,4}=0.11$, $p=0.74$).

Claw

Back claws were more enriched in $\delta^{13}C$ than front claws (lmer: $\chi^2_{1,5}=14.89$, $p<0.001$), but only by 0.19‰, which is just outside measurement error of the scute standard for $\delta^{13}C$ (0.17‰). Front claws were also more enriched in $\delta^{15}N$ than back claws (lmer: $\chi^2_{1,5}=14.11$, $p<0.001$) by a mean of 0.39‰, which is again just outside measurement error of the scute standard (0.36‰). There was no difference in δD of front and back claws (lmer: $\chi^2_{1,5}=0.99$, $p=0.32$)

Position of claw (front/ back) did not affect the relationship between $\delta^{13}C$ and number of days spent in the UK (lmer: $\chi^2_{1,7}=2.82$, $p<0.09$). However $\delta^{15}N$ of back claws became more depleted with increased time spent alive in the UK than did front claws (lmer: $\chi^2_{1,7}=6.32$, $p<0.05$; figure 6). There was also an interaction between number of days in the UK and claw position for δD (lmer: $\chi^2_{1,7}=8.02$, $p<0.01$; figure 6).

Discussion

In contrast to Murray and Wolf (2012), who sampled tortoises by lifting a strip of keratin from the scute, I sampled only the material at the surface. The sampling technique used by Murray & Wolf (2012) resulted in inclusion of material of non-target ageing periods. In this study I used weight of material taken as a proxy for depth of sampling. Deep samples are likely to include non-target ages of growth. The absence of a relationship between weight and signature despite differences in signature among ages suggests that I did not reach a great enough depth to include non-target ages.

However, this is not a robust method to test the age of material sampled and a diet-switch experiment would be required to definitively confirm these results. There does not seem to be a disadvantage to the sampling technique that I have used here as isotope values do not differ among scutes: significantly, material can be pooled from multiple scutes in order to obtain representative larger sample sizes.

The difference in $\delta^{13}C$, $\delta^{15}N$ and δD among scute keratin of different ages suggests that serial sampling of scutes may present an opportunity for resolving temporal patterns of signature in tortoises (figure 5). However the time taken for growth ring deposition in tortoises is not well understood and is likely to be influenced by changes in environment, behaviour and/ or physiology (Wilson et al 2003). It is known that tortoises in captivity are capable of laying down multiple rings in a year, for example desert tortoises kept in captivity by Murray and Wolf (2012) grew between two and five growth rings per year. The period of time represented by the newest growth of scute in this study is probably months rather than weeks prior to sampling as time spent in the UK before death had no effect on signature of the newest growth of scute.

Front and back claws differ quite markedly in size, with back claws being much shorter and narrower. They also differ in both $\delta^{13}C$ and $\delta^{15}N$, which suggests that they incorporate isotopic signatures from different time periods. It may therefore be possible to use claw material for longitudinal studies, with back claws presumably representing a more recent time period (figure 6). However, more research is needed on the growth pattern of claws and how this varies between wild and captive tortoises, before use of claw tissue for this purpose would be practicable.

Given current knowledge of metabolic turnover rates, discrimination factors and growth pattern of claws, the most promising method for resolving temporal patterns in isotopic signatures for tortoises is serial sampling of scute keratin. The remainder of my thesis will therefore focus on this approach to characterising the origin of tortoises.

CHAPTER 4

Stable isotope analysis as a potential method to distinguish between ranched and wild *T. horsfieldii*

Introduction

The wildlife trade can be a major driver of declines in wild populations (Hill 1990, Sodhi et al 2004, Schlaepfer et al 2005, Smith & Walpole 2005). Whilst the legal trade can be monitored and quotas based on these data, the same is not true for the illegal trade due to its often secretive nature. Whilst seizures of illegal goods can provide information on trade networks and the extent of, and temporal patterns in the illegal trade, (Wasser et al 2008, Rosen & Smith 2010, Shepherd & John 2010), this can be impeded by the difficulty of distinguishing between legal and illegal specimens. Such a difficulty may arise when specimens are traded under false identity, for example as being from a legal geographic or breeding origin, or being a different species (Baker 2008, Vinke & Vinke 2010, White et al 2011). An increase in knowledge of trade routes, which populations are under threat from over-exploitation, and trends in trade volume is highly desirable as this can then be used to target conservation resources more efficiently.

It is in the interest of both consumers and suppliers that specimens entering the market are legal and of known origin. Ignorance of true population size and extent of collection may lead to overexploitation and, in addition to population declines, may also lead to loss of income for suppliers and loss of supply to consumers. A further concern for consumers is that the behaviour and welfare of specimens may differ among sources: disease, parasite load, stress-level, and life expectancy all affect the value of specimens and can differ among sources (Cabezas et al 2012, Reed 2005, Theile 2000). In addition, specimens traded outside the legal framework are unlikely to be screened for diseases, which may then be unwittingly spread to captive populations.

Development of tools to determine the origin of specimens would benefit the trade and may provide valuable information on the extent of threat to wild populations. *T. horsfieldii* is an ideal organism with which to test the potential of stable isotope analysis to differentiate between populations of different breeding source: carapace keratin

provides a long-term temporal record, and both wild and ranched specimens of *T. horsfieldii* are legally traded in the UK. In the UK, ranched specimens are more popular, fetch higher prices than wild origin tortoises and are more likely to survive in captivity (Theile 2000, *pers. comm.* Rick Wilton, company director, Zoological International Ltd). It is legal for specimens of *T. horsfieldii* from all sources (wild/ captive/ ranched) to be traded, yet there are concerns that some wild specimens are being fraudulently claimed as ranched (*pers. comm.* Rick Wilton). If this is the case, it is possible that some batches of imported tortoises consist of both ranched specimens and wild specimens.

Currently expert opinion is used to determine whether a shipment or specimen is legal or illegal. Variation among specimens and disparities between paperwork and specimens (such as reported age and size) can be used as indicators of the legality of shipments. Variation in size and morphology within a batch, the degree of wear on the carapace, and parasite diversity are also indicators of breeding origin. In addition, the carapace of wild specimens should have relatively smooth carapaces compared to those of specimens raised in captivity, which are more likely to show signs of pyramiding. Pyramiding in Testudo tortoises is a pathological deformity of the carapace that gives the carapace a lumpy appearance and is difficult to completely avoid when raising tortoises in captivity. Pyramiding can be caused by improper diet or environment and is often used as an indicator of how well captive care approximates conditions seen in the wild (Gerlach 2004, Wiesner & Iben 2003).

In this chapter I examine the isotopic composition of a batch of imported *T. horsfieldii* specimens. Using degree of pyramiding as a proxy for source, I explore whether differences in signature may be attributable to differences in source and discuss whether signature, and the change in signature over time within individuals, could be used towards regulation of the tortoise trade.

Methods

I analysed 30 *T. horsfieldii* from a single batch re-exported from Ukraine. The specimens died on import. Country of origin and breeding source are unknown. Data for 15 of these specimens are also used in chapter 3. Methods of sampling and stable isotope analysis are as described in chapter 3, with the exception that I pooled material from multiple carapacial scutes for the specimens not used in chapter 3.

To determine degree of pyramiding I took three photographs of each specimen in profile (see figure 7 for protocol). I measured the length and greatest depth of the second, third and fourth vertebral scutes in Image-J (figure 8). In order to obtain a measurement of degree of pyramiding I divided length by depth for each scute and took the average of this measurement for all scutes across all photographs for each tortoise. I categorised tortoises in groups of 5 according to degree of pyramiding, from the five most pyramided to the five smoothest.

Statistical analysis

On visualisation of the data, two highly distinct groups were apparent in old age growth when I plotted $\delta^{13}C$ against $\delta^{15}N$ (figure 9). Isotope ratios of samples from a batch of tortoises consisting of a mixture of both ranched and wild origin specimens could be expected to fall into two major groups that remain consistent across ages of growth. I therefore assigned individuals to the groups seen in old age growth (henceforth I refer to them as group A and group B) and tested whether individuals clustered into groups A and B at all ages of growth. To do this I used the kmeans function in R to carry out partitioning cluster analysis for each age of growth for the three combinations of elements ($\delta^{13}C$ and $\delta^{15}N$, $\delta^{13}C$ and δD, $\delta^{15}N$ and δD). For these analyses I used the mean isotope ratio across scutes for each tortoise at each age.

In order to determine whether there were differences in isotope ratios between group A and B for each element I used general linear mixed models (GLMMs) in the package lme4. I specified group and age of growth as independent variables and included the interaction between them. I specified identity of tortoise and repeat number as random factors. Where applicable I conducted post-hoc tests to see at which ages of growth differences occurred.

I used mean values of $\delta^{13}C$, $\delta^{15}N$ and δD to calculate the total change in signature over time for group A and group B. I calculated this as the sum of the difference between juvenile and old growth and between old and recent growth. I compared the groups with an ANOVA.

Results

Samples that clustered in old growth also clustered in recent and juvenile growth in plots of $\delta^{13}C$ and $\delta^{15}N$ (figure 9). For juvenile growth, partition cluster analysis

assigned 13 of the 17 specimens in group A correctly and all of the specimens in group B correctly. For recent growth 14 of the 15 specimens in group A and 11 of the 12 specimens in group B, were assigned correctly. The addition of hydrogen did not improve clustering and increased the number of incorrect allocations at all ages (table 4).

Given the possibility that clustering may be due to differences in origin, I looked at whether clustering was related to degree of pyramiding. Measurements of pyramiding are shown in figure 10. The five smoothest specimens and five most pyramided specimens (a proxy for wild/ captive origin respectively) fell into separate clusters (Fisher's exact test, $p<0.05$): all the smooth specimens belonged to group B, whilst three of the pyramided specimens belonged to group A, and one to group B. The remaining pyramided specimen was not assigned to a group, as the old growth sample for this individual was lost. However, from plots of the juvenile and recent growth it appeared to also belong to group A (figure 9).

I conducted general linear mixed models to determine whether groups were significantly different to each other at each age for $\delta^{15}N$, $\delta^{13}C$, and δD. There was a significant interaction between age and group for $\delta^{13}C$ (lmer; $\chi^2_{2,7}=74.75$, $p<0.001$), $\delta^{15}N$ (lmer; $\chi^2_{2,7}=30.50$, $p<0.001$) and δD (lmer; $\chi^2_{2,7}=22.43$, $p<0.001$). I conducted post-hoc tests for each period of growth. There was a significant difference between group A and group B individuals at all ages for $\delta^{15}N$ (lmer; Juvenile: $\chi^2_{1,4}=19.77$, $p<0.001$, Old: $\chi^2_{1,4}= 64.40$, $p<0.001$, Recent: $\chi^2_{1,4}=27.08$, $p<0.001$), but this difference was apparent only in old growth for $\delta^{13}C$ (lmer; Juvenile: $\chi^2_{1,4}=0.0004$, $p = 0.99$, Old: $\chi^2_{1,4}=25.75$, $p<0.001$ Recent: $\chi^2_{1,4}=0.06$, $p = 0.81$), and δD (lmer; Juvenile: $\chi^2_{1,4}=0.0005$, $p = 0.98$, Old: $\chi^2_{1,4}=11.60$, $p<0.001$, Recent: $\chi^2_{1,4}=0.15$, $p = 0.70$) (figure 11).

As clusters remained conserved across ages of growth I assumed that they were true groups and compared them to see if there were differences in total change over time. The total change in $\delta^{13}C$ of group A specimens was larger than that of group B specimens (ANOVA; $F_{1,25}= 4.45$, $p < 0.05$), however there was no difference between the groups for either nitrogen (ANOVA; $F_{1,25}= 2.83$, $p = 0.10$), or hydrogen (ANOVA; $F_{1,21}= 0.08$, $p = 0.78$).

Discussion

This study indicates that there may be potential for stable isotope analysis to be used as a tool to explore the composition of batches of specimens. Specimens in a single batch that is claimed to be consistent in source can be expected to have similar signatures and similar temporal trends in signature. Where there is deviation from this expectation, specimens in the batch must vary in some way, for example in diet, location, or age. The specimens sampled in this study were from a single batch but could be placed into two distinct groups (figure 9). That the most and least pyramided tortoises fell into separate groups suggests that in this case the clustering may be due to differences in breeding source, with one group being of wild origin and the other of ranched (see Gerlach 2004). If this is the case, stable isotope may be useful as a tool to identify batches of specimens in which illegally-sourced specimens have been included among legally-sourced individuals.

However, without further studies on known-origin specimens, these results remain equivocal. It is possible that individual preferences in diet could cause both differing signature and differing degree of pyramiding. In addition it is possible that the specimens were all ranched but from more than one breeding program, or from different cohorts. Alternatively, all specimens from this batch may be of wild origin but from multiple locations: individuals are likely to vary both in diet and in susceptibility to pyramiding due to different habitat and environmental conditions (Gerlach 2004, Wiesner & Iben 2003).

Nevertheless, if it is assumed for the present that the differences in signature are due to differences in breeding source, these data present an opportunity to explore the potential of using stable isotope analysis to distinguish between wild and captive tortoises. There are clear differences in signature between the two groups, particularly in $\delta^{15}N$ (figure 11). I also found that the carbon signature of 'captive-raised' specimens changes to a greater degree over time than that of 'wild' specimens. However, the potential for stable isotope analysis to infer the source a single specimen, or a small group of specimens is questionable as signatures of wild populations and captive populations are likely to overlap. The same is true of degree of change over time as this is likely to vary within and among both wild and captive specimens depending on, for example, between year differences in climate in the wild, and source of food supply in captivity. Extensive sampling of different populations would be needed to assess whether there was a true difference in signature.

In summary, stable isotope analysis has potential to be used as a tool to infer the composition, and possibly source, of batches of tortoises. Whilst I found differences in signature between two nominal groups, were these not side by side within a single batch, it is unlikely they could be used to infer anything about breeding source. The results of this study are based on the assumption that the correspondence of degree of pyramiding and clustering in isotopic space reveals a difference in breeding source. Further studies using known origin samples are required to confirm or refute these tenuous findings.

CHAPTER 5

Longitudinal isotope record in carapacial scutes: a potential method for determining the source of tortoises?

Introduction

In chapter 4 I consider the potential of stable isotope analysis to infer breeding source of newly imported tortoises. However, in some cases determination of the origin of specimens that have spent some time in the importing country is desired. For example, *T. graeca* and *T. hermanni* specimens can be easily and cheaply acquired from tourist markets in several countries (RSPCA 2001). These tortoises are illegally taken from the wild and are not accompanied by Article 10 certificates. In order to legally trade these species in the EU, an Article 10 certificate stating age and origin must accompany each specimen, so specimens from tourist markets should not be in trade in the UK. However, certificates can be fraudulently applied for by people claiming to have bred the tortoises themselves. It is thus possible that wild specimens collected abroad or bought on tourist markets are entering the trade under the guise of UK-captive bred specimens. Enforcement- and investigative- officers can check that the age on the certificate tallies with the size of the specimen, however reliance on such subjective judgement is not ideal.

Stable isotope analysis may have potential to be used as evidence of non-UK origin (Kelly et al 2002). As an indicator of geographic origin, hydrogen isotope ratios (δD) in particular may be useful (e.g. Bearhop et al 2006, Fox et al 2010). If the UK signature is consistently different to that of the natural range of the species, analysis of recently grown keratin could be used to help infer origin of a specimen by identification of individuals whose isotope signature lies outside the known-UK signature. A similar approach was used by Hobson et al (2004b) who measured feather δD of American redstarts (*Setophaga ruticilla)* and ovenbirds (*Seiurus aurocapillus*) at their breeding grounds and used outliers as an indication of molt at other breeding sites, thereby identifying birds that had undergone long distance dispersal. However, we do not yet know the range of isotope signature in captive UK tortoises so cannot currently identify outliers.

An alternative method is to use longitudinal sampling to identify shifts in signature within an individual. For example Cerling et al (2006) identified range shifts in elephants associated with crop raiding by sampling along the length of tail hair. They corroborated isotopic data with GPS data. Through sampling carapace keratin of multiple ages within an individual, it may then be possible to infer long distance movement, i.e. import, in tortoises, regardless of whether wild and UK isotope signatures overlap. In this chapter I longitudinally sample a number of specimens and examine whether a shift in signature occurs between the oldest and most recent growth periods.

Methods

I obtained samples from 8 *T. graeca*, and 4 *T. hermanni* adult specimens that, due to their age, are likely to be pre-ban wild caught imports, however their history is undocumented so this may not be the case. I also sampled one adult *T. graeca* that is known to have spent several decades in the UK after having been wild-caught and imported from Morocco and two juvenile *T. hermanni* imported from Macedonia that grew for several months in the UK before death.

I took a number of equally spaced keratin samples across the carapacial scutes of each specimen, from the juvenile scute (present before hatching) to the most recent growth. Where growth bars were visible each sample comprised of material from two growth bars. I pooled samples from multiple scutes. I prepared and analysed all samples following the methods described in chapter 2. I excluded all data from two plates of hydrogen samples due to unacceptable standard values. This included all samples from one of the two known-history juvenile *T. hermanni* specimens.

I used general linear mixed models to compare the oldest post-hatching growth and newest growth of specimens. I specified isotope ratio as the dependent variable, and age of growth and species as independent variables with an interaction term between them. I specified identity of tortoise and repeat number as random factors.

Results

The relationship between age of growth and isotope signature differed between species for δD (*T. hermanni* n = 4, *T. graeca* n = 9; lmer; $\chi^2_{1,6}=18.68$, $p<0.001$), $\delta^{13}C$ (lmer; $\chi^2_{1,6}=6.19$, $p<0.05$) and $\delta^{15}N$ (lmer; $\chi^2_{1,6}=14.26$, $p<0.001$). There was no difference in signature between old and recent growth for *T. hermanni* (lmer; δD:

$\chi^2_{1,4}$=0.75, p=0.39; $\delta^{13}C$: $\chi^2_{1,4}$=0.99, p=0.32; $\delta^{15}N$: $\chi^2_{1,4}$=0.93, p=0.33; figure 12). However for *T. graeca* recent growth was depleted by 12.12‰ in δD compared to old growth (lmer: $\chi^2_{1,4}$=46.36, p<0.001). Recent growth was also depleted in $\delta^{13}C$ (lmer: $\chi^2_{1,4}$=6.79, p<0.01) and enriched in $\delta^{15}N$ (lmer: $\chi^2_{1,4}$=28.43, p<0.001; figure 12).

Temporal changes in signature for the three specimens I know to have been imported are given in figure 13: all could be argued to have a shift in hydrogen signature. For the *T. hermanni* specimen there is a concordant shift in both nitrogen and carbon signatures.

Discussion

These results suggest that it may be possible to use temporal change in signature as an indication of import, particularly for *T. graeca* as there is a marked shift in hydrogen signature between old and recent growth for this species (figure 12). Whilst the same does not apply for *T. hermanni* specimens in general, there is an apparent shift in δD for the individual known to have been imported and to have grown for some time in the UK (figure 13). In this individual, at the same point in time that δD shifts, there is a corresponding change in $\delta^{13}C$ and $\delta^{15}N$. This corroborates the idea that the shift in δD is indicative of import as diet would also be expected to change with new ownership (figure 13).

In addition to marked shifts in δD, change is also seen in $\delta^{13}C$ and $\delta^{15}N$ of *T. graeca* specimens but not of *T. hermanni*. The difference between the two species may be due to a number of reasons; the four unknown origin *T. hermanni* may have been bred in the UK, or they may have been imported to the UK once they had stopped growing. Alternatively it may be that the isotopic signature of wild *T. hermanni* is more similar to that of UK captive tortoises than the signature of wild *T. graeca*.

Whilst there are shifts in all three signatures for *T. graeca*, δD is the most robust test of import as the large shifts in hydrogen signature imply large-scale movement (Hobson 2005), whereas shifts in $\delta^{13}C$ and $\delta^{15}N$ could indicate a change in husbandry within the UK. Unfortunately, it was not possible to test if shifts in isotope signature were due to import or some other factor, as I do not have prior knowledge of country of origin or of when (or even if) the majority of these specimens moved to the UK. It is possible that the shifts in signature seen in this study are due to factors other than import, such as change in relative allotment of nutrients to maintenance and growth. However, I could

not find reference to such effects on hydrogen signature in the literature and there are conflicting results on the effect of growth rate on nitrogen discrimination (see del Rio et al 2009 for review of the evidence). In addition, the shift in δD seen at a young age in the juvenile *T. hermanni* specimen of known-origin provides some support that physiological changes with age are not responsible for the marked depletion in δD over time. In contrast, shifts in $\delta^{13}C$ and $\delta^{15}N$ could easily be as a result of a diet switch due new ownership or change in advised tortoise diet, rather than change from a wild diet to a captive diet.

In conclusion, it may be possible to infer that a group of tortoises has been imported by looking at change in δD between old and recent growth, though with these data it is not currently possible to ascertain the degree of change in signature that is required to infer import. In order to use longitudinal sampling of this kind to infer import it will be necessary to sample a large number of UK captive-bred and imported specimens with known histories.

CHAPTER 6

GENERAL DISCUSSION

Tortoise carapacial and plastral keratin can be sampled non-invasively to obtain longitudinal isotopic information about individual tortoises, and about batches of tortoises. Although it is not conclusive, this thesis provides some evidence that stable isotope analysis of tortoise keratin may have potential as a tool for regulation of the tortoise trade. In particular it could be useful for determining whether illegally sourced specimens have been included in batches of legal specimens, and for detecting wild tortoises that have been fraudulently claimed as UK captive-bred. In the former case multiple breeding sources may be indicated by clustering of specimens in carbon/ nitrogen isotopic space (figure 9). This is an application that seems promising, but has not been considered in previous isotope studies focussed on trade regulation. In the latter case, a marked shift in signature (particularly δD) within an individual may suggest large-scale movement, which, given the sedentary behaviour of tortoises, would imply importation (figure 12). However, it should be noted that tortoises that have been born and raised in captivity may also experience significant shifts in signature if diet or water source changes to include non-UK sourced items.

Marked shift in signature within an individual can provide more conclusive evidence of movement than a single signature from an individual (see Fox et al 2007). Nevertheless, signature of a single sample may be enough to infer origin where there is a pronounced difference between the isoscape experienced by populations of opposing origins (e.g. Kelly et al 2007, Bearhop et al 2006). I found little overlap between nitrogen signature of *T. horsfieldii* assumed to be captive-reared and those assumed to be wild (figure 11). This suggests that there may be potential for stable isotope analysis to identify the breeding source of *T. horsfieldii* individuals imported to the UK. A reason for difference in nitrogen signature between wild and ranched individuals may be that ranched specimens are (at least in some cases) given supplementary protein to increase growth rate (pers. comm. Evgeniy Peregontsev, UzZooComplex). It is possible that the range of nitrogen signature in protein-supplemented individuals is outside that found in wild conditions. Nonetheless, particular caution should be applied when assessing origin of specimens on these grounds as differences may only pertain to individuals from certain habitats, or breeding centres, or in a particular year: wild

tortoises from one location may be distinguishable from ranched tortoises, whilst those from another location may not.

I did not directly address the question of whether stable isotope analysis could be used to tell if a *T. hermanni* or *T. graeca* specimen was wild-caught and laundered through a large scale captive-breeding operation such as that found in Slovenia or Macedonia. It is possible that a difference in signature such as that seen in *T. horsfieldii* specimens may also exist between wild-caught specimens and those raised in breeding farms. The signature of specimens in breeding farms could theoretically be characterised and used to test suspect specimens, much like genetic signatures are currently used (Ogden et al 2009). A specimen with a signature outside the range found in breeding farms would be suspect. Conversely, captive-origin could not be assumed of a specimen within the range seen in farms as testing all potential wild populations would be extremely difficult. However, I must give a further note of caution against this approach as there is an additional problem with comparison of signature for the purpose of inferring breeding origin, rather than geographic location: whereas signature of wild populations is likely to persist across years, the signature of captive populations may not due to vagaries in husbandry. For example, commercial pressures to obtain competitively priced sources of supplementary protein could affect nitrogen isotope signatures. Therefore, differences seen in one year may not exist in another and captive specimens given a different diet to those on which captive isotope signature was characterised may be erroneously categorised as wild.

Unfortunately, I was unable to sample more than three specimens of known origin so my results are largely based on assumptions and conjecture. Consequently, I cannot draw any firm conclusions of whether stable isotope analysis is applicable as a tool for regulation of the tortoise trade. Where I have observed differences in isotopic signature, these may be due to a number of factors other than true difference in origin. For instance, in chapter 4 I observed clustering of specimens that I attributed to differing breeding origin (figure 9). However, this may alternatively be explained by differences in metabolic routing between males and females, for example female desert tortoises (*Gopherus agassizii*) draw on body nutrient reserves to produce eggs Henen (2002). That the clusters I observed could be associated with a known indicator of captive/ wild status (degree of pyramiding) does however give some confidence to my assumption in this case. Similarly, for the majority of *T. graeca* and *T. hermanni* specimens I analysed in chapter 5, I do not know for sure that they were imported or at what age they were imported. They may for example have been fully grown on import

and therefore not have any UK growth. The lack of change in δD of *T. hermanni* may be due to this or to smaller differences between natural range and the UK than for *T. graeca*. On the other hand, depletion in δD over time in *T. graeca* may be caused by factors other than import.

Future studies to develop understanding of how stable isotope analysis might be used as a tool for regulation of the tortoise trade should only be made if it is possible to test specimens of known origin. If such specimens are available the following questions are of particular interest given the results of this thesis: 1) Does clustering occur in batches of tortoises from the same source; 2) Are there consistent differences in signature between wild and captive-raised specimens (and does protein supplement given to ranched tortoises cause a more enriched signature than is found in wild populations)?; 3) Is a temporal shift in δD seen in wild tortoises that have not been imported?; 4) What is the range of signature seen in UK captive-bred specimens and how large does a change in δD have to be to confidently class a specimen as imported?

The results of this thesis suggest that, with further work, stable isotope analysis has potential to facilitate the detection of illegally imported tortoises. If this is indeed the case it may promote understanding of the scale of illegal trade in tortoises in the UK, and inform authorities of potential hotspots of illegal activity to which resources can be directed.

FIGURES

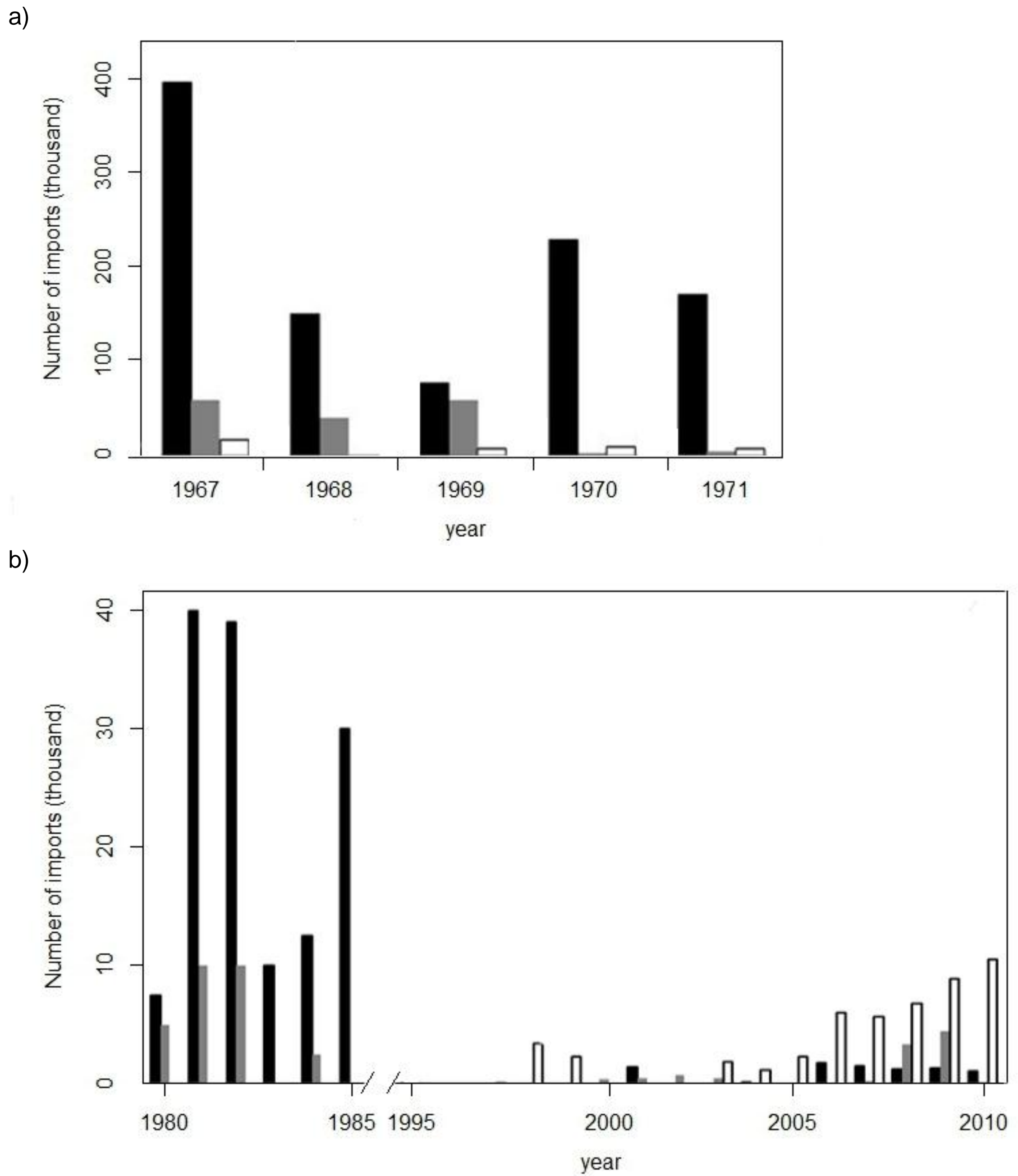

Figure 1. Number of imports (thousands) of T. graeca (black), T. hermanni (grey) and T. horsfieldii (white) between a) 1967 and 1971, and b) 1979 and 2010. a is constructed from data in Honegger et al 1974. b is constructed from CITES import data: import quantities of live specimens for trade. Note the difference in y-axis.

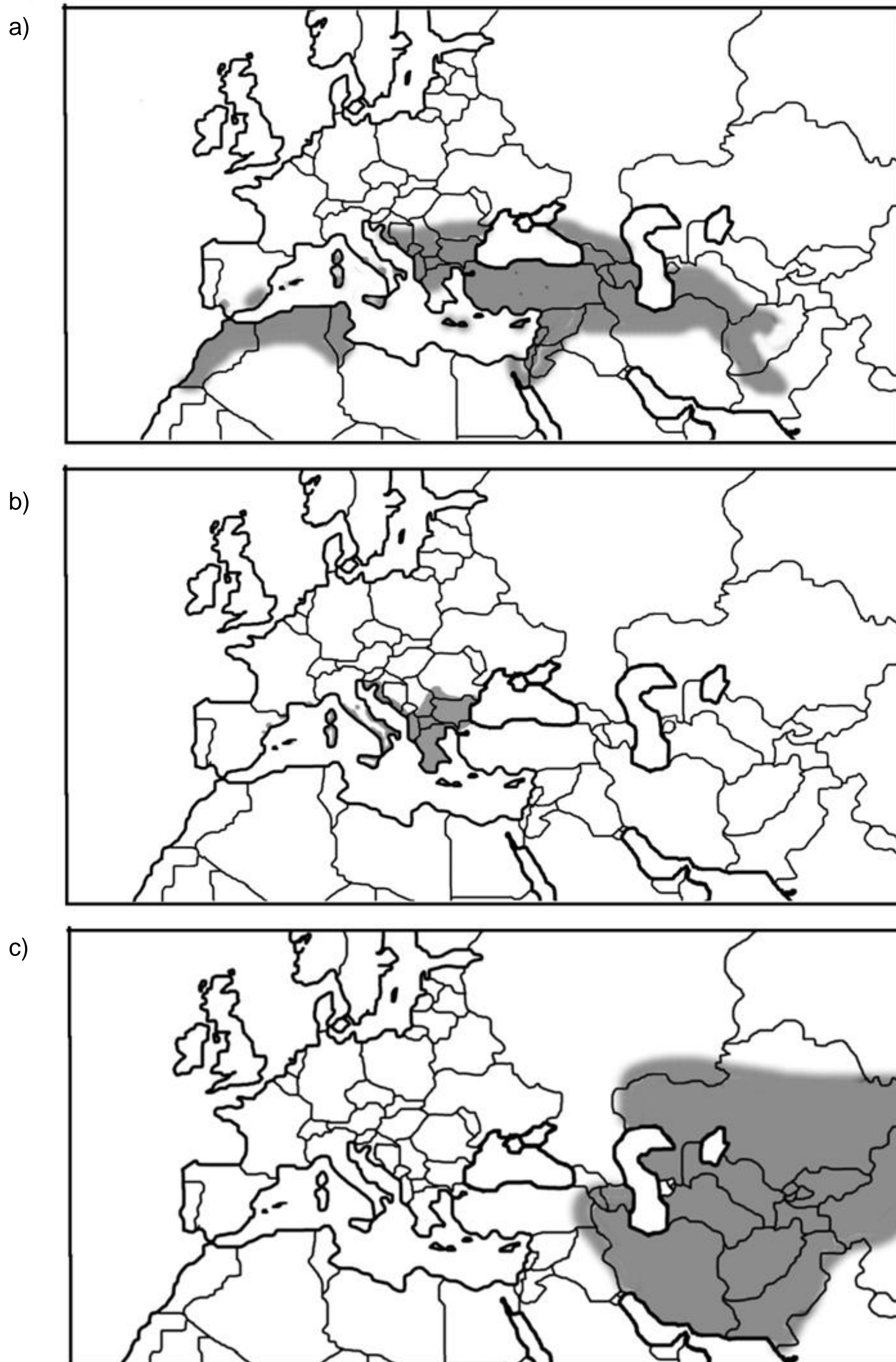

Figure 2. Maps of approximate ranges of a) *Testudo graeca* b) *Testudo hermanni* and c) *Testudo horsfieldii*

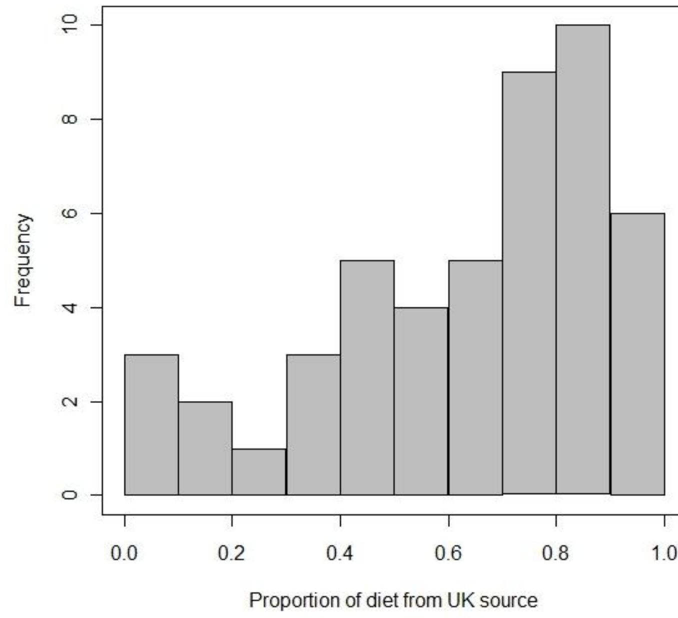

Figure 3. Proportion of diet of captive UK tortoises consisting of food from a definite UK source. Frequency is number of tortoise owners surveyed.

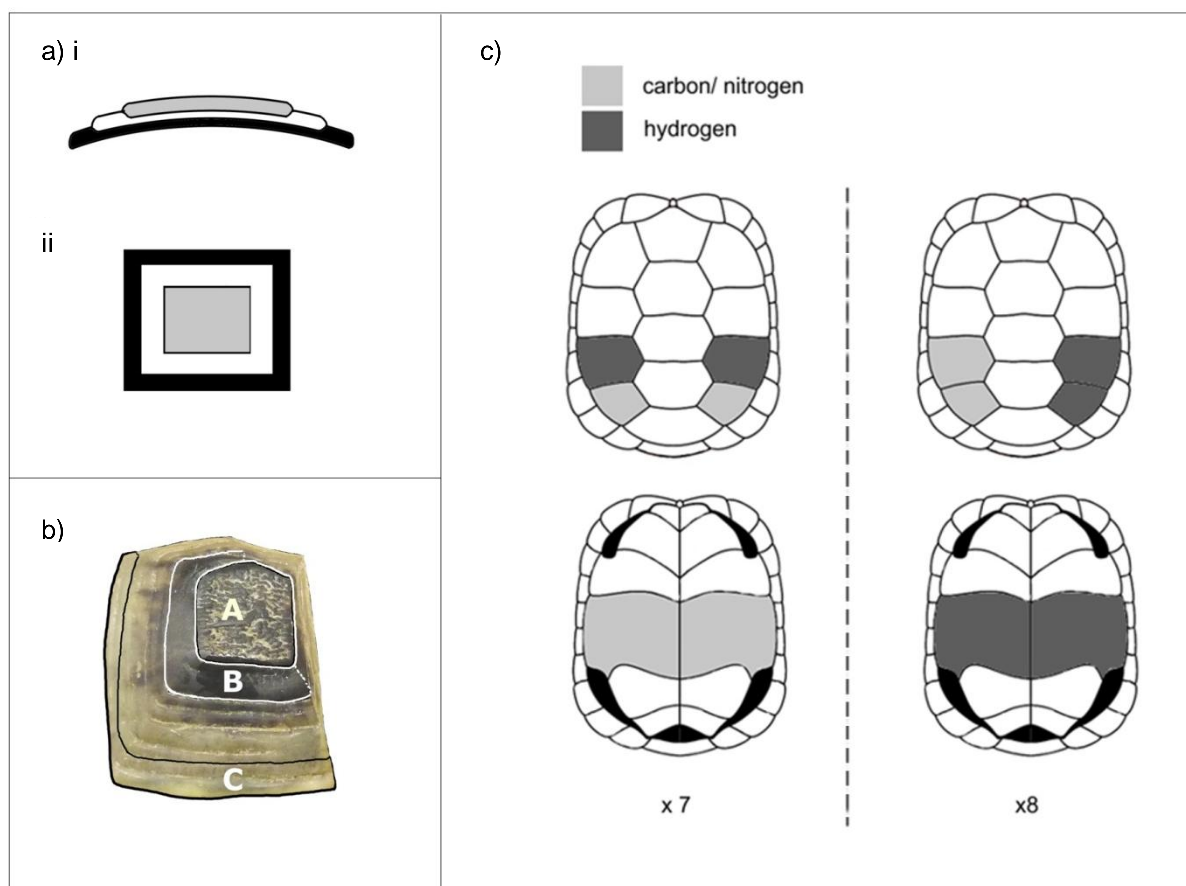

Figure 4. a) i: Schematic of scute growth in cross section and ii: from above, showing juvenile scute present at hatching (grey), early growth (white), and recent growth (black). b) photograph of a single scute showing approximate areas of sampling. A) juvenile scute; B) early growth; and C) most recent growth. c) Location of carapacial (top) and plastral scutes (bottom) sampled are shown in grey. Due to low sample weights, specimens were split into two groups as shown above. In 7 specimens (left) I analysed the left and right fourth pleural scutes of the carapace and both abdominal scutes of the plastron for carbon and nitrogen (light grey), and the left and right third pleural scutes of the carapace for hydrogen (dark grey). In 8 specimens (right) I analysed the left third and fourth pleural scutes of the carapace for carbon and nitrogen (light grey), and the right third and fourth pleural scutes of the carapace and both abdominal scutes for hydrogen (dark grey).

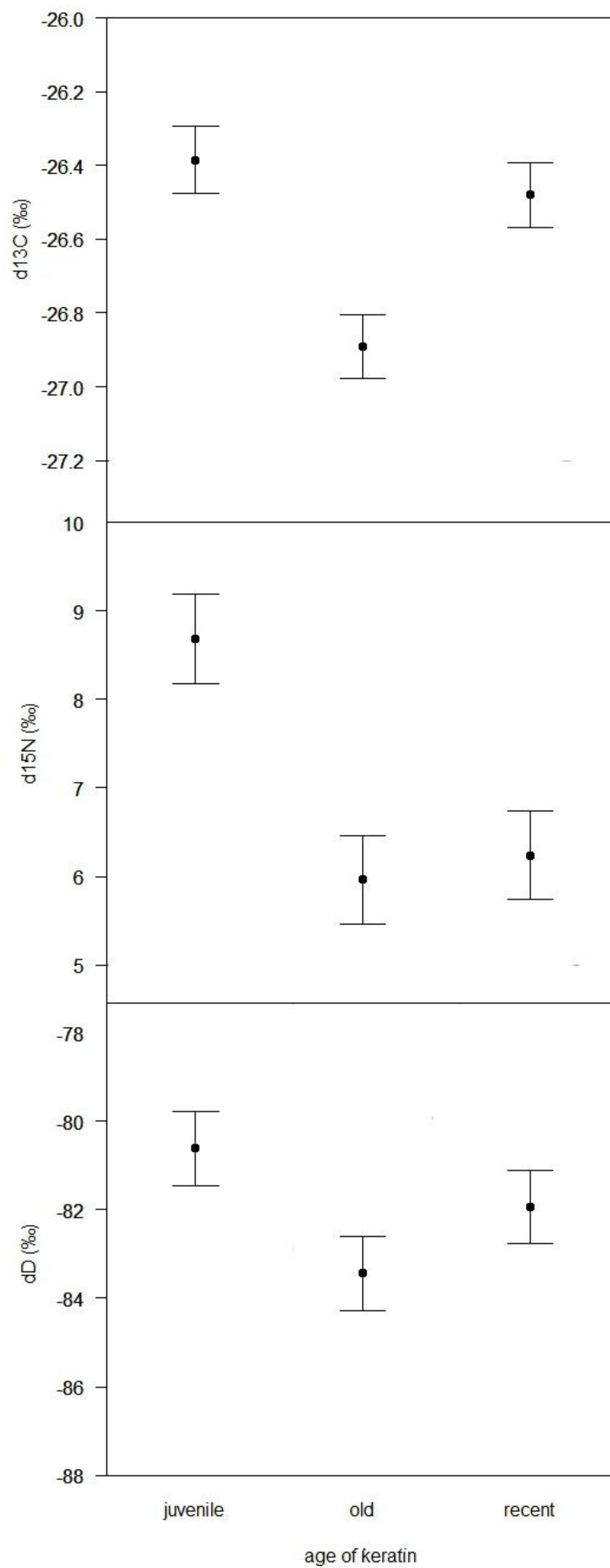

Figure 5: Plots showing model fitted values (mean ± 2SE) for carbon, nitrogen and hydrogen stable isotope signatures of different ages of scute keratin.

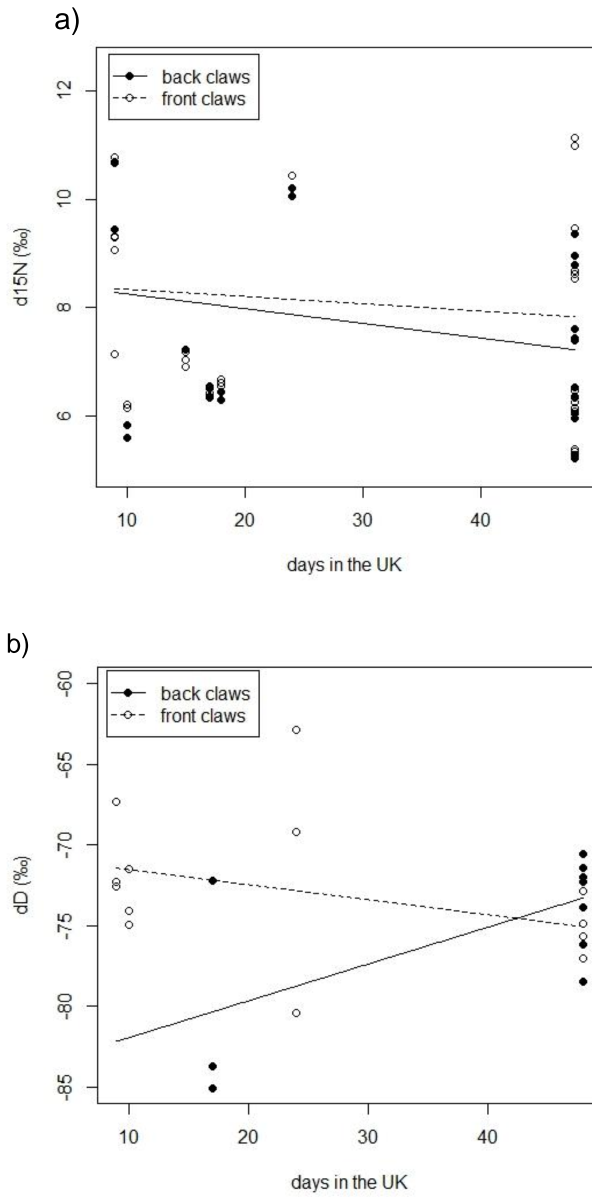

Figure 6. Showing the interaction between position of claws (front/ back) and the number of days spent in the UK before death for a) $\delta^{15}N$ (lmer: $\chi^2_{1,7}=6.32$, $p<0.05$) and b) δD (lmer: $\chi^2_{1,7}=8.02$, $p<0.01$). All tortoises were fed the same diet between import and death.

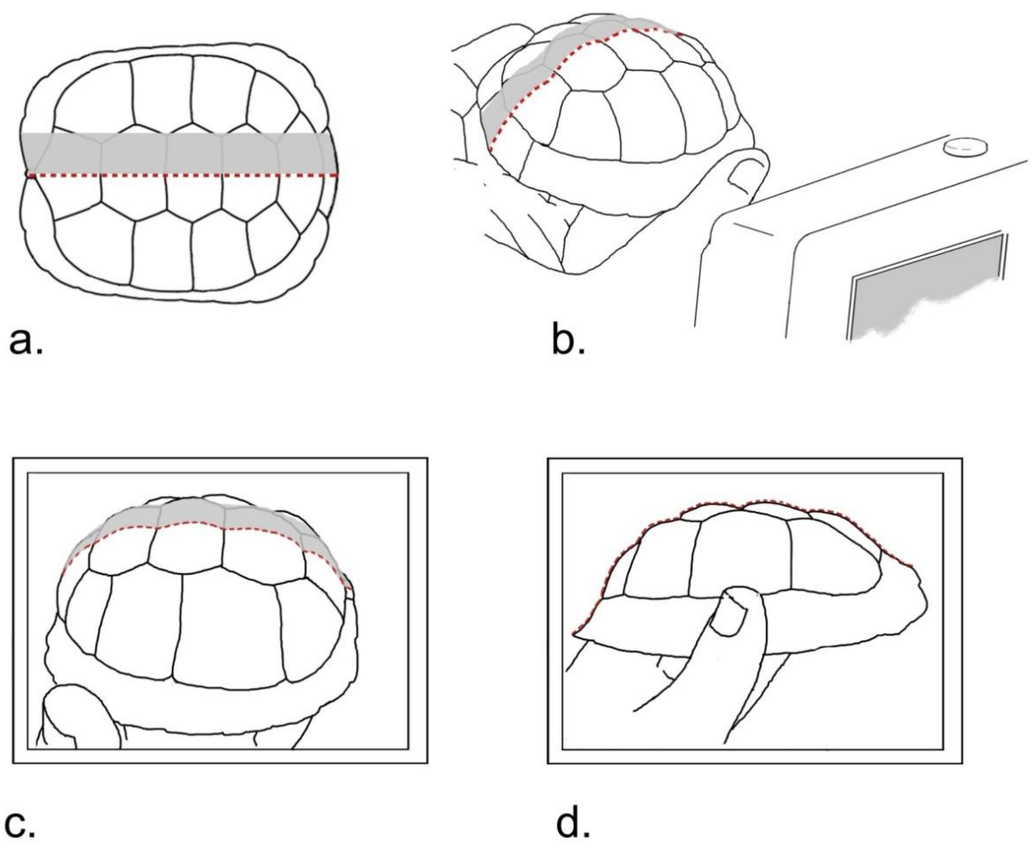

figure 7. Method for photographing specimens for measurement of pyramiding . a) Masking tape placed with close edge along the centre of the vertebral suctes; b to d) tortoise viewed through the screen of a camera and tilted away until the point at which the masking tape disappeared from view; d) photo taken.

figure 8. Profile photographs of a pyramided (top) and a smooth (bottom) carapace, showing lines of measurement taken.

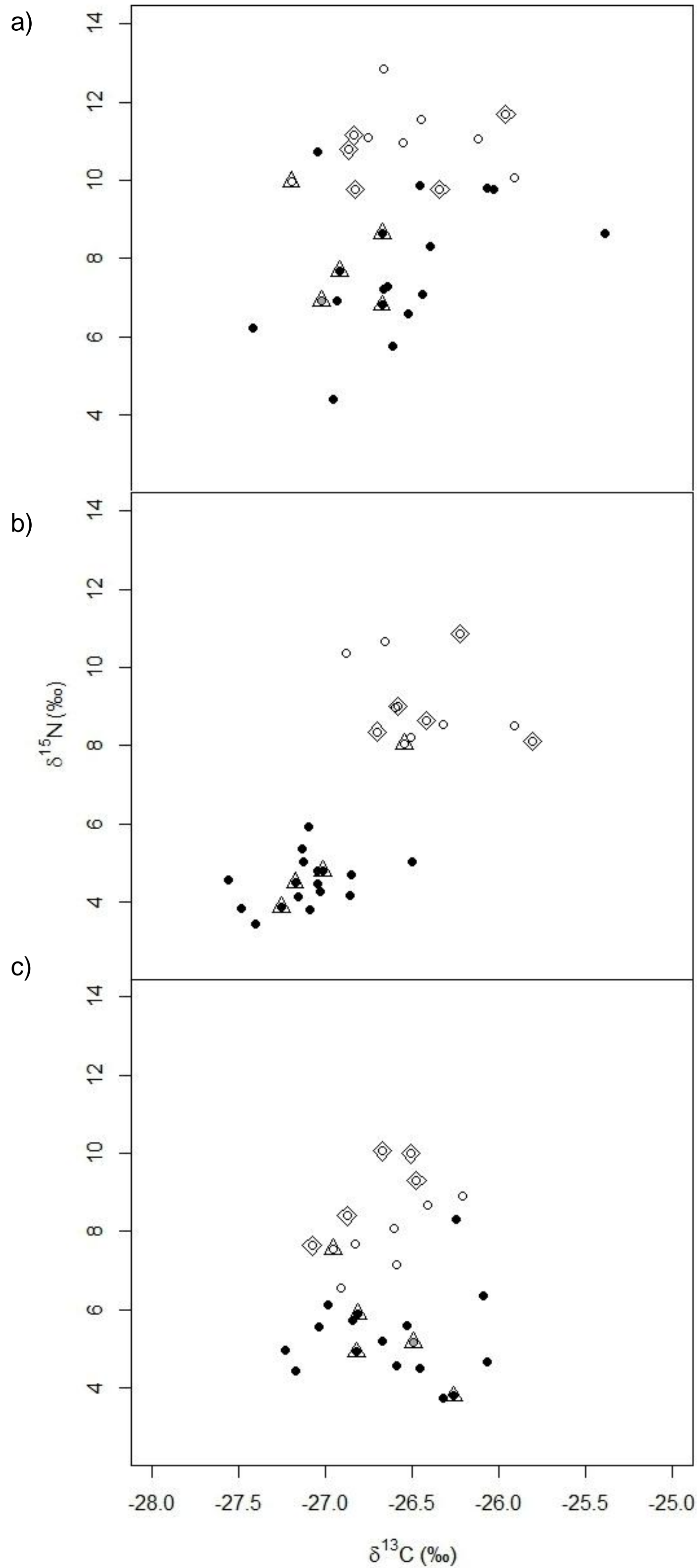

Figure 9. Clustering of *T. horsfieldii* specimens in δ13C and δ15N isotopic space for a) juvenile growth, b) old growth and c) recent growth. I grouped specimens according to the distinct clusters in panel b: group A (closed circles) are associated with the most pyramided specimens (triangles); group B (open circles) are associated with the least pyramided specimens (diamonds). The old growth sample of one of the most pyramided specimens was missing from the dataset, this individual in represented by a grey circle.

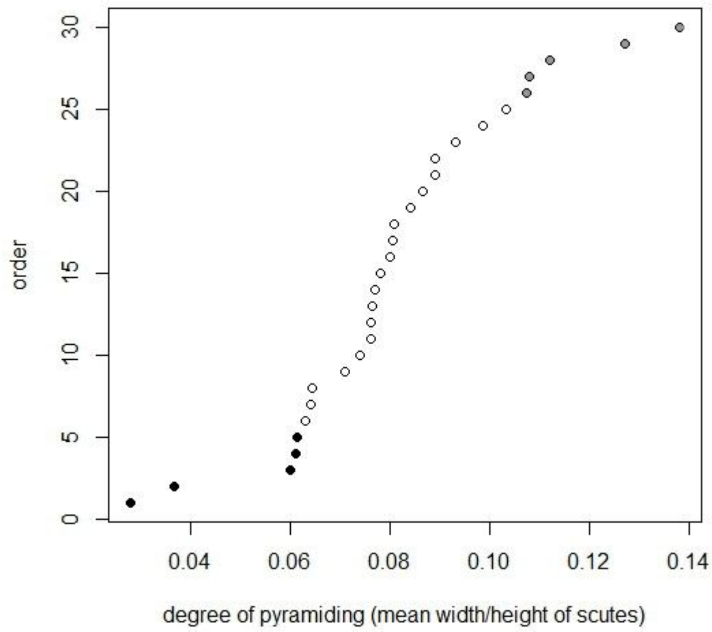

Figure 10. Showing measurement of pyramiding for individual *T. horsfieldii* specimens in order of least to most pyramided. I measured degree of pyramiding as the mean width/height ratio of the second, third and fourth vertebral scutes. Black filled circles: five smoothest specimens; grey filled circles: five most pyramided specimens.

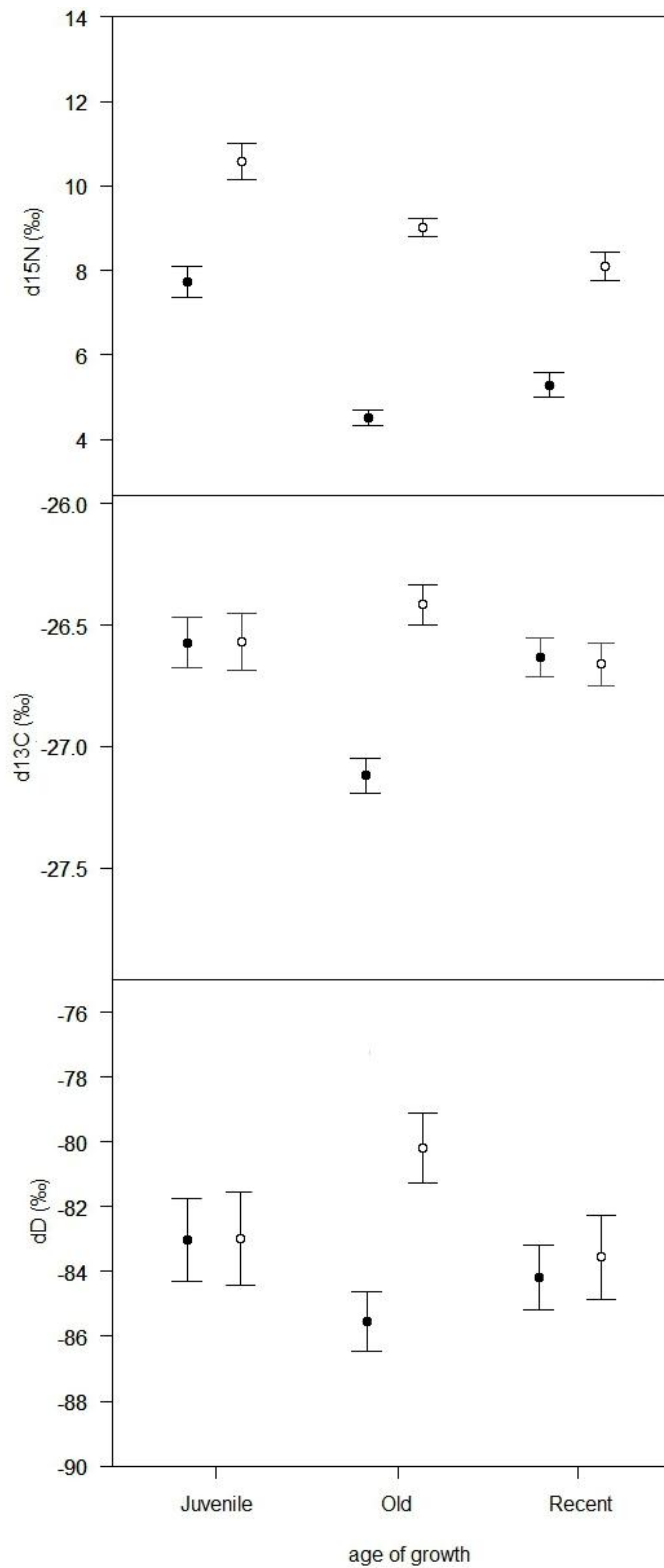

Figure 11. Mean ± SE nitrogen, carbon, and hydrogen isotopic signature for tortoises in group A (associated with pyramided/ 'captive-raised' tortoises; closed circles) and tortoises in group B (associated with smooth/ 'wild' tortoises; open circles) over time. Group A and B differed significantly in δ15N at all ages (lmer; J: $\chi^2_{1,4}$=19.77, p<0.001, M: $\chi^2_{1,4}$= 64.40, p<0.001, N: $\chi^2_{1,4}$=27.08, p<0.001), but only differed in old growth for $\delta^{13}C$ (lmer; $\chi^2_{1,4}$=25.75, p<0.001) and δD (lmer; $\chi^2_{1,4}$=11.60, p<0.001).

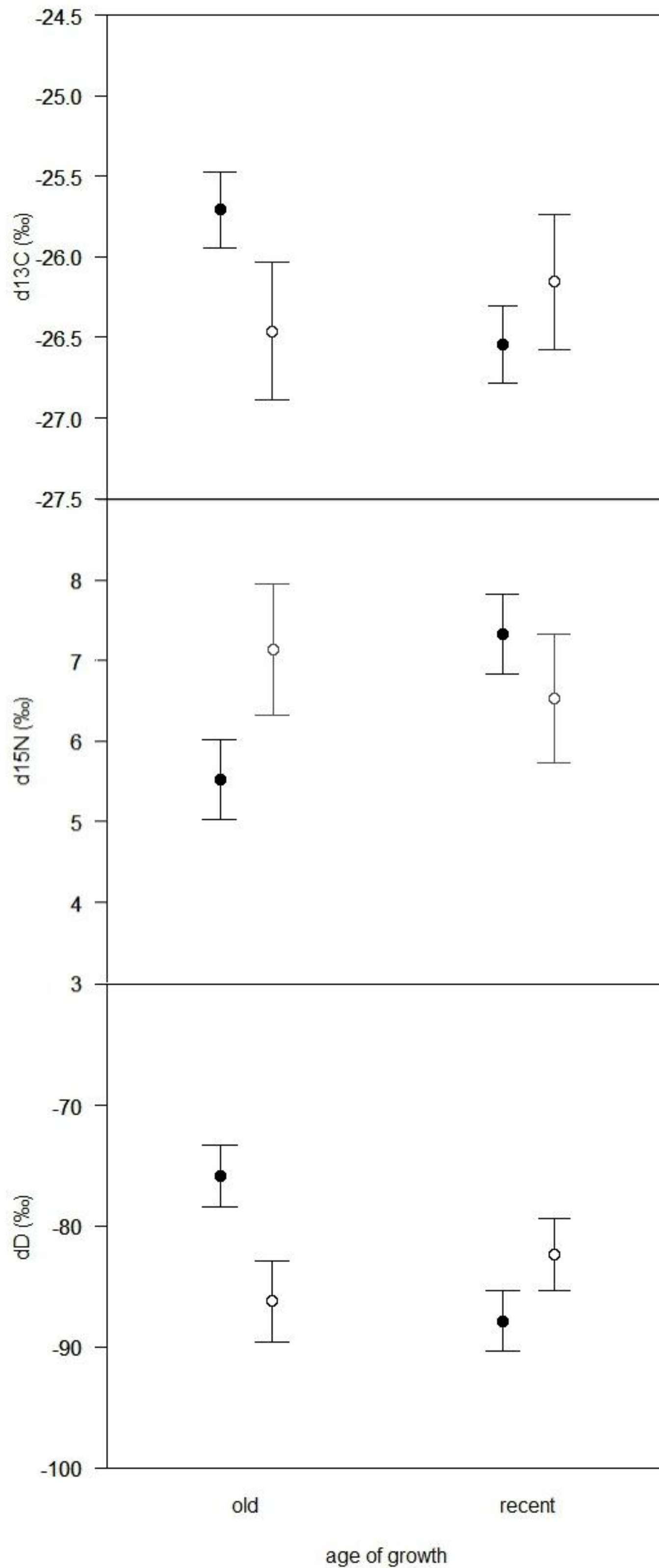

Figure 12. Mean change (± 1SE) in isotope signature between oldest and most recent growth for *Testudo graeca* (closed circles) and *Testudo hermanni* (open circles) specimens. There was a significant difference between old and recent growth for *T. graeca* for all elements (lmer: δD: $\chi^2_{1,4}$=46.36, p<0.001, $\delta^{13}C$: $\chi^2_{1,4}$=6.79, p<0.01, $\delta^{15}N$: $\chi^2_{1,4}$=28.43, p<0.001), but no difference between ages for *T. hermanni*.

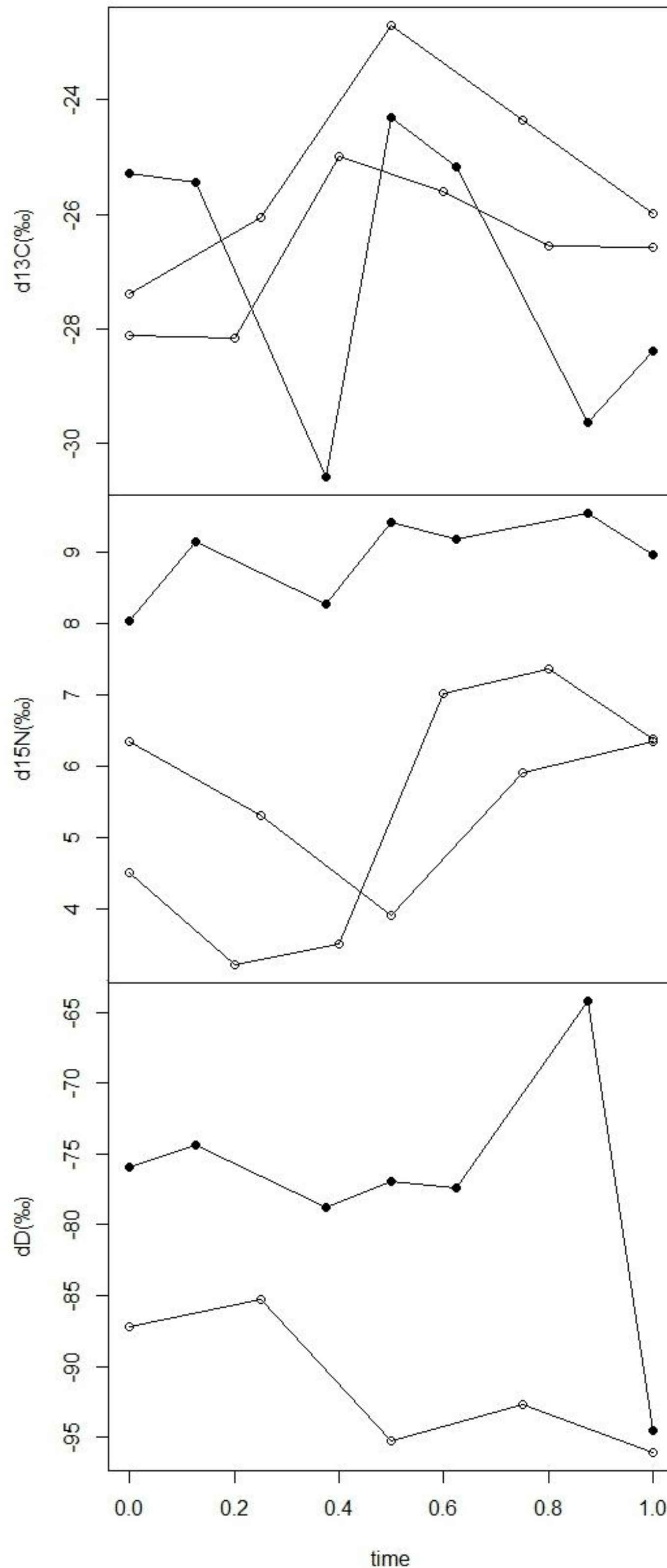

figure 13. Change in isotope signature over time for a *T. graeca* specimen known to be imported from Morocco (closed circles) and two known-history *T. hermanni* specimens imported from Macedonia (open circles). The x-axis gives the position at which the scute was sampled as a proportion of the width of all growth bars, with 0 being the oldest growth and 1 the most recent.

TABLES

Table 1. Country of export of *T. graeca* and *T. hermanni* specimens between 2001 and 2011. Constructed using data from CITES trade database: import quantities of live specimens for the purpose of trade. Imports from EU countries are not included. *Exports of *T. hermanni* from Ukraine are re-exports with the origin given as Slovenia.

country of export	*T. hermanni*	*T. graeca*
Turkey	750	7,125
Macedonia	7,200	-
Slovenia	1,717	*
Lebanon	-	1,494
Ukraine	250	210*
Jersey	20	-

Table 2. Number of tortoise breeders and species bred in the UK. Compiled from lists available on tortoise advice websites.

Species	Number of breeders
Testudo hermanni	35
T. graeca	30
T. horsfieldii	16
T. marginata	7
Geochelone carbonaria	7
G. elegans	3
G. denticulata	2
G. elongata	2
G. pardalis	2
Manouria emys	1
Malacochersus tornieri	1

Table 3. Dietary items given to tortoises by survey respondents (n=48). * denotes items that definitely bear a UK signature.

Dietary item	Number of respondents
Garden weeds*	44
Supermarket greens	35
Home-grown greens*	21
Supermarket fruit	14
Packaged tortoise food	10
Supermarket root vegetables	7
Home-grown fruit*	6
Home-grown root vegetables*	2

Table 4. I allocated individuals to groups A and B depending on which cluster they fell into in the plot of $\delta^{13}C$ and $\delta^{15}N$ of old growth. I used partition cluster analysis to assign individuals to clusters at all ages for all plots (C and N; C and H; N and H). This table shows the number of cluster assignments that matched the allocated group. Clusters were conserved across ages of growth for plots of $\delta^{13}C$ and $\delta^{15}N$, but less so for plots of δD.

Allocated group	Number of correct assignments / total number of specimens		
	Juvenile growth	Old growth	Recent growth
C,N			
A	13/17	(17/17)	14/15
B	12/12	(12/12)	12/12
C,H			
A	9/16	10/16	11/16
B	7/11	8/10	5/9
N,H			
A	9/16	11/16	11/16
B	7/11	8/10	5/9

APPENDIX A

Survey 1. Given to owners attending post hibernation veterinary care sessions in Bristol

Account Logout

I am a research student with the University of Exeter and Fera (the Food and Environment Research Agency). We are trying to establish whether chemical analysis can be used to distinguish between wild and captive-bred tortoises from different regions. Hopefully this will lead to the development of a new forensic tool that will help in regulating trade in tortoises. We are currently only looking at these three species: Hermann's tortoise (T. hermanni), Spur-thighed tortoise (T. graeca) and the Russian/ Horsfield's tortoise (T. horsfieldii). The technique is based on what animals have eaten and where the food is from geographically. I'd therefore like to get a better idea of what tortoises in the UK are fed and would be very grateful if you could complete the short survey below.

Click here to see the project website for more information.
Your details will be kept confidential and not passed on to third parties.

Question 1

Which of these species have you had?

Hermann's tortoise (T. hermanni)
Spur-thighed tortoise (T. graeca)
Russian (Horsfield's) tortoise (T. horsfieldii)

Question 2

Which of the following have you had in your collection (only including the species in Q1)?

hatchling
juvenile
adult

Question 3

Do you breed tortoises?

Yes
No

Question 4

Roughly what percentage of the following make up the diet of your Hermann's, Spur-thighed and/or Horsfield's tortoises
(don't worry about being exact, I'd just like a rough idea):

Note 1: If you feed hatchlings/ juveniles and adults differently please answer for adults here and also answer question 5.
Note 2: If you have more than one of the above species and feed them on different diets please answer for just one and then answer question 6.
.

	0%	1-10%	11-25%	26-50%	51-75%	76-100%
garden weeds						
home-grown greens						
supermarket greens						
home-grown root vegetables						
supermarket root vegetables						
home-grown fruit						
supermarket fruit						
packaged tortoise food						
other						

If 'other' please give details. If 'packaged tortoise food' what brand and type (eg. pellets/ dried herbs)?

Question 5

Only answer if you feed adults and hatchlings/ juveniles on different diets
In what way does the diet of your hatchlings/ juveniles differ to that of your adults?

Question 6

Only answer if you have more than one of the above species and feed them different diets
Which species did you answer for in question number 4?

Question 7

What does your tortoise drink?

tap water
rainwater
bottled water

Question 8

This space is for any other comments you may have -
for example if you used to feed your tortoises a completely different diet what was it and when did you change?

Survey 2. Given to owners attending post hibernation veterinary care sessions in Bristol

Date:		**Tortoise number:**

Species	T. hermanni / T. graeca / T. horsfieldii
Source	Captive bred / wild / ranched
Country of Origin	
Age	
Sex	
Location (town/ county)	
Has the tortoise moved location in the past 4 years? **If yes please give details**	
Approx date claws were last clipped	
Does the owner breed torotises?	

What percentage of the following makes up the diet of this tortoise

	percentage
garden weeds	
home-grown greens	
home-grown root vegetables	
home-grown fruit	
supermarket greens	
supermarket root vegetables	
supermarket fruit	
packaged tortoise food	
other (if so, what?)	

Note: I only need rough percentages to get a vague idea of proportion of each type of food source

Has the diet changed at all over the past 4 years? **If so, how?**	

sample number		**Position**	Front
sample number		**Position**	Back

APPENDIX B

Signatures of T. horsfieldii soft tissues

Methods

I dissected the ten freshest specimens and sampled liver and shoulder muscle from all and heart blood from eight. I freeze dried these samples and removed lipids by the following method. I added 20ml hexane to every 1g of tissue, homogenised the mixture for 30 seconds with a vortex mixer and left it in a sonic bath for 20 minutes, after which I removed the liquid. I repeated this process twice for each sample.

In order to gain an understanding of time period covered by different tissues I performed a series of Spearman's rho correlations between pairs of tissues. I applied a Bonferroni adjustment to reduce the chance of type I errors (significant p value of less than 0.004), though it should be noted that given the small sample sizes and large number of correlations (n=14), this is extremely conservative and the chance of type II errors is inflated. I also performed Spearman's rho correlations between tissues and the number of days the specimen was alive in the UK, again applying a Bonferroni adjustment to correlations (significant p value of less than 0.008).

Results and discussion

There was no correlation between $\delta^{15}N$ or $\delta^{13}C$ of blood, liver or muscle and number of days in the UK. However, the correlation between $\delta^{13}C$ of liver and number of days in the UK approached significance (spearman's rho: r_s= -0.75, df=8, p=0.013; figure A1), which supports the expectation that of the tissues sampled, liver should have the satest turnover rate.

Blood, liver and muscle values are presented in figure A2.Correlations between tissues provide an idea of relative time periods represented by different tissues, with strong correlations suggesting incorporation of isotopes occurred over a similar timescale. However, my results are not significant (table A1) and larger sample sizes would be required to elucidate relationships between tissues.

Table A1. Spearman's rho correlation coefficients for a) $\delta^{13}C$ and b) $\delta^{15}N$ with degrees of freedom given in brackets. With Bonferroni adjustment $p<0.007$ is significant at the 10% level ('.'), and $p<0.004$ at the 5% level ('*').

a)	blood	muscle	front claw	back claw	new scute
liver	0.64 (6)	0.44 (8)	-0.25 (8)	0.95 (7)	0.57 (8)
blood		0.69 (6)	0.36 (6)	0.21 (5)	0.34 (6)
muscle			0.49 (8)	0.52 (7)	0.52 (8)
front claw				-	0.46 (13)
back claw					0.56 (11)

b)	blood	muscle	front claw	back claw	new scute
liver	0.71 (6)	0.77 (8)	**0.82 . (8)**	0.83 (7)	0.35 (8)
blood		0.81 (6)	**0.90 . (6)**	0.75 (5)	0.13 (8)
muscle			**0.83 . (8)**	0.90 (7)	0.33 (8)
front claw				-	0.61 (13)
back claw					0.66 (11)

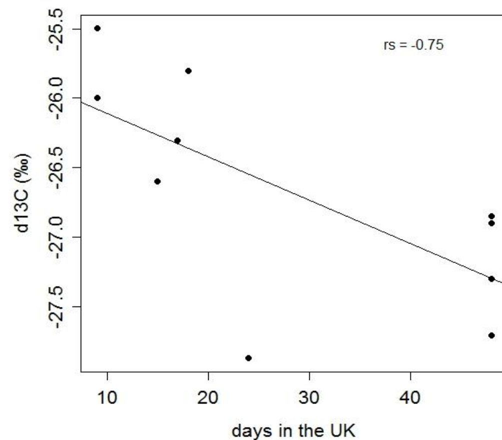

figure A1. The effect of number of days alive in the UK on $\delta^{13}C$ for liver ($p=0.013$).

a. b.

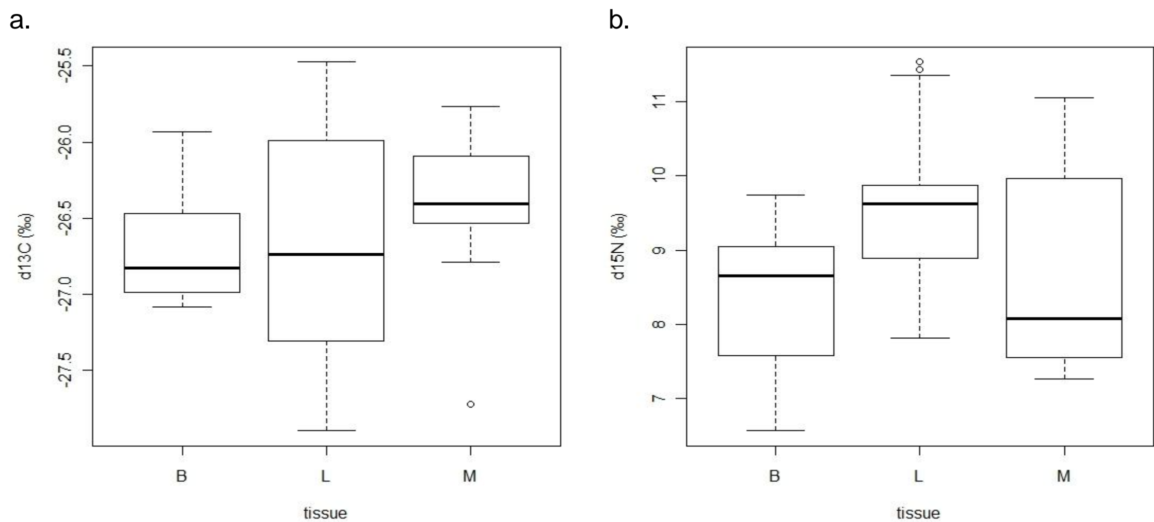

figure A2. Boxplots of a) $\delta^{13}C$ and b) $\delta^{15}N$ of *T.horsfieldii* blood (B; n=8), liver (L; n=10), and muscle (M; n=10), showing median, inter-quartile range, and maximum and minimum values.

APPENDIX C

Table A2) Mean δD values with 1 standard deviation for in-house scute keratin standard from a T. graeca specimen of unknown history. For each weight samples were run in both duplicate and triplicate.

weight (mg)	triplicate (T) / duplicate (D)	δD‰ ± 1sd
1.0	T	-88.16 ± 2.23
1.0	D	-94.05 ± 6.05
0.5	T	-88.19 ± 3.29
0.5	D	-92.59 ± 6.48

Table A3) *$\delta^{13}C$, $\delta^{15}N$ and δD values with 1 standard deviation for standards*, *and the tissues for which standards were used (K keratin; B blood; L liver; M muscle).*

Standard	tissue	$\delta^{13}C$ (‰) ± sd	$\delta^{15}N$ (‰) ± sd	δD (‰) ± sd
Trace casein	K,B,L,M	-23.37 ± 0.21	6.15 ± 1.3	-113 ± 3.2
Trace porcine collagen	K,B,L,M	-17.98 ± 0.14	6.12 ± 0.2	-65.9 ±8
IsoLab GmbH Bavaria horse hair	K	-	-	-85 ± 3
IsoLab GmbH Paraguay horse hair	K	-	-	-58 ± 4
NIST bovine muscle CRM 8414	B,L,M	-	-	-146.4 ± 4
NIST bovine liver CRM 1577b	B,L,M	-	-	-136.1 ± 3.5
(Tortoise scute keratin)	K, B,L,M	-25.94 ± 0.17	5.43 ± 0.36	-87.11 ± 4.6

REFERENCES

Agrobs (no date) URL: http://www.agrobs.de (accessed 12 June 2012).

Alibardi L (2005) Proliferation in the epidermis of chelonians and growth of the horny scutes. J. Morphol. 265: 52-69.

Anadón JD, Gimenez A, Perez I, Martinez M& Esteve MA (2006) Habitat selection by the spur-thighed tortoise *Testudo graeca* in a multisuccessional landscape: implications for habitat management. Biodivers. Conserv. 15:2287-2299.

Baker CS (2008) A truer measure of the market: the molecular ecology of fisheries and wildlife trade. Mol. Ecol. 17:985-3998.

Balling HP & Roßmann A (2004) Countering fraud via isotope analysis - case report. Kriminalistik 58:44-47.

BCG (2012) Care of Mediterranean tortoises. URL: http://www.britishcheloniagroup.org.uk/caresheets/caremed (accessed 10 June 2012).

Beard BI & Johnson CM (2000) Strontium isotope composition of skeletal material can determine the birth place and geographic mobility of humans and animals. J. Forensic Sci. 45:1049-1061.

Bearhop S, Thompson DR, Waldron S, Russell IC, Alexander G & Furness RW (1999) Stable isotopes indicate the extent of freshwater feeding by cormorants *Phalacrocorax carbo* shot at inland fisheries in England. J. Appl. Ecol. 36:75-84.

Bearhop S, Waldron S, Votier SC & Furness RW (2002) Factors that influence assimilation rates and fractionation of nitrogen and carbon stable isotopes in avian blood and feathers. Physiol. Biochem. Zool. 75:451–458.

Bearhop S, Furness RW, Hilton GM, Votier SC & Waldron S (2003) A forensic approach to understanding diet and habitat use from stable isotope analysis of (avian) claw material. Funct. Ecol. 17:270-275.

Bearhop S, Fiedler W, Furness RW, Votier SC, Waldron S, Newton J, Bowen GJ, Berthold P & Farnsworth K (2006) Assortative mating as a mechanism for rapid evolution of a migratory divide. Science 310:502-504.

Betini GS, Hobson KA, Wassenaar LI & Norris DR (2009) Stable hydrogen isotope dD values in songbird nestlings: effects of diet, temperature and body size. Can. J. Zool. 87:767-772.

Bhattacharya SK, Gupta SK & Krishnamurthy RV (1985) Oxygen and hydrogen isotopic ratios in groundwaters and river waters from India. J. Earth Syst. Sci. 94:283-295.

Bodey TW, Bearhop S, Sugoto SR, Newton J & McDonald RA (2010) Behavioural responses of invasive American mink *Neovison vison* to an eradication campaign, revealed by stable isotope analysis. J. Appl. Ecol. 47:114-120.

Bol R & Pflieger C (2002) Stable isotope analysis (^{13}C, ^{15}N and ^{34}S) of the hair of modern humans and their domestic animals. Rapid Commun. Mass Spectrom. 16:2195-2200.

Bowen GJ & Wilkinson B (2002) Spatial distribution of d18O in meteoric precipitation. Geology 30:315–318.

Bowen GJ, Wassenaar LI & Hobson KA (2005) Global application of stable hydrogen and oxygen isotopes to wildlife forensics. Oecologica 143(3): 337-348.

Britzke ER, Loeb SC, Hobson KA, Romanek CS & Vonhof MJ (2009) Assessing origins of bats using stable hydrogen isotope analysis: an investigation of four species in the eastern United States. J Mammal. 90:743–751.

Cabezas S, Carrete M, Tella JL, Marchant TA & Bortolotti GR (2012) Differences in acute stress responses between wild-caught and captive-bred birds: a physiological mechanism contributing to current avian invasions? Biol Invasions. DOI 10.1007/s10530-012-0304-z

Cerling TE, Wittemyer, Rasmussen HB, Vollrath F, Cerling CE, Robinson TJ & Douglas-Hamilton I (2006) Stable isotopes in elephant hair document migration patterns and diet changes. P. Natl. Acad. Sci. 103(2):371-373.

Chelazzi G & Calzolai R (1986) Thermal benefits from familiarity with the environment in a reptile. Oecologica 68:557-558.

Cherel Y, Kernaléguen L, Richard P & Guinet C (2009) Whisker isotopic signature depicts migration patterns and multi-year and inter- and intra-individual foraging strategies in fur seals. Biol. Lett. 5:830-832.

Cobo M & Andreu AC (1988) Seed consumption and dispersal by the spur-thighed tortoise *Testudo graeca*. Oikos 51:267-273.

Cooney R & Jepson P (2006) The international wild bird trade: what's wrong with blanket bans? Oryx 40:18-23.

Craig H (1961) Isotopic variations in meteoric water. Science 133:1702–1703.

Crawford K, McDonald RA & Bearhop S (2008) Applications of stable isotope techniques to the ecology of mammals. Mammal Rev. 38:87-107.

Cryan PM, Bogan MA, Rye RO, Landis GP & Kester CL (2004) Stable hydrogen isotope analysis of bat hair as evidence for seasonal molt and long-distance migration. J. Mammal. 85:995–1001.

Dalerum F & Angerbjörn (2005) Resolving temporal information in vertebrate diets using naturally occurring stable isotopes. Oecologica 144: 647-658.

Dansgaard W (1964) Stable isotopes in precipitation. Tellus 16:436–468.

Del Rio CM, Wolf N, Carleton SA & Gannes LZ (2009) Isotopic ecology ten years after a call for more laboratory experiments. Biol. Rev. 84:91-111.

Del Rio CM & Carleton SA (2012) How fast and how faithful: the dynamics of isotopic incorporation into animal tissues. J. Mammal. 93:353-359.

Del Vecchio S, Burke RL, Rugiero L, Capula M & Luiselli L (*2011*) Seasonal Changes in the Diet of *Testudo hermanni hermanni* in Central Italy. Herpetologica. 67: 236-249.

Ehleringer JR, Bowen GJ, Chesson LA, West AG, Podlesak DW & Cerling TE (2008) Hydrogen and oxygen isotopes ratios in human hair are related to geography. P. Natl. Acad. Sci. 105:2788-2793.

El Mouden EH, Slimani T, Kaddour KB, Lagarde F, Ouhammou A & Bonnet X (2006) *Testudo graeca graeca* feeding ecology in an arid and overgrazed zone in Morocco. J. Arid Environ. 64:422-435.

Engler M & Parry-Jones R (2007) Opportunity or threat: the role of the European Union in global wildlife trade. TRAFFIC Europe – Brussels, Belgium.

Fox TAD, Christensen TK, Bearhop S & Newton J (2007) Using stable isotope analysis of multiple feather tracts to identify moulting provenance of vagrant birds: a case study of Baikal Teal *Anas Formosa* in Denmark. Ibis 149:622-625.

Fox TAD, Hobson KA, Ekins G, Grantham M & Green AJ (2010) Isotope forensic analysis does not support vagrancy for a Marbled Duck shot in Essex. Brit. Birds 103:464-467.

France RL (1995a) Differentiation between littoral and pelagic food webs in lakes using stable carbon isotopes. Limnol. Oceanogr. 40:1310-1313.

France RL (1995b) Carbon-13 enrichment in benthic compared to planktonic alagae: foodweb applications. Mar. Ecol. Prog. Ser. 124:307-312.

Fraser I, Meier-Augenstein W & Kalin RM (2006) The role of stable isotopes in human identification: a longitudinal study into the variability of isotopic signals in human hair and nails. Rapid Commun. Mass Spectrom. 20:1109-1116.

Gannes LZ, Martínez del Rio C & Koch P (1998) Natural abundance variations in stable isotopes and their potential uses in animal physiological ecology. Comp. Biochem. Phys. 119: 725–737.

Garten CTJ (1993) Variation in foliar ^{15}N abundance and the availability of soil nitrogen on Walker Branch watershed. Ecology 74: 2098-2113.

Gerlach J (2004) Effects of diet on the systematic utility of the tortoise carapace. *Afr. J. Herpetol.* 53: 77-85.

Graves GR, Romanek CS & Navarro AR (2002) Stable isotope signature of philopatry and dispersal in a migratory songbird. P. Natl. Acad. Sci. USA 99:8096–8100.

Heaton THE (1987) The $^{15}N/^{14}N$ ratios of plants in South Africa and Namibia: relationship to climate and coastal/saline environments. Oecologica 74:236-246.

Hebert CE & Wassenaar LI (2001) Stable nitrogen isotopes in waterfowl feathers reflect agricultural land use in western Canada. Environ. Sci. Technol. 35:3482–3487.

Henen BT, Nagy KA, Bonnet X & Lagarde F (2000) Reproductive output of female Central Asian tortoises (*Testudo horsfieldi*). In: Abstracts of the 24th Annual Meeting and Symposium of the Desert Tortoise Council, St. George, Utah, 5-8 March 1999. pp. 44-45. [Abstr.]

Henen BT (2004) Capital and income breeding in two species of desert tortoise. T. Roy. Soc. S. Afr. 59:65-71.

Highfield AC (1992) The Horsfield's tortoise: *Testudo horsfieldi* (Gray) 1844 - A brief review of its biology, ecology and captive breeding URL: http://www.tortoisetrust.org/articles/horsfield2.html (accessed 07 Feb 2012).

Highfield AC (2002) Feeding tortoises: A practical guide to avoiding dietary disasters. URL: http://www.tortoisetrust.org/articles/webdiet.html (accessed 10 June 2012)

Hill KD (1990) The Convention on International Trade in Endangered Species: Fifteen years later. 13 Loy. L.A. Int'l & Comp. L. Rev. 231. (Available at: http://digitalcommons.lmu.edu/ilr/vol13/iss2/1).

Hobson KA (1993) Trophic relationships among high Arctic seabirds: insights from tissue-dependent stable-isotope models. Mar. Ecol. Prog. Ser. 95:7-18.

Hobson KA (1990) Stable isotope analysis of marbled murrelets: Evidence for freshwater feeding and determination of trophic level. Condor 92:897-903.

Hobson KA (2005) Using stable isotopes to trace longdistance dispersal in birds and other taxa. Diversity Distrib. 11:157-164.

Hobson KA & Clark RG (1992) Assessing avian diets using stable isotopes I: turnover of C-13 in tissues. Condor 94: 181-188.

Hobson KA, Alisauskas RT & Clark RG (1993) Stable-nitrogen isotope enrichment in avian tissues due to fasting and nutritional stress: implications for isotopic analyses of diet. Condor 95:388-394.

Hobson KA, Piatt JF & Pitocchelli J (1994) Using stable isotopes to determine seabird trophic relationships. J. Anim. Ecol. 63:786-798.

Hobson KA & Wassenaar LI (1997) Linking breeding and wintering grounds of neotropical migrant songbirds using stable hydrogen isotopic analysis of feathers. Oecologica 109:142-148.

Hobson KA, Wassenaar LI, Taylor OR (1998) Natal origins of migratory monarch butterflies at wintering colonies in Mexico: New isotopic evidence. Proc. Natl. acad. Sci. USA 95:15436-15439.

Hobson KA, Wassenaar LI, Taylor OR (1999a) Stable isotopes (dD and d13C) are geographic indicators of natal origins of monarch butterflies in eastern North America. Oecologia 120:397–404.

Hobson KA, Drever MC & Kaiser GW (1999b) Norway rats as predators of burrow-nesting seabirds: insights from stable isotope analyses. J. Wildlife Manage. 63:14-25.

Hobson KA, Atwell L & Wassenaar LI (1999c) Influence of drinking water and diet on the stable-hydrogen isotope ratios of animal tissues. P. Natl. Acad. Sci. USA, 96: 8003-8006.

Hobson KA, Bowen GJ, Wassenaar LI, Ferrand Y & Lormee H (2004a) Using stable hydrogen and oxygen isotope measurements of feathers to infer geographical origins of migrating European birds. Oecologia 141: 477-488.

Hobson KA, Wassenaar LI & Bayne E (2004b) Using isotopic variance to detect long-distance dispersal and philopatry in birds: An example with ovenbirds and American redstarts. The Condor 106:732–743.

Hobson KA, Van Wilgenburg S, Wassenaar LI, Moore, F & Farington J (2007) Estimating origins of three species of neotropical migrant songbirds at a gulf coast stopover site: Combining stable isotope and GIS tools. Condor 109:256-267.

Hobson KA, Lormée H, Van Wilgenburg SL, Wassenaar LI & Boutin JM (2009a) Stable isotopes (δD) delineate the origins and migratory connectivity of harvested animals: the case of European woodpigeons. J. of Applied Ecol. 46:572-581.

Hobson KA, Van Wilgenburg SL, Larsen K & Wassenaar LI (2009b) A feather hydrogen isoscape for Mexico. J. Geochem. Explor. 102:63-70.

Hobson KA, Wunder MB, Van Wilgenburg SL, Clark RG, Wassenaar LI (2009c) A method for investigating population declines of migratory birds using stable isotopes: Origins of harvested lesser scaup in North America. PLoS ONE 4: e7915.

Inger R & Bearhop S (2008) Applications of stable isotope analysis to avian ecology. Ibis 150:447-461.

Ishibashi H, Díaz-Fernández R, Carrillo E & Koike H (2000) $\delta^{15}N$ and $\delta^{13}C$ measurements from the hawksbill turtle, *Eretmochelys imbricata*, used for scute sourcing. Bull. Grad. Sch. Social Cultural Stud. Kyushu Univ. 6:37-45.

IUCN (2000) A report by IUCN – The World Conservation Union on the effectiveness of trade measures contained in the Convention of International Trade in Endangered Species of Wild Fauna and Flora (CITES). Prepared for The Economics, Trade and Environment Unit United Nations Environment Programme.

Kelly JF, Atudorei V Sharp ZD & Finch DM (2002) Insights into Wilson's warbler migration from analyses of hydrogen stable-isotope ratios. Oecologia 130:216-221.

Kelly S, Heston K & Hoogewerff J (2005) Tracing the geographical origin of food: the application of multi-element and multi-isotope analysis. Trends Food Sci. Tech.16: 555–567.

Kelly A, Thomson R & Newton J (2007) Stable hydrogen isotope analysis as a method to identify illegally trapped songbirds. J. Forensic Sci. Soc. 48:67-70.

Kempster B, Zanette L, Longstaffe FJ, MacDougall-Shackleton SA, Wingfield JC & Clinchy M (2007) Do stable isotopes reflect nutritional stress? Results from a laboratory experiment on song sparrows. Oecologia 151:365-371.

Körner Ch, Farquhar GD & Wong SC (1991) Carbon isotope discrimination by plants follows latitudinal and altitudinal trends. Oecologia 88:30-40.

Lagarde F, Bonnet X, Nagy K, Henen B, Corbin J & Naulleau G (2002) A short spring before a long jump: the ecological challenge to the steppe tortoise (*Testudo horsfieldi*). Can. J. Zoolog. 80:493–502.

Lagarde F, Bonnet X, Corbin J, Henen B, Nagy K, Mardonov B & Naulleau G (2003) Foraging behaviour and diet of an ectothermic herbivore: *Testudo horsfieldii.* Ecography 26: 236-242.

Langin KM, Reudink MW, Marra PP, Norris DR, Kyser TK & Ratcliffe LM (2006) Hydrogen isotopic variation in migratory bird tissues of known origin: implications for geographic assignment. Oecologica 152:449-457.

Lott CA, Meehan TD & Heath JA (2003) Estimating the latitudinal origins of migratory birds using stable hydrogen and sulfur isotopes in feathers: influences of marine prey base. Oecologia 134: 505–510.

Lott CA & Smith JP (2006) A Geographic-Information-System approach to estimating the origin of migratory raptors in North America using stable hydrogen isotope ratios in feathers. The Auk: 123(3):822-835.

Lyons JA & Natusch DJD (2011) Wildlife laundering through breeding farms: Illegal harvest, population declines and a means of regulating the trade of green pythons (*Morelia viridis*) from Indonesia. Biol. Conserv. 144:3073-3081.

Manel S, Berthier P & Luikart G (2002) Detecting wildlife poaching: identifying the origin of individuals with Bayesian assignment tests and multilocus genotypes. Conserv. Biol. 16:650-659.

Marra PP, Hobson KA & Holmes RT (1998) Linking winter and summer events in a migratory bird using stable carbon isotopes. Science 282:1884-1886.

McCue MD & Pollock ED (2008) Stable isotopes may provide evidence for starvation in reptiles. Rapid Commun. Mass Spec. 22:2307-2314.

McKechnie AE, Wolf BO & del Rio CM (2004) Deuterium stable-isotope ratios as tracers of water use: an experimental test with rock doves. Oecologia 140:191–200.

Meehan TD, Rosenfield R, Atudorei VN, Bielefeld J, Rosenfield LJ, Stewart AC, Stout WE & Bozek MA (2003) Variation in hydrogen stable isotope ratios between adult and nestling Cooper's Hawks. The Condor 105:567-572.

Meehan TD, Giermakowski JT & Cryan PM (2004) GIS-based model of stable hydrogen isotope ratios in North American growing-season precipitation for use in animal movement studies. Isotopes Environ. Health Stud. 40(4):291-300.

Meek R & Innskeep R (1980) Aspects of the field biology of a population of Hermann's tortoise (*Testudo hermanni*) in Southern Yugoslavia. Brit. J. Herpetol. 6:159-164.

Michener RH & Kaufman L (2007). Stable isotope ratios as tracers in marine foodwebs: An update. In Michener R & Lajtha K (eds) *Stable Isotopes in Ecology and Environmental Sciences*: 238–282. Oxford: Blackwell Publishing.

Murray IW & Wolf BO (2012) Tissue carbon incorporation rates and diet-to-tissue discrimination in ectotherms: tortoises are really slow. Physiol. Biochem. Zool. 85:96-105.

Nardoto GB, Silva S, Kendall C, Ehleringer J, Chesson LA, Ferraz ESB, Moreira MZ, Ometto JPHB & Martinelli LA (2006) Geographical patterns of human diet derived from stable-isotope analysis of fingernails. Am. J. Phys. Anthropol. 131:137-146.

Nijman V & Shepherd CR (2009) Wildlife trade from ASEAN to the EU: Issues with the trade in captive-bred reptiles from Indonesia. TRAFFIC Europe Report for the European Commission, Brussels, Belgium.

Ogden R, Dawnay N & McEwing R (2009) Widlife DNA forensics – bridging the gap between conservation genetics and law enforcement. Endang. Species Res. 9(3):179-195.

O'Leary MH (1981) Carbon isotope fractionation in plants. Phytochemistry 20:553-67.

Olsen J, Seierstad I, Vinther B, Johnsen S & Heinemeier J (2006) Memory effect by deuterium analysis by continuous flow isotope ratio meausurement. Int. J. Mass Spectrom. 254:44-52.

Palsbøll PJ, Bérubé M, Skaug HJ & Raymakers C (2005) DNA registers of legally obtained wildlife and derived products as a means to identify illegal takes. Conserv. Biol. 20(4): 1284-1293.

Perälä J (2003) *Testudo kleinmanni*. In: IUCN 2012. IUCN Red List of Threatened Species. Version 2012.1. URL: http://www.iucnredlist.org (accessed 13 June 2012).

Peterson BJ, Howarth RW & Garritt (1985) Multiple stable isotopes used to trace the flow of organic matter in estuarine food webs. Science 227:1361-1363.

Pietzsch M, Hillyard PD, Medlock JM & Leach S (2006) Importation of exotic ticks into the United Kingdom via the international trade in reptiles. Exp. Appl. Acarol. 38:59-65.

Polischuk SC, Hobson KA & Ramsay MA (2001) Use of stable-carbon and -nitrogen isotopes to assess weaning and fasting in female polar bears and their cubs. Can J. Zool. 79:499-511.

Post DM (2002) Using stable isotopes to estimate trophic position: models, methods, and assumptions. Ecology 83(3):703-718.

Powell LA & Hobson KA (2006) Enriched feather hydrogen isotope values for Wood Thrushes sampled in Georgia, USA, during the breeding season: implications for quantifying dispersal. Can. J. Zool. 84:1331–1338.

Pyankov VI, Ziegler H, Akhani H, Deigele C & Lüttge U (2010) European plants with C-4 photosynthesis: geographical and taxonomic distribution and relations to climate parameters. Bot. J. Lin. Soc. 163:283-304.

Reed RN (2005) An ecological risk assessment of non-native boas and pythons as potentially invasive species in the United States. Risk Anal. 25:753-766.

Reich KJ, Bjorndal KA & Bolten AB (2007) The ‘lost years’ of green turtles: using stable isotopes to study cryptic lifestages. Biol. Lett. 3:712–714.

Reich KR, Bjorndal KA & del Rio (2008) Effects of growth and tissue type on the kinetics of 13C and 15N incorporation in a rapidly growing ectotherm. Oecologia 155:651-663.

Rep-Cal (no date) URL: http:// repcal.com/Tort.htm (accessed 12 June 2012).

Rocque DA, Ben-David M, Barry RP, Winker K (2006) Assigning birds to wintering and breeding grounds using stable isotopes: lessons from two feather generations among three intercontinental migrants. J. Ornithol. 147:395-404.

Rosen GE, Smith KF (2010) Summarizing the evidence on the international trade in illegal wildlife. EcoHealth 7:24–32.

Roth JD (2002) Temporal variability in arctic fox diet as reflected in stable-carbon isotopes; the importance of sea ice. Oecologica 133(1):70-77.

RSPCA (2001) Shell shock: the continuing illegal trade in tortoises.

Rugiero L & Luiselli L (2006) Ecological modelling of habitat use and the annual activity patterns in an urban population of the tortoise, *Testudo hermanni.* Ital. J. Zool. 73:219-225.

Schlaepfer P.M, Hoover C, & Dodd CK (2005) Challenges in evaluating the impact of the trade in amphibians and reptiles on wild populations. BioScience 55:256-264.

Seltzer MD & Berry KH (2005) Laser ablation ICP-MS profiling and semiquantitative determination of trace element concentrations in desert tortoise shells: documenting the uptake of elemental toxicants. Sci. Total. Environ. 339: 253-265.

Shepherd CR & John E (2010) Monitoring the Large Trade in Clouded Monitors in Peninsular Malaysia. In Broad S & Lochen K (eds) *TRAFFIC Bulletin*: 22(3) p.103.

Smith AD & Dufty AM (2005) Variation in the stable-hydrogen isotope composition of Northern goshawk feathers: relevance to the study of migratory origins. The Condor 107:547-558.

Smith RJ & Walpole J (2005) Should conservationists pay more attention to corruption? Oryx 39:251-256.

Sodhi NS, Kho LP, Brook BW & Ng PKL (2004) Southeast Asian biodiversity: an impending disaster. TRENDS Ecol. Evol. 19:654-660.

Stubbs D (1989a) *Testudo hermanni*, Hermann's tortoise. in I.R.Swingland and M.W.Klemens, (ed.), The conservation biology of tortoises. Occasional Papers of the IUCN Species Survival Commission No. 5.Gland, Switzerland: IUCN. 34-36.

Stubbs D (1989b) *Testudo horsfieldii*, Horsfield's tortoise. in Swingland IR & Klemens MW (ed.), The conservation biology of tortoises. Occasional Papers of the IUCN Species Survival Commission No. 5.Gland, Switzerland: IUCN. 37-38.

Stubbs D (1989c) *Testudo graeca*, Spur-thighed tortoise. in Swingland IR & Klemens MW (ed.), The conservation biology of tortoises. Occasional Papers of the IUCN Species Survival Commission No. 5.Gland, Switzerland: IUCN. 31-33.

Stuiver M & Braziunas TF (1987) Tree cellulose $^{13}C/^{12}C$ isotope ratios and climate change. Nature 328:58-60.

The Tortoise Table (no date) URL: http://www.thetortoisetable.org.uk/site/tortoise_home_1.asp (accessed 10 June 2012).

Theile S (2000) Ranching and breeding Testudo horsfieldii in Uzbekistan. TRAFFIC, Europe.

Theile S, Steiner A & Kecse-Nagy K (2004) Expanding borders: new challenges for wildlife trade controls in the European Union. TRAFFIC, Europe, Brussels, Belgium.

Tiny Tortoises (no date) Tortoise diet. URL: http://tinytortoises.co.uk/tortoise%20diet.html (accessed 10 June 2012).

Tortoise.org.uk (no date) Tortoise care: How to keep your tortoise happy and healthy. URL: http://www.tortoise.org.uk (accessed 10 June 2012).

TRAFFIC (2011) TRAFFIC Bulletin: Seizures and prosecutions March 1997- April 2011. TRAFFIC, Europe, Brussels, Belgium.

UNEP-WCMC (2010) Review of species selected on the basis of a new or increased export quota in 2010. UNEP-WCMC, Cambridge.

UNEP-WCMC (no date) CITES trade data dashboard. URL: hhtp://www.dashboards.cites.org/global?id=Reptiles (accessed 10 June 2012).

van der Kuyl AC, Ballasina DLP & Zorgdrager F (2005) Mitochondrial haplotype diversity in the tortoise species Testudo graeca from North Africa and the Middle East. BMC Evol. Biol. 5:29.

van der Merwe NJ, Lee-Thorp JA, Thackeray JF, Hall-Martin A, Kruger FJ, Coetzee H, Bell RHV & Lindeque M (1990) Source-area determination of elephant ivory by isotopic analysis. Nature 346:744-746.

van Dijk PP, Corti C, Mellado VP & Cheylan M (2004) *Testudo hermanni*. In: IUCN 2012. IUCN Red List of Threatened Species. Version 2012.1. URL: http://www.iucnredlist.org (accessed 13 June 2012).

Vander Zanden HB, Bjorndal KA, Reich KJ & Bolten AB (2010) Individual specialists in a generalist population: Results from a long-term stable isotope series. Biol. Lett. 6:711-714.

Vanderklift MA & Ponsard S (2003) Sources of variation in consumer-diet δ^{15}N enrichment: a meta-analysis. Oecologica 136:169-182.

Vinke T & Vinke S (2010) Do breeding facilities for chelonians threaten their stability in the wild? Schildkröten im Fokus online, Bergheim 1:1-18.

Vogel JC, Eglington B & Auret JM (1990) Isotope fingerprints in elephant bone and ivory. Nature 346:747-749.

Wassenaar LI & Hobson KA (2000) Stable-carbon and hydrogen isotope ratios reveal breeding origins of red-winged blackbirds. Ecol. Appl. 10:911–916.

Wassenaar LI & Hobson KA (2003) Comparative Equilibration and online technique for determination of non-exchangeable hydrogen of keratins for use in animal migration studies. Isotopes Environ. Health Stud. 39(3):211-217.

Wasser SK, Mailand C, Booth R, Mutayuba B, Kisamo ES, Clark B & Stephens M (2007) Using DNA to track the origin of the largest ivory seizure since the 1989 trade ban. Proc. Natl. Acad. Sci. 104:4228-4233.

Wasser SK, Clark WJ, Drori O, Kisamo ES, Mailand C, Mutayoba B & Stephens M (2008) Combating the illegal trade in African elephant ivory with DNA forensics. Conserv. Biol. 22:1065-1071.

White NE, Dawson R, Coghlan ML, Tridico SR, Mawson PR, Haile J & Bunce M (2011) Application of STR markers in wildlife forensic casework involving Australian black-cockatoos (*Calyptorhynchus* spp.) Forensic Science International: Genetics. 6:664-670.

Wiesner CS & Iben C (2003) Influence of environmental humidity and dietary protein on pyramidal growth of carapaces in African spurred tortoises (*Geochelone sulcata*). *J. Anim. Physiol. Anim. Nutr.* 87: 66-74.

Williams CT, Buck CL, Sears J & Kitaysky AS (2007) Effects of nutritional restriction on nitrogen and carbon stable isotopes in growing seabirds. Oecologica 153:11-18.

Wilson DS, Tracy CR & Tracy R (2003) Estimating age of turtles from growth rings: a critical evaluation of the technique. Herpetologica 59: 178-194.

Wright J, Steer E & Hailey A (1988) Habitat separation in tortoises and the consequences for activity and thermoregulation. Can. J. Zool. 66:1537-1544.

Wunder MB, Kester CL, Knopf FL & Rye RO (2005) A test of geographic assignment using isotope tracers in feathers. Oecologica 144:607-617.

Wunder MB & Norris DR (2008) Improved estimates of certainty in stable-isotope-based methods for tracking migratory animals. Ecol. Appl. 18:449-459.

Ziegler S, Merker S & Jacob D (2012) Geographic variation of stable isotopes in African elephant ivory. Geophysical Research Abstracts 14: EGU2012-1884, EGU General Assembly 2012.