Chapter 6

Synthesis

In the previous four chapters, a range of isotope methods were successfully applied in a variety of settings, demonstrating that the bones and teeth of vertebrates can function as valuable and durable geochemical archives. I have explored various ways in which isotope geochemistry can test and expand on paleontological findings. Spatial niche partitioning in the Late Cretaceous marine turtle *Allopleuron hofmanni* led us to hypothesize that adults of this species were herbivores feeding on seagrass; subsequent carbon isotope analysis suggested that this would only be possibly if seagrass $\delta^{13}C$ values were depleted compared to those of most extant species. Isotope results for Indonesian fossil sites corroborated and refined earlier paleoenvironmental interpretations based on faunal assemblage composition, improving geochronological constraints. Isotope geochemistry allows us to add a new layer to our understanding of past ecosystems.

The versatility of isotope proxies is especially evident in the broad range of conceptual scales in which they are able to provide insight. At one end of the spectrum, a single tooth of a muntjac deer may give us information about the behavior and preferences of that specific individual. At the other end, it may help us to gain more knowledge of large-scale changes in its ecosystem and climate; processes whose spatial and temporal scope exceed the deer's home range and life span by orders of magnitude.

As I carried out this research I encountered technical challenges, as well as opportunities for further refinement. Both are important to acknowledge and to explore. Especially with regard to diagenesis, this chapter will discuss optimal ways to mitigate its effects and suggests solutions that will improve future research. Most of these solutions are not just applicable to isotope research, but are relevant to geochemical methods as a whole.

The central question I ask in this thesis is; how can isotope tools best be embedded in the effort to reconstruct ancient habitats and the behavior of their inhabitants? This final chapter attempts to answer that question on the basis of the research I have conducted. It examines the current and potential role of isotope geochemistry in paleontology, offers different perspectives on the interpretation of carbon, oxygen and strontium isotope results, and discusses various ways in they can be effectively combined with other (geochemical, as well as more traditional) paleontological techniques.

6.1 Diagenesis: an inevitable and ineradicable factor

A major problem of working with fossils is the ever-present possibility that they have been diagenetically altered. Diagenesis can entail the precipitation of secondary minerals, exchange of atoms with the surrounding sediments, and even complete recrystallization of the original apatite crystal. Any of these modifications may result in partial or complete overprint of the original isotopic composition, thus corrupting the paleo-proxy record.

6.1.1 Improving our diagenetic screening protocol

The research in this thesis utilizes an array of different methods to screen for diagenetic alteration, from macroscopic visual inspection, to comparing the isotope values of leached and unleached samples. Many of these tests have the potential to unveil an aspect of diagenetic alteration. For instance, cathodoluminescence (CL) microscopy gives us an indication of the extent in which trace elements substitute for calcium in the apatite crystal lattice, a general indication of chemical alteration (e.g. Kohn et al., 1999; Sponheimer and Lee-Thorp, 2006). Comparing the stable oxygen isotope values in the structural carbonate versus phosphate enamel fraction tests the preservation of original oxygen isotope values (Tudge, 1960; Iacumin et al., 1996; Zazzo et al., 2004). As described in Chapter 3 of this thesis (van Baal et al., 2013), oxygen isotope values in these components are strongly correlated unless the less diagenetically resistant carbonate oxygen has been altered (Bryant et al., 1996; Iacumin et al., 1996; Martin et al., 2008).

Note, however, that we compared carbonate and phosphate oxygen only in the shark teeth of Chapter 3, but not in the Sundaland herbivores of Chapter 4 and 5. Conversely, CL microscopy was applied on the latter set of specimens, but not on the former. Other useful additions to the diagenetic screening process would have been the microscopic analyses of thin sections of the shark and ray teeth, and the comparison of structural carbonate and phosphate oxygen isotopes in a selection of bovid and cervid teeth. I believe our research on both the Maastrichtian type area fossils and the Quaternary fossils from the Indonesian archipelago would have potentially benefited from further diagenetic testing. These diagenetic tests cannot be used interchangeably, because each method shows only certain types of alterations (e.g., cathodoluminescence is a measure of trace element alteration; phosphate versus carbonate oxygen isotope composition primarily informs about alteration of carbonate oxygen) which are not guaranteed to correspond to alteration of other fractions. Rather, screening tests must be seen as complementing each other, with each method independently providing clues as to the state of preservation.

Taking into account their tendency to be destructive and to require significant amounts of sample material, as well as the time and resources required to implement them, we must strive to make selective and effective use of multiple screening techniques in each case study. Furthermore, the results of screening should not solely be used as a tentative and unquantified indication of diagenetic action. Instead, I would propose using a systematic protocol in which possible indications of diagenesis (as well as signs that a specimen originated from other layers or sites, i.e. was mislabeled) are evaluated using a standardized scoring table. Ideally, these assessments are performed before any isotopic results are available, in order not to influence the evaluation. Characteristics that are quickly and non-destructively appraised (e.g. deviations in color and sheen, the amount of cracking in the enamel, type of attached matrix, whether the label or other examiners profess some doubt about the taxon or provenance, etc.) should be evaluated for each specimen sampled for isotope analysis. More extensive testing (e.g. examining thin sections with CL microscopy, analyzing phosphate oxygen isotopes) can be done on a representative subset of specimens, and always on a site-by-site basis. On the one hand, we ideally want to select a rather large amount of samples for extensive diagenetic testing, while on the other hand we don't want to do more damage than necessary. The outcome of weighing up these considerations will be different for every case study. The bottom line is that it is essential to get a good grip on diagenesis in order to trust and thus utilize the actual results, and in the future we may want to test more extensively than we did in the case studies featured in this thesis.

Having composed a quantitative scoring table, based on a significantly large set of specimens, we can come back to it whenever we have a suspicion about an outlier in our isotope data. But in addition, we can go one step further towards a more objective approach, and analyze statistically whether there are correlations between diagenetic markers and the measured isotope values. This method allows us to go beyond just noting the obvious exceptions (like the C_3-eating Trinil bovid in Chapter 4) towards a more nuanced evaluation of the integrity of our sample collection.

6.1.2 Expanding our repertoire of screening techniques

There are some additional screening methods I suggest we add to our screening protocol. Notably, I advocate analyzing the dentin isotopic compositions for teeth of which enamel is taken as a primary sample (Ayliffe et al., 1994; Budd et al., 2000; Sharp et al., 2000; Copeland et al., 2010). Because dentin is less mineralized, it is also less diagenetically robust than enamel. Dentin isotopes may thus give an indication of the 'direction' of diagenesis. Sampling dentin is generally less invasive with regard to important deterministic features of tooth morphology and overall appearance: in some cases it would even be possible to sample the

dentin directly behind the enamel sampling spot, thus avoiding additional surface area damage. Furthermore, carbonate stable isotope analyses on bioapatite are relatively time- and cost-friendly. I would therefore propose to analyze the dentin of a large portion of any sample set (except in the case of very small or fragile teeth).

Another type of screening method worth considering is to investigate whether fossil specimens have been diagenetically enriched in trace elements (see for instance Kohn et al., 1999; Dauphin and Williams, 2004; Sponheimer and Lee-Thorp, 2006). Alteration of U, F, and rare-earth element distributions would be especially relevant when we are interested in the Sr isotope ratios of fossil specimens, or in other elements that substitute in the Ca and OH sites of bioapatites (Kohn et al., 1999), although there will not be a linear correlation between the diagenetic incorporation of Sr and other elements. Trace elements also shed some light on the integrity of oxygen isotopes in the carbonate fraction (here, alteration of the OH site could produce diagenetic offsets of ~1‰ $\delta^{18}O$; Kohn et al., 1999) but they are probably less informative with regards to oxygen isotopes in the phosphate fraction. A primary advantage is that trace element concentrations can be measured in the phosphoric acid residues remaining after carbonate stable isotope analysis, and thus require no additional sample material.

It is important to note, however, that trace element alteration can only be established after the original concentrations can be estimated with sufficient certainty. Unfortunately the *in vivo* concentrations of many of these trace elements vary considerably within species, between species, and per region (Price et al., 1985; Price et al., 1986; Sillen, 1988; Sponheimer et al., 2005). Using elements like Sr, Ba, Pb and Zn as an absolute indicator for diagenesis would require analyzing large sample sets for each species at each site, comprising not only fossil specimens but also modern equivalents. So far, no work has been carried out into establishing a modern baseline for Indonesian ecosystems, and so we are still far removed from applying such methods. Such an approach exceeds the practical scope of the kind of studies featured in this thesis.

Analysis of U and Th distributions provides better opportunities for our research, as the *in vivo* concentrations of these trace elements in bioapatite are so low that high concentrations are a readily recognizable sign of diagenetic overprinting. Systematic mapping of the distribution of these elements across a particular specimen (using laser ablation-ICPMS) would allow us to determine which zone is least altered and thus most suitable for sampling (Trueman et al., 2008; Willmes et al., 2016). Even for elements with *in vivo* concentrations that may be quite variable, such as Zn, such mapping would allow us to establish the relative, if not absolute, degree of alteration over the entire specimen.

The challenges and possibilities of applying vibrational spectroscopy - Fourier transform infrared (FTIR), attenuated total reflection (ATR), and diffuse reflectance infrared Fourier transform (DRIFT) - are in some ways similar to trace element screening methods.

These techniques offer powerful tools for detecting secondary carbonates and structural alterations in fossil bone and enamel (Lee-Thorp and van der Merwe, 1991; Ildefonse and Morin, 1995; Sponheimer and Lee-Thorp, 1999; Beasley et al., 2014), but their results show significant variation even in modern teeth. In addition, vibrational spectroscopy requires considerable amounts of sample powder (1-2 mg) and thus additional destruction of fossil specimens. With this technique, as with trace element analysis, I would argue that application to large sample sets would be too costly in terms of time and resources. Both methods, however, could be exceedingly useful in situations where accurate isotope ratios from particular (perhaps very valuable and rare) specimens potentially make a lot of difference in our interpretations.

6.1.3 Chemical pre-treatment: towards an optimal approach

In an effort to mitigate some of the possible effects of diagenesis, we make use of chemical pre-treatment. The case studies in this thesis apply the procedure described by Bocherens et al. (1996). In it, samples are soaked in 2-3% NaOCl for 20 hours to extract organic residues, and subsequently leached in 1 M acetic acid -calcium acetate buffer for 20 hours to remove the more soluble carbonates. The reasoning behind this second step is that the more labile carbonates are most likely diagenetic accretions of secondary carbonate. and that the more stable, original structural carbonate bound in the bioapatite crystal lattice will remain intact. However, this assumption may not always be valid.

In Chapter 4 (Janssen et al., 2016), I analyzed splits of 26 samples from Quaternary fossil bovid enamel: one split of each sample was pre-treated with the method described by Bocherens et al. (1996) and one split was untreated. I found non-systematic offsets for several of the represented sites, including the Pleistocene Trinil site where all specimens originate from a single layer at a single location (suggesting a similar diagenetic setting). The offsets were in the order of 1-2 per mille, which is significant, but not large enough to compromise paleodietary and paleoenvironmental interpretations in this particular study. Because it was unclear what the exact mechanisms were that produced the observed offsets, we could not determine which values best represented the *in vivo* isotope ratios, and thus decided to base our interpretations on isotope data from untreated tooth enamel. An important factor in this consideration was the fact that some forms of diagenetic apatites will be stable enough to resist the acid leaching process, while under some conditions leaching may induce artifacts: recrystallization, the creation of new chemical compounds, and isotopic fractionation in the original compounds (Koch et al., 1997; Lee-Thorp, 2002).

The ‘Bocherens method’ is not the only form of chemical pre-treatment used on enamel and bone. Notably, the strength of the acid solution varies across the methods

employed. While Bocherens et al. (1996) applies with 1.0 M acetic acid-Ca acetate buffer, those who follow the technique developed by Lee-Thorp (1989) use 1.0 M unbuffered acetic acid. Balasse et al. (2002) and others have used a more dilute acetic acid solution of 0.1 M, in effect trading a reduction in the risk of producing chemical artifacts with an increase in the probability that not all secondary carbonates are removed from the sample. How should we decide on the most appropriate method?

Koch et al. (1997) compare both treatments on enamel, dentin and bone (of similar age as our Indonesian samples) and conclude that either may be used, but that the use of unbuffered 1.0 M acetic acid should be avoided. They suggest that the stronger acid solution may even be preferable as some samples experience recrystallization even when treated with the weaker acid solution. Importantly, this problematic response specifically happens in modern samples, an observation corroborated by Garvie-Lok et al. (2004) in their pre-treatment comparison; recrystallization may be less of a risk in working with paleontological samples. At the same time, the observation that even in modern samples the isotope ratios change in response to weak acid leaching suggests that unintended isotopic effects are inevitable when applying any form of acid pretreatment. Similar to Koch et al. (1997), Garvie-Lok et al. (2004) advises against the use of 1.0 M (unbuffered) acetic acid; a buffered solution was not included in their comparison. Regarding the period of time that samples are soaked in acid, several studies suggest that a shorter reaction time of 4 hours is sufficient to dissolve a similar amount of labile carbonate while it decreases the potential for recrystallization (Lee-Thorp and van der Merwe, 1991; Balasse et al., 2002; Garvie-Lok et al., 2004). I would therefore recommend leaching samples in 1.0 M acetic acid-Ca acetate buffer for 4 hours instead of 20 hours.

Based on these studies I would conclude that, to quote Koch et al. (1997), "no treatment gives an unambiguous 'right' answer." Weak acid leaching probably results in incomplete removal of diagenetic carbonates. Strong acid leaching may cause isotopic artifacts. I think that our response should be to stop seeing chemical pre-treatment as a way to undo the effects of diagenesis, and instead to see it as a diagenetic screening method: an approach that potentially reveals something about the extent and isotopic direction of diagenesis. In interpreting isotope data we should simply accept the fact there is an inevitable offset between the measured values and the original *in vivo* values, whether it be due to diagenesis or due to our own pre-treatments. In other words, rather than focusing on the question, "can we take these measured values to represent original values?", we should ask, "how large do we expect the maximum offset between measured and original values to be, and how would such an error influence our interpretations?"

6.2 Valuable collections, valuable knowledge

6.2.1 Reducing sample size

The measurement of isotope ratios in bioapatites is inherently destructive. The most common analysis applied in this thesis - the measurement of the carbon and oxygen isotopic composition of structural carbonate using the GasBench II preparation device - currently requires samples containing 6 - 12 µg of pure carbonate to produce reliable results. However, only a fraction of bone and tooth enamel is carbonate: it occurs as one part of carbonated hydroxylapatite, the primary mineral from which these tissues are composed. Thus more than 300 µg (0.3 mg) of enamel, dentin or bone needs to be sampled for each measurement, an amount equivalent to a couple of sugar grains. Strontium analysis on bioapatite requires similar sample sizes, but chemical pre-treatment invariably results in considerable sample loss. This is both due to the dissolution of diagenetic and organic material (this is the intended effect of the treatment) and as an unintended consequence of having to rinse the samples 6 - 9 times in this process. In practice, sampling a bone or tooth for both these analyses entails drilling a circular hole of 1-2 mm in width. In taxa with very thin enamel, it's not possible to drill deep, and therefore a larger surface area has to be damaged. Measurements on the phosphate fraction of enamel require samples of ~7 mg, necessitating damage to an even larger surface area.

The reasons for wanting to minimize the necessary sample sizes for these analyses are obvious: the fossil specimens in museum collections examined in this thesis are of great paleontological and historical significance, and need to be preserved for future generations and future research. At the same time, they contain a wealth of information that can only be accessed through research that is destructive to some extent. Specimens thus have to be chosen wisely, always striving to inflict minimal damage. This is especially important when dealing with fossils that are very well-preserved, fragile, or represent rare taxa. Smaller minimum sample sizes also make it possible to analyze very small specimens in the first place (e.g. the thin enamel of small fish and rodent teeth, otoliths), to perform incremental intra-tooth sampling at a finer resolution, and to target specific small subsections of a specimen (such as a particular accretion of a diagenetic mineral).

At our lab, the stable isotope laboratory of the FALW department at VU University Amsterdam, we are currently looking into several ways to reduce the necessary sample size for bioapatite. Recent tests by Melanie During, Suzan Verdegaal and Jeroen van der Lubbe have shown that we can significantly reduce the necessary sample size for bioapatite stable isotope analysis without compromising the accuracy and precision of the resulting data, by

more sharply defining the lowest level of output voltage at which the resulting isotope values remain stable. In the near future we expect to be able to produce reliable results even below this level. The deviations that arise when analyzing samples smaller than ~0.15 mg are consistent in direction and magnitude, and can thus be carefully corrected if these samples are measured interspersed with calcite standards and CO_2 reference gas injections in exactly the right amounts, producing voltage outputs closely approximating the voltage output of the samples. To be able to produce optimal reference gas peaks, our lab aims to acquire a DIPcon® device (as developed by Hans Heinrich Cordt of IRMS-ctrl) which is able to adjust the reference gas quantities in response to the magnitude of the sample peaks. I am confident that combining these methods will allow us to reduce the necessary sample size from 0.3 mg to approximately 0.1 mg.

An alternative to drilling samples is laser ablation. It works by firing a pulsed laser beam at a particular spot on the surface of a specimen, thereby ionizing a micro-sample which can then be analyzed in a mass spectrometer. Laser ablation has been successfully applied to measure the isotopes of C, O, Sr, and many other elements. Ablation pits may be as small as ~250 µm wide and ~100 µm deep, although a series of pits are needed for one measurement (Passey and Cerling, 2006; Garcia et al., 2015). The precision and accuracy of laser ablation is currently somewhat less than that of conventional methods. But it is more than sufficient for most research questions, including those of this thesis: it can be used to distinguish between the teeth of C_3 browsers and C_4 grazers (e.g. Passey and Cerling, 2006) and investigating provenance in most parts of the world (Copeland et al., 2008). However, there are several drawbacks to this technique which make it less suitable for our purposes than the conventional methods used in the research of this thesis. First, it is not possible to pretreat samples, except to clean the intact enamel surface before sampling. Second, specimens with a low carbonate content or high organic content cannot be reliably analyzed (Passey and Cerling, 2006; Henry et al., 2012; Patterson et al., 2016). Also, laser ablation measures both the oxygen isotope ratio in the phosphate fraction and the carbonate fraction of bioapatite, and due to the high temperature at which decarbonation takes place in the measurement process the resulting oxygen isotope values are mainly a reflection of the phosphate oxygen isotopic composition (Cerling and Sharp, 1996; Sharp and Cerling, 1996). And lastly, due to technical challenges related to the CO_2 outgassing of specimens, this method cannot be used on large specimens such as the ungulate molars I analyzed in this thesis (Passey and Cerling, 2006). Although laser ablation will be an extremely useful alternative if we are to sample for instance rodent teeth or *Homo erectus* teeth in the future, for most other applications the conventional (non-laser) methods are still the better option.

6.2.2 On the importance of rigorous record keeping

Besides a continuous effort to further reduce sample size, we can further minimize unnecessary damage to museum specimens by taking steps to ensure that the scientific community makes optimal use of our sampling. Notably, I recommend that this will include: (a) leaving a well-organized record of their activities in both collections and scientific literature, and (b) saving excess sampled material for future use.

Our research on the Dubois, von Koenigswald and Selenka collections was noted down in the museum records, and for many of the specimens we also appended a note detailing our names and the analyses for which we intended to use the sampled material. Notwithstanding the current enthusiasm for (and undeniable usefulness of) digitalization, paper labels in a collection drawer may yet prove more enduring than digital records - and they are difficult to overlook for a future researcher examining a specimen kept in the same box. Regarding the publishing of results, researchers performing destructive analyses should take extra care in mentioning ambiguous and 'null' results in their publications. When an experiment does not lead to immediately interpretable or relevant results, this is also worth mentioning. If there is no correlation between two variables, this is also a result. We need to know what doesn't work in order to move forward to an approach that does work. For workers examining an abundant resource such as atmospheric air, or the leaf water of a handful of C_4 grass, publishing these 'disappointing' results may at the very least help others avoid using the same approach. For those of us who work with valuable and unique samples, preventing unfruitful repetition is even more important. In line with the need for information sharing, our group plans to upload part of the data generated in the research for this thesis to a scientific data repository. An open access online archive will maximize the access of other researchers to our datasets, encouraging more efficient science and allowing verification. Uploaded data also functions as a remote backup, which will preserve the products of our work even when the primary data storage medium is unintentionally lost or damaged. The extensive Sundaland isotopic datasets we generated are ideally suited for sharing via a data repository, as I expect interpretations based on this data to benefit tremendously from further comparisons across taxa, faunal assemblages, geographical sites, and analytical techniques.

Just as it is important to appreciate the value of the original fossil specimens we use and the data generated from these specimens, we should also take care not to overlook the potential value of the sample material that is left over after having conducted our analyses. This lesson was impressed upon me particularly because it was how the most unique sample material analyzed in this thesis was obtained: powdered and fragmented *Homo erectus* bone samples, left over from fluorine and SEM analyses in the 1950's and 1970's. Instead of being discarded or forgotten in a university storage room, these samples were returned into the

museum collection, where our team found them over half a century later. They provided us with a unique research opportunity, at no further cost to the original specimens, and even now there remains enough material of these samples for future analyses. For the faunal specimens I sampled myself, too, I am left with small quantities of excess material, and these remains might very well have useful current and future applications. I want to make sure that these samples remain the subject of meticulous record keeping, and that, after our research on these samples draws to a close, they are returned to the museum collections. Who knows of what use they may be in half a century?

The rigorous and systematic record keeping that I stress here may appear to be nothing more than a rather drab and self-evident exercise. I admit that it is not one of the most inspiring and exciting aspects of this type of research, but I do think it is a fundamental necessity that all too often falls through the cracks during the day-to-day work of scientific research.

6.3 Paleovegetation and climate dynamics

Paleodietary reconstructions centered on herbivores are particularly vital in helping us to understand past ecosystems. Vegetation is an exceedingly important factor in many aspects of ecosystem functioning, as well as a highly sensitive indicator of environmental change. Another reason why herbivores (and in particular, bovids) have been one of the most utilized paleoenvironmental indicators in Quaternary settings is because their fossil teeth are a common find, at sites representing a wide range of paleoenvironments. When a tooth belongs to a still extant species which only lives in a certain habitat, it may be deduced that its occurrence in a fossil fauna assemblage indicates the presence of this same habitat type. Such inferences are more difficult to make in the case of a species that has a flexible diet or has gone extinct; here isotope analysis is one of the approaches that demonstrates its strong utility.

Our investigation into the Punung fauna is a good example of the nuance that $\delta^{13}C$ data can add to paleoenvironmental reconstruction. Based on faunal assemblage composition, the Punung fauna is a typical rainforest fauna. But our isotope results for Punung also identify one bovid (species undetermined) that has grazed exclusively on grass, suggesting either the nearby presence of grasslands, or alternatively that this tooth originated from another layer. Unfortunately I have so far only been able to analyze two bovids, one suid, and one tapir from Punung, leaving us with insufficient data to isotopically characterize the C_3/C_4 vegetation balance for this site, and underscoring that successful paleoenvironmental reconstruction using enamel carbon isotope compositions requires large data sets (>15 specimens per site).

But the isotope approach does not need to focus solely on the vegetational component of an ecosystem as seen through the eyes (or, more accurately, through the teeth) of its herbivores. We may also concentrate on a species in itself, because our understanding of its biology will ultimately contribute to understanding the system in which it thrives. Take the extinct bovid *Duboisia santeng*, which was found in various sites on Java as part of the Trinil Hauptknochenschicht (HK) and Kedung Brubus faunal assemblages. What does the presence of *D. santeng* in a particular paleoenvironment signify? Dental wear analysis by Rozzi et al. (2013) indicates "browsing feeding regime mostly typical for a forest dweller feeding on leaves, occasionally including abrasive vegetation to a minor extent." Our $\delta^{13}C$ analyses on *D. santeng* from Trinil paint a different picture: all three specimens are interpreted to have solely grazed on C_4 grasses. It would be very interesting to further explore the paleodiet of *D. santeng* by constructing a more extensive data set, comparing the results of dental analysis and isotopic analysis on the same specimens from a number of sites, to develop a better understanding of why they exhibit certain dietary preferences in certain habitats. More generally, it could be argued that integrating evidence from different paleodietary data sources ($\delta^{13}C$ composition, mesowear, microwear, ecomorphology, etc.) into a multi-proxy approach will produce more robust dietary interpretations. It must be said, however, that the risk of misinterpreting $\delta^{13}C$ data is much smaller when looking at diet in terms C_3 versus C_4 vegetation as opposed to diet in an all-C_3 ecosystem (as in Rawlence et al., 2016). Although it was of less concern in revealing inter-site vegetation contrasts, I would argue that when looking at specific species it is of importance to consistently sample M3 molars to be sure that the obtained values represent the adult diet; tooth enamel mineralized before weaning may have lower $\delta^{13}C$ values due to the fact that a large portion of this carbon is derived from lipids (DeNiro and Epstein, 1978).

One example of a species which I would suggest as the focus of such an exercise is *Muntiacus muntjak*, or the Indian muntjac. Modern-day Indian muntjacs feed on a diet of both C_3 and C_4 foodstuffs (Kitchener, 1990; Oka, 2000; Ilyas and Khan, 2003; Brodie and Brockelman, 2009). Two fossil specimens from the Wajak site I measured give $\delta^{13}C$ values of -4.2‰ and -9.8‰, compatible with a (quite flexible) mixed diet. However, I was not able to characterize the Wajak vegetation balance in Chapter 4 due to the small quantity of specimens available to us at the time, as well as doubt over whether all Wajak specimens did have the same age (and thus, whether they all originate from the same type of habitat). It is therefore unclear whether these muntjacs have a strong preference for a mixed C_3-C_4 diet, or if feeding behavior varies per environment. Will muntjacs always seek out C_3 sources, even in very open savanna habitats, or will their enamel composition shift towards higher $\delta^{13}C$ values in these types of environments? Because muntjacs have also been found in the Trinil HK layer, Kedung Brubus, and Punung faunal assemblages, we can directly check their feeding behavior in contrasting environments.

A group of animals that has so far been all but absent in our exploration of Sundaland ecosystems is that of the carnivores. These taxa will inevitably provide a weaker reflection of the vegetation balance in a certain paleohabitat, and as such are not very useful for characterizing it, as was one of the main objectives in Chapter 4. However, carnivores are a key element in ecosystems, even indirectly influencing the vegetation balance through controlling herbivore populations, and knowledge of the trophic relationships between species is crucial in understanding ecosystem functioning. Unfortunately, none of the isotope systems I have so far applied are particularly useful in demonstrating trophic relationships. Take for instance the Sundaland carnivores I have sampled, all originating from Trinil. The tiger (*Panthera tigris*) has a $\delta^{13}C$ value of -5.6‰. Carbon stable isotopes in animal soft tissues do show a trophic level effect, but tooth enamel does not (see Chapter 1, Fig. 4). Thus, the $\delta^{13}C$ value of tiger enamel only reflects the mix between vegetation types at the base of its food chain (in this case a mixed C_3/C_4 base): Its diet may have consisted of a mix of ungulate grazers and exclusive C_3 browsers (although I have not yet encountered Trinil taxa in the latter category in our isotope research, and one may question if enough C_3 vegetation is present for this scenario), but hypothetically could have also been based mainly on the omnivorous pig (or hominin!) population. The same goes for possible dietary interpretations regarding *Gavialis bengawanicus* (-6.6‰ $\delta^{13}C$) and *Crocodylus ossifragus* (-3.0‰ $\delta^{13}C$).

If we want to increase our understanding of the complex pathways in a paleo-foodweb, we must turn to other techniques. The most frequently used biogeochemical method used in modern-day ecosystem research and archaeological research is to look at nitrogen isotopes ($\delta^{15}N$) in collagen. In some cases, collagen as old as the Early Pleistocene has been found in reasonably good condition (e.g. Palmqvist et al., 2003; Buckley and Collins, 2011) but preservation of this protein can vary greatly from site to site. I consider it unlikely for collagen to have survived in the fossils of Early and Middle Pleistocene open-air sites on Java (e.g. Trinil, Sangiran), although the conditions for preservation have undoubtedly been better in the younger dry cave sites (Punung, Wajak, Hoekgrot). At any rate, it would be interesting to attempt collagen extraction on a small selection of samples. Other proxies that may contribute to our understanding of trophic relationships are Ba/Ca and Sr/Ca ratios (Elias et al., 1982; Sealy and Sillen, 1988; Burton et al., 1999; Blum et al., 2000), non-radiogenic strontium isotopes (Knudson et al., 2010), calcium isotopes (Heuser et al., 2011), and other non-traditional isotope systems (Jaouen et al., 2013; Martin et al., 2014). Finding ways to further explore the subject of dietary ecology in Pleistocene Java will be especially crucial if we wish to place the omnivore *Homo erectus* within the foodwebs of its ecosystem.

We have seen that faunal $\delta^{13}C$ values can be used to examine the feeding behavior of a certain taxon across sites, and that the aggregate of $\delta^{13}C$ values from all taxa at one particular site can be used to characterize its paleohabitat and the trophic relations (i.e. herbivorous

versus carnivorous) in its ecosystem. I have taken my interpretations one step further, and compared the general vegetation pattern between Sundaland sites of various ages and linking the observed contrasts to different climatic conditions. In Chapter 4 I hypothesize that the strong clustering in faunal $\delta^{13}C$ values (with almost no values indicating mixed feeding) is closely tied to the apparent abruptness of the transitions between glacial and interglacial climate states. In addition to contributing to the understanding of climate-driven ecosystem change in Sundaland, we have now created a framework to which data from additional sites may be compared.

In constructing and refining this framework of glacial cycle-driven vegetation change, the integration with other relevant proxies would be of great value. As Levin (2015) asserts, "proxies from fluvial, lacustrine, and marine records integrate data from different temporal and spatial scales and therefore provide different kinds of information about environmental and climate change." Unfortunately - and in stark contrast to that other paleo-anthropological hotspot, Africa - no $\delta^{13}C$ record from plant leaf waxes, soil organic matter, or pedogenic carbonates from Indonesia reaches back further than the most recent glacial period. Thus we cannot presently match our tooth enamel $\delta^{13}C$ records to other paleovegetation proxies, a comparison which would show how the herbivore $\delta^{13}C$ vegetation signal relates to the actual proportion of C_3 and C_4 vegetation available. For almost every fossil site we investigated, herbivores focused either on C_3 or C_4 vegetation: comparison with other paleovegetation proxies could determine if we were correct in our suggestion that this reflects the abruptness of the glacial-interglacial changeover in the vegetation balance. Pollen records from earlier glacial periods would allow us to ascertain that C_3 grasses have played a minimal role in the ecosystems of past glacial periods in Sundaland, something we are assuming based on their scarce occurrence in the last glacial period and in the tropics in general.

I think it is imperative that we look for additional proxies in order to more robustly reconstruct Sundaland glacial environments. Although the geographical and topographical situation in this region would have remained quite similar throughout glacial periods, we cannot be certain that subtle variations in climatic parameters (such as temperature, aridity, timing of the rainy season, atmospheric carbon dioxide pressure) and changes in ecosystem dynamics haven't resulted in environments that are significantly different from those in the last glacial period - especially with regard to C_4 grass abundance. Large herbivores and hominins are 'ecological engineers'; the extent to which they have shaped the landscape would not have remained constant as species compositions and population densities changed through time. Undoubtedly, recovery of older palynological, leaf wax or paleosol records from this region would represent the single biggest contribution to advancing our understanding of Sundaland glacial-interglacial climate dynamics.

6.4 Ranging patterns and migration

In Chapter 5 of this thesis, we applied strontium isotope ($^{87}Sr/^{86}Sr$) analysis to faunal specimens from five fossil localities on Java, Indonesia, revealing the types of substrate on which each animal ranged at the time that the sampled portion of enamel was being formed. We see that ungulates found at Hoekgrot and Wajak ranged predominantly on volcanic substrates, rather than on the limestone terrain located at close proximity to these sites. In contrast, Trinil and Sangiran ungulates predominantly ranged on limestone-rich substrates, as are present in the low-lying area north of both sites. The observation that C_4 feeders exhibit a broad variety of $^{87}Sr/^{86}Sr$ values also strengthens the interpretation put forward in Chapter 4, that the dominant C_4 signal in Trinil and Sangiran herbivore teeth represents the overall vegetation balance in this area. Could we further increase the resolution of our understanding by refining our methodology?

For instance, a considerable portion of samples featured in Chapter 4 of this thesis were taken from museum specimens which were too fragmented to identify tooth type (e.g. M2, M3) or species. This approach was well-suited to our stable isotope study: the fact that we did not need such detailed information to demonstrate large-scale vegetation contrasts enabled us, in conjunction with collection curators, to select a large sample set without unnecessarily submitting more complete specimens to destructive sampling. In a further bid for efficient sample use, we used the remains of a subset of these samples for Sr isotope analysis. Here, however, interpretations necessarily were less detailed as a result of incomplete taxon and tooth type information. For instance, if we know that a particular specimen belongs to the species *Muntiacus muntjak* we can expect that the represented range is relatively small and has not varied greatly between the wet and dry season. In contrast, larger deer species would have had a larger home range and may have migrated seasonally. Knowing to which species of deer a specimen belongs would increase our understanding of what its Sr isotope value represents. Knowing which exact tooth we've sampled constrains the period of life represented by the Sr isotope value. If research questions pertaining the ranging behavior of specific taxa were to arise, complete taxon and tooth type information would certainly be a primary requirement in our selection of specimens.

In comparing our faunal Sr isotope results to the substrate geology isoscapes we constructed, a major caveat is that the $^{87}Sr/^{86}Sr$ values of bioavailable Sr in a certain location are determined by more than just the bulk $^{87}Sr/^{86}Sr$ of the geological substrate underneath. Differences may be caused by contrasts in Sr concentration and weathering rates (Fritz et al., 1992; Blum et al., 1993; Capo et al., 1998; Bentley, 2006), as well as non-geologic sources (Graustein and Armstrong, 1983; Kennedy et al., 1998; Chadwick et al., 1999; Vitousek et al., 1999; Whipkey et al., 2000). In my view, it is unlikely that our provenance interpretations are

impacted by unexpected influences from the latter category. I don't expect the deposition of eolian dust on Java in significant amounts to offset bioavailable $^{87}Sr/^{86}Sr$ ratios, and in Chapter 5 we show that river water $^{87}Sr/^{86}Sr$ at Trinil and Sangiran is similar to expected local substrate $^{87}Sr/^{86}Sr$ values. We account separately for the possibility of an effect from sea spray. Given the dominant lithologies, very large differences in the Sr concentrations of substrate rocks and their weathering rates seem unlikely (see Capo et al., 1998; Bentley, 2006). I do think a better understanding of the possible influence of these factors would allow for increased accuracy and nuance in our comparisons. The most effective approach to increase our knowledge in this area would be to target modern bioavailable Sr directly by sampling soils and plants on a range of different substrate types (cf. Evans et al., 2009; Poszwa et al., 2009; Copeland et al., 2011; Maurer et al., 2012).This will also circumvent the inaccuracies introduced by our previous estimations of substrate $^{87}Sr/^{86}Sr$ values.

Many provenance studies measuring Sr or O isotopes perform serial sampling on the chronological 'time lines' of each tooth. As the bioapatite in tooth enamel is laid down progressively, high-resolution sampling of the full length of a bovid M3 molar promises to give detailed provenance information over a period of approximately a year, which would allow us to detect seasonal patterns (in the case of Sr, seasonal migration) and would potentially enable us to compare every individual animal over the same season. In fact, some low-resolution incremental Sr analyses on M2 and M3 molars from five bovids (one *Bubalus palaeokerabau* and four individuals not determined to species level) and one cervid (*Axis lydekkeri*) from Trinil has been performed by myself and by Jose Joordens, with 2-4 samples taken from over most of the length of the tooth. Intra-tooth variation proved to be on average 0.000258 $^{87}Sr/^{86}Sr$, and at the most 0.000502 $^{87}Sr/^{86}Sr$ (for one bovid specimen). When comparing this to an estimated substrate Sr isotope variability of ~0.0045 in the direct vicinity (<2 km) of the Trinil site, we must conclude that the observed intra-tooth Sr isotope variation is indeed very small. Does this indicate that these animals did not migrate within the represented time span?

As we've seen in section 4.5, it must be taken into account that the elements incorporated in enamel are drawn from a body pool that averages the ingested input signal over a certain time period (Montgomery et al., 2010). The situation is complicated further by uncertainties over whether enamel mineralization, the phase during which most of the Sr is actually incorporated, progresses via the same chronological time-lines in which the enamel matrix is deposited (Suga, 1982, 1989; Fisher and Fox, 1998; Hoppe et al., 2004; Tafforeau et al., 2007). It does appear possible to obtain chronological profiles from herbivore teeth for oxygen isotopes (Fricke and O'Neil, 1996; Fricke et al., 1998; Balasse et al., 2002; Zazzo et al., 2002), but animals whose enamel matures slowly - such as large bovids - may not be good recorders of the relatively short-term variability of seasonal isotopic changes (Fricke et al., 1998; Passey and Cerling, 2002; Kohn, 2004; Zazzo et al., 2005, 2006; Tafforeau et al., 2007).

Even if we take a very small and discrete sample from the M3 molar of a large bovid, the period over which carbon and oxygen were incorporated into this particular portion is ~6 months, and for strontium it may be more than 12 months (Montgomery et al., 2010). To summarize, the absence of significant Sr isotope variation in the bovid teeth may very well be expected, even if the Sr isotope signal of these animals' food and water has varied during its formation. For the much smaller *Axis lydekkeri* deer, strontium isotope profiles in teeth should be able to pick up changes in isotopic input at a higher temporal resolution, and the lack of variation in Sr isotope ratios may indeed indicate that this animal did not range on significantly different substrates throughout the year.

One 'negative conclusion' to be drawn from the Sr isotope research presented in this thesis is that it is not able to show regional-scale migration on Java. This is because in contrast to some other regions, there are large substrate $^{87}Sr/^{86}Sr$ variations relatively close to any locality in Java. If all high-$^{87}Sr/^{86}Sr$ substrates were located in a certain subregion of Java, and we'd find high-$^{87}Sr/^{86}Sr$ fossil teeth in another subregion, we could confidently interpret this as a sign that these animals migrated the considerable distance in between. But if high-$^{87}Sr/^{86}Sr$ substrates were scattered across every subregion, as is the case on Java (see Chapter 5, Fig. 2), we cannot distinguish if high values in animals that potentially migrate large distances are the cause of grazing on terrain 5 km or 500 km from the site where they have been found. If we want to test for regional-scale migration in such fossil remains, we will have to consider if other isotope systems (such as Pb, Nd) are better up to the task.

6.5 Isotope ratios as determined by a complex interplay of processes

Isotopically, ecosystems and animals display a considerable amount of heterogeneity. Large datasets are often a prerequisite for capturing some of the subtleties and complexities of the systems that determine isotope ratios in faunal tissues. Extending our grasp of these intricate systems is essential to optimize the application of the isotopic toolbox; more research into the underlying biochemical processes that affect stable isotopes is sorely needed.

From refining diagenetic screening and paleovegetation reconstructions, to uncovering trophic relationships and migration, we have seen that a single proxy can be inconclusive or misleading. CL microscopy shows us a different aspect of diagenesis than phosphate oxygen isotopes do. Taking long dives and having a carnivorous diet involve different isotope-modifying processes but can lead to the same isotope ratios. The ultimate solution is to expand the limited scope of a single isotope system by applying it in conjunction with other geochemical tools. In this chapter I have suggested a wide range of methods that

might be used in combination with enamel $\delta^{13}C$, $\delta^{18}O$, and $^{87}Sr/^{86}Sr$ data. In many instances, little work has yet been done on these methods, but it is clear that further exploration of multi-proxy approaches should have high priority in future work.

Quoting astronomer Carl Sagan, “nature is always more subtle, more intricate, more elegant than what we are able to imagine”. But as time capsules of less than a nanometer in size, atoms in fossilized remains have the potential to show us at least a glimpse of the world as it was millions of years ago. Reconstructing the dynamics of ancient ecosystems and climate ultimately allows us to better understand and predict global change and its consequences, a highly relevant feat in today’s world.