Using captive seabirds to assess knowledge gaps in stable isotope analysis of diets

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Isabel Andrea Micklem

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Abstract

Stable isotope (SI) ratios of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) are now widely used as biomarkers in ecological studies to provide information about food web structuring. However, understanding trophic relationships using SI analysis requires not only knowledge of SI values of predator and prey, but also accurate discrimination factors (DFs), which can differ among species and by physiological state.

This thesis examined three questions using captive birds from the South African Foundation for the Conservation of Coastal Birds (SANCCOB). First, the effects of ontogeny on $\delta^{13}C$ and $\delta^{15}N$ ratios of African penguins (*Spheniscus demersus*) were assessed. Blood samples were collected from penguins in four age classes (P3 chicks, blues, juveniles and adults) concurrently with their diet (sardine (*Sardinops sagax*) and formula). Second, to assess the influence of breeding physiology on SI ratios, the blood of ten breeding pairs of penguins was sampled over a five-month period from June to October 2016. Following laying, each pair was categorised into one of three (four for whole blood) egg production phases (initial yolk deposition, rapid yolk deposition and post-laying) and their influences on SI ratios were tested. Third, species differences in DFs were evaluated for African penguins, kelp and Hartlaub's gulls (*Larus dominicanus* and *L. hartlaubii*), greater crested terns (*Thalasseus bergii*) and Cape cormorants (*Phalacrocorax capensis*). Flying birds were mostly fed sardine with a small but unknown amount of sardinella (*Sardinella aurita*), DFs were therefore estimated for a 50:50 sardine:sardinella diet, a 75:25 sardine:sardinella diet and a 100% sardine diet for each flying bird species. The DFs were assessed for the whole blood (WB), red blood cells (RBC), plasma (PL) and delipidated plasma of the penguins, and only WB for the flying birds as well as flesh, whole fish, delipidated flesh and delipidated whole fish for fish species, and for formula.

Results indicated that age influenced both the $\delta^{13}C$ and $\delta^{15}N$ of WB, only the $\delta^{15}N$ of RBC and the $\delta^{13}C$ of delipidated PL. The assessment of breeding physiology yielded a significant interaction between the effects of egg production phase and sex on the $\delta^{13}C$ of WB; females had significantly lower $\delta^{13}C$ in the rapid yolk deposition phase than the other two phases and all males. The $\delta^{13}C$ of PL was affected only by sex, with females having a significantly lower $\delta^{13}C$ value than males. Neither physiological state nor sex influenced the other blood components.

Differences were found among the three DFs in the non-penguin species, but not for all consumer – prey tissue combinations. There were also significant differences among species with a DF calculated from a diet with the most probable prey proportions eaten. Depending on the combination of consumer and prey tissue used to calculate the DF, a different conclusion regarding trophic information can be reached. A literature review updated with the present data showed that no

general pattern or grouping of similar species with regards to DF values could be drawn, highlighting the importance of determining species- and tissue-specific DFs.

Thus age, egg production, tissue and species all influenced the SI values of bird blood and therefore their DFs. Not all physiological conditions affect all blood components in the same way, making different components more or less sensitive to physiological influences. Though their influence is at a small enough scale that it is unlikely to hamper correct conclusion in ecological studies, it is crucial that these factors are considered when using SI analysis (SIA). When uncertainties exist for some coefficients in wild studies, SIA should therefore be combined to other dietary techniques to determine the food web structure as best as possible.

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Table of Acronyms

Acronyms	Full words	symbols
ANOVA	analysis of variance	
ANCOVA	Analysis of covariance	
C	carbon	
CRU	chick rearing unit	
DF	discrimination factor	Δ
DDDF	diet dependent discrimination factor	
EP	egg production	
EPP	egg production phase	
FL	flesh	
GLM	general linear model	
N	nitrogen	
PL	plasma	
RBC	red blood cells	
SANCCOB SF	the South African Foundation for the Conservation of Coastal Birds Cape St Francis	
SANCCOB TV	the South African Foundation for the Conservation of Coastal Birds Table View	
SI	stable isotope	δ
SIA	stable isotope analysis	
WB	whole blood	
WF	whole fish	

Chapter 1 General introduction

Ecosystems provide "services" necessary for human life. These include the sequestering of carbon dioxide and production of oxygen, the provisioning of food resources, and more (Fisher et al. 2009). Marine ecosystems collectively produce approximately half of the total oxygen in the atmosphere (Emerson et al. 1991). The oceans are not in a good state of health (Doney et al. 2012). Our oceans are constantly being plundered to satisfy the appetite of fisheries, oil companies, transporters, and many other industries making use of the ocean. The extent to which humans are utilising the ocean is ever increasing (Halpern et al. 2008). This has led to a decline in the health of our oceans (Jackson et al. 2001). This decline in health is seen in the collapse of fisheries (Lotze et al. 2006, Lotze & Worm 2009), the increase in plastic in the ocean (Robards et al. 1995, Derraik 2002, Ellen MacArthur Foundation 2016), the increase in dead zones (Diaz & Rosenberg 2008), and the decrease in the populations of many seabird species (Croxall et al. 2012). Though ecosystems will continue to function, through the filling of empty niches by replacement species, they may change in ways that are not beneficial to humans.

Thus, to prevent altering ecosystems to the point where they are no longer suitable for human use, they need to be managed sustainably. Fish are a highly utilised resource of the ocean, but if sustainable fishing practices are not implemented, fisheries can crash as seen in the often-cited example of the cod fisheries along Georges Bank (Jackson et al. 2001). In this case, the trophic level of the cod was taken over by dogfish which are not as economically valuable as the cod were. Once the niche has been filled by a replacement species, it is often very difficult for the ecosystem to revert to the earlier conditions. This means that the situation of the utilised stock needs to be monitored to put the best management practices in place to prevent a collapse, which could arise in an undesired stock shift.

It is because of the importance of ecosystems that management strategies to maintain ecosystems are implemented (Brussard et al. 1998). To employ management strategies which achieve the desired outcome, the ecology of the system being conserved needs to be well understood (Botsford et al. 1997, Murawski et al. 2010). This has promoted the development of various methods for monitoring ecosystems or specific populations. Some of these include looking at past catch rates and trying to extrapolate relationships between catch rate and fish stock, looking at the population size of other species utilising a specific stock as well as monitoring other processes which may give an indication of the state of an ecosystem. Another method implemented for monitoring ecosystems, is

the use of indicator species. When changes in the environment occur, organisms respond to the change and their effects filter through the food web.

1.1 Indicator species

The more information that is accumulated about various ecosystems the more we appreciate their interconnectivity (Pace et al. 1999). This has led to the idea that to assess the health of an environment, an indicator species could be monitored to provide insight into the functioning of an ecosystem. Indicator species act either as early warning systems or as indicators of specific disturbances (Caro & O'Doherty 1999, Frederiksen et al. 2007, Parsons et al. 2008). Organisms which are often considered ideal as indicator species, are those which change their behaviour when environmental conditions change as well as those influenced by changes in the ecosystem in a measurable way (Carignan & Villard 2002).

The indicator species and the changes to be monitored need to be carefully chosen to provide timely information for management (Carignan & Villard 2002). It would be ineffective to look at long term changes (versus short term changes) in a population when trying to implement short-term management strategies. For example, looking at long-term changes in the number of birds within a population while trying to manage annual quotas for fishing could result in the implementation of an ineffective management strategy (Smith et al. 2011). The use of indicator species to assess the status of an ecosystem has some caveats but due to logistical constraints this is still generally the most feasible way of monitoring an ecosystem (Carignan & Villard 2002), especially marine environments which are often less accessible or less easily observed than terrestrial environments.

Some species possess life history traits which make them good to use as indicator species, while others do not (Carignan & Villard 2002). Some parasites are considered very good indicators of changing environments (MacKenzie 1999). Helminths, for instance, have complex life cycles, which at various times are sensitive to multiple factors, subjecting their populations to multiple influences which could decrease their numbers (MacKenzie 1999). Another group considered as good indicators are top predators as they can be directly influenced by prey availability (Einoder 2009). They often rely heavily on the same food source as humans, therefore, subject to anthropogenic influence. A single universal taxon which reflects all changes, or points to specific causes, does not exist. In some ecosystems or biomes, a range of taxa may be used to monitor a specific change, while in others it may be possible to detect a range of changes by monitoring a few populations of one taxon (Jackson et al. 2001, Mallory et al. 2010, Croxall et al. 2012).

Top predators have often been used as proxies for changes in the environment as they can be easier to monitor than other organisms, and because of the way they respond to prey availability (Einoder

2009). Seabirds are considered credible indicator species which can be used for assessing the health of an ecosystem because they represent the top of the food chain and because all seabird species come to land to reproduce, making them accessible (Caro & O'Doherty 1999, Carignan & Villard 2002, Parsons et al. 2008, Einoder 2009). By assessing breeding success, the duration of the foraging trip, the diet, and many other variables, the seabird population in question can be used to determine the productivity and potential prey availability shifts of the ocean system the birds are utilising (Parsons et al. 2008).

Harding et al. (2005) have complied a list of studies which used seabirds as indicator species to monitor various changes in the marine environment. Some examples include the number of seabird carcasses found along a beach showing a decline in oil pollution (Camphuysen 1998), reproductive success indicating short and long term changes in the environment (Hunt et al. 1996, Kitaysky & Golubova 2000, Gaston & Smith 2001, Sydeman et al. 2001, Gjerdrum et al. 2003, Gaston et al. 2005, Doney et al. 2012, Kowalczyk et al. 2014) or prey utilisation (Rindorf et al. 2000, Hennicke & Culik 2005), and diet indicating shifts in the food web or prey availability (Montevecchi & Myers 1996, Croxall et al. 1999, Barrett 2002, Gaston et al. 2003) or the effects of climate change (Hunt et al. 1996, Hedd et al. 2002, Pinaud et al. 2005).

Different variables are influenced by the environment over different time scales. It is therefore very important to choose the most applicable variable for the time scale for which information is needed. Many seabirds are long lived and do not breed in every season, thereby potentially skewing population counts. Using population turnover or fluctuations may not yield useful information about changes in the fish-stock on an annual basis (Lack 1967 and Wein 1989 in Montevecchi 1993, Parsons et al. 2008). Changes in feeding behaviour, foraging area, trip duration and/or diet are more likely to provide information over a briefer time scale (Velarde et al. 1994, Einoder 2009). The diet of indicator species is often monitored as it gives an indication of where and on what birds feed (Barrett et al. 2007). If certain prey items are scarce, the more generalist seabird species quickly change to other prey items, while other, more specialised foragers are likely to extend their foraging range to ensure they gather enough preferred prey (Blight et al. 2015). Knowing the area or range birds use, may allow better management practices to be implemented. The protection of areas which are high in biodiversity or are utilised by many species or individuals has been shown to be beneficial for population conservation (Hooker & Gerber 2004, Piatt & Sydeman 2007, Croxall et al. 2012).

Considering birds diets can sometimes provide information about where the birds are foraging and can give an indication of prey availability or stocks (Montevecchi & Myers 1996, Barrett 2002, Bunce

2004). Different techniques of divulging dietary information have been used over the years including shooting birds to dissect their stomach contents, observing the food being carried in the birds' beak, and looking at the regurgitated hard parts (Duffy & Jackson 1986, Barrett et al. 2007). In more recent years, biochemical techniques have been utilised more regularly, as they are often less invasive and can be repeated over short time periods without causing harm to the individual or population (Bond & Jones 2009). Such approaches include analysing the DNA of prey in guano, looking at the stable isotope (SI) ratios of $^{13}C/^{12}C$ and $^{15}N/^{14}N$ in various tissues, as well as looking at the fatty acid (FA) profiles of a range of tissues (Barrett et al. 2007, Cherel & Hobson 2007). For this study, seabirds were used to assess the effect of physiology on SI values as well as to increase our knowledge of the difference between predator and prey tissue – the discrimination factor (DF).

1.2 Diet studies in marine predators

An aspect of ecosystem dynamics which is studied to gain more understanding on the ecosystem is trophic relationships. To assess trophic relationships, diet studies are often conducted to make more accurate inferences about the food web, including where and on what organisms are feeding (Montevecchi & Myers 1996, Barrett 2002, Bunce 2004). Many techniques of diet analysis exist, with some methods working better for some organisms or in specific environments. All investigative techniques have positive and negative qualities, some of which may involve long lasting effects. In the past, collection of data on feeding ecology was conducted by very destructive methods, the most common being the killing of animals to glean a wide range of data (Duffy & Jackson 1986). Killing animals is in most cases no longer deemed ethical or necessary. This is especially true if the organisms are experiencing, or are at risk of, population declines, and so less destructive methods of gleaning useful information are being implemented (Barrett et al. 2007).

A good example of progression away from destructive methods of diet analysis is stomach flushing. This technique was developed to negate the need to kill birds to determine diet while still yielding the desired information. Stomach flushing works by inserting a tube down the bird's throat into its stomach and pumping water into the stomach so that its contents are displaced when the bird is held upside-down (Wilson 1984). Using only stomach contents in diet studies is, however, biased against quickly digested prey and only shows a limited number of hours of information (Jackson 1992, Barrett et al. 2007). Stomach flushing in penguins, for instance, severely underestimates the value of gelatinous prey while only yielding information of the last foraging trip (Hyslop 1980). Causing an animal to regurgitate means that the animal or its offspring does not reap the benefits of its sampling trip. This could have negative consequences on the population, especially if the technique is implemented over a large sample size and for an extended period, especially during a year of low food availability (Stein et al. 2017). To overcome only seeing a short window of the diet,

other techniques which yield information about longer periods of foraging are utilised either on their own or in combination with others. In more recent years, biochemical techniques, such as stable isotope analysis (SIA), fatty acid signature analysis (FASA) or DNA assessment of faeces, have been utilised more regularly, as they are generally less invasive and can often be repeated over short time periods without causing harm to the individual or population (Pasquaud et al. 2007, Bond & Jones 2009).

Before using a diet analysis technique, its benefits and limitations should be understood (Barrett et al. 2007). Gathering scats for DNA analysis can be simple, non-invasive, and can give information about the recent diet of an animal (Jarman et al. 2013). This can be useful for species that are monitored continually. A limitation, however, is that a database of potential prey DNA needs to be available as a reference for DNA found in scats (Jarman et al. 2013). FA make up most of the lipids found in organisms and can be characteristic to species, making them traceable through a food chain (Barrett et al. 2007, Karnovsky et al. 2012). They are passed through the food web, often unaltered or by a 'predicted' alteration, and can therefore be used as trophic markers (Dalsgaard et al. 2003, Wang et al. 2010). It is necessary, however, to know some information about the prey species and their FA because the FA signature of the consumer is often complex and integrates multiple species. It is a good technique to use together with other trophic markers such as SIs.

1.3 Stable isotopes

1.3.1 Introduction

Elements occur in various natural forms called isotopes. Isotopes are derived from variations in the number of neutrons present in the nucleus of the atom (Michener & Lajtha 2007). This affects the weight of the atom and results in heavier and lighter isotopes of the same element. Because lighter molecules require less energy in chemical reactions, they are processed slightly faster than heavier molecules (Michener & Lajtha 2007). This results in a change in the ratio of heavy to light isotopes within substances. This ratio of heavy to light isotopes of elements can be measured using isotope ratio mass spectrometry (Michener & Lajtha 2007). Comparing this ratio to the global reference ratio for the same element gives a SI value which is denoted by δ in parts per thousand (‰) relative to the standard (Bond & Hobson 2012). Using the SI values, various inferences about the assessed substance can be made, as discussed below (Craig 1953, Ehleringer et al. 1986, Wolen 1986, Peterson & Fry 1987, Fry 2006, Michener & Lajtha 2007).

1.3.2 History and uses

The idea that elements can appear in different forms with the same properties, was first hypothesised by Frederick Soddy. He first introduced the term "isotope" in 1913 and went on to be

awarded the 1921 Nobel Prize in Chemistry for his "investigation into the origin and nature of isotopes". Francis W Ashton built a mass spectrometer in 1919 which allowed the detection of multiple isotopes of neon. He was later awarded a Nobel Prize in Chemistry in 1922, after discovering that multiple elements, not just neon, have isotopes (Fry 2006). In the past, the analysis of SIs has been limited by the technology and the cost of running analyses. This is also confounded by the need for different machinery to assess the SI values of lighter elements such as hydrogen, oxygen, nitrogen and carbon compared to heavier elements such as potassium, calcium and manganese (Michener & Lajtha 2007). Assessing the heavy to light SI ratios of elements within a substance is termed stable isotope analysis (SIA) and this technique has been used widely from c. 1970s (Ehleringer & Rundel 1989, Michener & Lajtha 2007).

SIA has been used in many fields of study such as palaeontology, pharmaceuticals, archaeology and ecology (Wolen 1986, Schramm 1991, Fry 2006, Schellekens et al. 2011, Meier-Augenstein et al. 2013). Different elements are used in different fields as each element has properties that yield different information about the substance assessed. Lead, for example, is used in archaeology to identify ancient trade routes (Shortland 2006), while boron and sulphur can be used to determine the source of pollution (International Atomic Energy Agency 1998, Han et al. 2016). In the biological realm, SIA has become widely used because of its versatility and ease of use.

In ecology, SIA was initially used as a biomarking technique along with other chemical markers (Garatun-Tjeldstø et al. 2006). Water containing the heavy isotopes of one hydrogen atom and of oxygen ($^{2}H_{2}{}^{18}O$), called doubly labelled water, was injected into a bird so that its water pool was labelled (Nagy 1980). The assumption that the ^{2}H would only leave the body via water excretion and that ^{18}O would leave the body by water and carbon dioxide (CO_2), was used to calculate the rate of CO_2 exhaled. The turnover of the doubly labelled water and the respiration rate were then used to calculate the amount of energy the animal expended. This allowed scientists to develop theories about how birds allocate their energy budget with regards to various behaviours, and which prey would be more beneficial and likely for different life stages (Kooyman et al. 1982, Klaassen et al. 1989). The problem with this assumption was that it didn't account for the potential incorporation of either isotope, ^{2}H or ^{18}O, into tissues, as may have been the case with growing chicks (Weathers & Sullivan 1991). The technique of marking the water pool with doubly labelled water was later used to determine the degree to which marine birds rely on salt water (Green & Gales 1990). The functioning of the nasal salt glands was fairly well understood before this technique was used, but since seabirds also eat prey with high salt loads, a better picture of the osmoregulation of seabirds was gained by using doubly-labelled water (Green & Brothers 1989). More and more the use of natural biomarkers is replacing chemically labelled molecules to answer questions about physiology.

1.3.3 Uses in trophic studies

Stable isotope analysis has been used by plant physiologists for many decades to gather information on the global oxygen cycle (Guy et al. 1993) as well as to differentiate between C_3, C_4 and CAM plants (Farquhar et al. 1989, Alonso-Cantabrana & Von Caemmerer 2016). The ability to differentiate between different primary producers led to the idea that because plants are at the base of food chains, and nutrients move up the food chain, assessing the SI value of consumers would provide insight into their diet (DeNiro & Epstein 1978, 1981). Carbon (C) and nitrogen (N) together are the chemical elements that are most commonly used for diet analysis. They are used to determine source of production, as well as to identify potential foraging areas and trophic level respectively (Mizutani et al. 1991).

The SIs of C exhibit very distinct broad spatial patterns, so $\delta^{13}C$ values can be associated with different locations and habitats, such as fresh water, marine or terrestrial, and pelagic versus benthic in the marine setting (Hobson 1999, Magozzi et al. 2017). Within an ecosystem, N moves up trophic levels as proteins are assimilated into tissue from food (DeNiro & Epstein 1978, 1981). The SI value of N increases predictably by 2 – 5 ‰ per trophic level because of the biological processes involved in nutrient routing (Minagawa & Wada 1984, Vander Zanden & Rasmussen 1999, Post 2002, Vanderklift & Ponsard 2003). Though there is a general increase in $\delta^{15}N$ of 2 – 5 ‰ per trophic level, to accurately infer specifics of a diet from the $\delta^{15}N$ of a consumer, the exact difference between predator and prey needs to be known as does information about factors which may influence the results (Bond & Jones 2009).

1.3.3.1 Influence of physiology on SI values

Some of the mechanistic sources of variation in the SI values of tissues are shaped by physiology, because of the processes involved in nutrient assimilation, use and/or storage (Boecklen et al. 2011). Some processes may preferentially take up lighter molecules while other processes preferentially remove lighter molecules, resulting in variation of SI values that depends on the physiological condition of an organism (Michener & Lajtha 2007). For example, important factors include the method of nitrogenous waste excretion, body size, isotopic routing, diet and more (Fry & Arnold 1982, Schwarcz 1991, Bearhop et al. 2000, Vanderklift & Ponsard 2003). Many studies and reviews have been done to try to obtain a clearer understanding of which physiological conditions and to which degree influence SI values (Fry & Arnold 1982, Bearhop et al. 2000, Cherel et al. 2005a, Boecklen et al. 2011). The $\delta^{15}N$ value may, for example, be affected by numerous conditions such as food limitation, waste excretion mechanism and growth, while the $\delta^{13}C$ can be influenced by lipid and lipoprotein contents in the tissue and/or diet (Vanderklift & Ponsard 2003, Post et al. 2007).

Food limitation is a physiological condition many wild species experience and its effects have been questioned in numerous studies. Penguins, for example, remain ashore and do not eat for the entire period of moulting, but instead rely on endogenous reserves. Birds then start utilising the proteins of their muscles to maintain the critical physiological pathways. This is thought to cause an increase in the $\delta^{15}N$ value due to the preferential excretion of lighter ^{14}N molecules (Hobson & Clark 1992a, Cherel et al. 2005a). Hobson and Clark (1993) go on to show that this is the case in starving (to the point of no growth) chicks and fasting birds. Williams et al. (2007) and Kempster et al. (2007), however, did not find the same results when subjecting birds to nutritional stress of 50-55% reduction and 35% reduction in food intake (in the respective studies) which would naturally occur during rearing.

Another physiological condition which may affect the $\delta^{15}N$ value is growth and nitrogenous waste excretion. The catabolism of proteins during growth produces uric acid as waste which is then transported in the blood for excretion (Marieb 2009). Bearhop et al. (2000) show that the uric acid levels in the birds' blood may decrease the final $\delta^{15}N$ results as lighter molecules are catabolised first. Excretion of nitrogenous waste will also remove lighter molecules first and could in turn, depending on the mechanisms of excretion, increase the $\delta^{15}N$ values of specific tissues (Vanderklift & Ponsard 2003). This combination of potential physiological effects on $\delta^{15}N$ values, shows how important it is to understand the physiological condition of an organism when interpreting SI values.

While pregnancy in mammals resembles the physiology of growth (Fuller et al. 2004), this is not seen in birds producing eggs. Instead birds need to deposit a yolk filled with lipids and proteins (Burley & Vadehra 1989a). Lipids are rich in carbon-based molecules which are depleted in ^{13}C (McConnaughey & McRoy 1979) and are produced in the liver and transported by the blood to the ovaries where the lipids are deposited into the maturing oocytes (Burley & Vadehra 1989a, b). If the $\delta^{13}C$ of tissue with higher lipid contents, such as liver or blood during egg formation, is assessed, the $\delta^{13}C$ result may be artificially lowered. The true $\delta^{13}C$ value of the tissue may be masked by that of the lipids (DeNiro & Epstein 1977).

1.3.3.2 Tissue turnover

The increase of SI values between trophic levels arises due to the way nutrients are assimilated into tissue from digestion. The time it takes for all the cells of a specific tissue to be replaced by the body is the turnover time (Vander Zanden et al. 2015). While some tissue types, like liver and plasma (PL), exhibit rapid cell division for maintenance, other tissue types, like bone, take a much longer time to turnover during cell maintenance (Schoeller 1999). This difference in turnover time means that tissues will reflect shorter or longer periods of the organism's diet (DeNiro & Epstein 1978). Thus,

assessing the SI values of various tissues from the same individual, can add temporal information to the dietary knowledge.

A comprehensive understanding of the turnover rate of different tissues is best accomplished by controlled feeding experiments. Many studies on captive seabirds have been conducted to assess the turnover times of various tissues (Bearhop et al. 2002, Evans Ogden et al. 2004, Madigan et al. 2012, Barquete et al. 2013). The way turnover is presented is in days for half-life, which is the number of days that half the tissue reflects the changed diet (Vander Zanden et al. 2015). A shorter half-life indicates a faster turnover of tissue and reflects a shorter period of the past diet, while a longer half-life, with a slower tissue turnover gives an indication of an extended period of the past diet (Ambrose & DeNiro 1986). Some tissue types will also not reflect all dietary shifts as certain molecules are not utilised in all tissues, resulting in an SI value which has little variation (Gannes et al. 1997). It is important to consider the half-life of the tissues selected for studies to ensure the period of diet incorporated into the tissue is known.

A general trend of tissue turnover that seems to be shown in the literature, is that the tissue with the shortest half-life is PL, followed by liver, blood, and muscle with the longest (Tieszen et al. 1983, Hobson & Clark 1993, Hobson & Bairlein 2003, Evans Ogden et al. 2004). Bone collagen is also sometimes sampled, especially in organisms which have died or which are excavated (DeNiro & Epstein 1981). Because bone collagen has a very slow turnover that often reflects the organisms' general diet over its whole life (Ambrose & DeNiro 1986, Kelly 2000). Diet shifts over a few weeks are not expected to be accurately reflected in tissue with turnover longer than that, such as penguin claws, though with improving technology, methods and understanding of analysis, this may become possible (Barquete et al. 2013, Phillips et al. 2014). Though controlled feeding studies are the easiest way of determining turnover, it is not always possible. Thomas & Crowther (2015) and Martínez del Rio et al. (2009b) compiled many of these studies and have designed methods to enable the estimation of turnover times of various tissues of various species, based on the size of the organism as well as its thermoregulation strategy. This will aid interpretation of SI values especially where controlled studies have not been conducted. To utilise the potential of SIA, a sound understanding of the physiological processes influencing nutrient assimilation is important.

1.3.3.3 Discrimination factors

The increase in the SI value from prey to consumer, or the difference in the SI value between a predator and its prey, is called the discrimination factor (DF - sometimes incorrectly referred to as fractionation value) and is represented by the symbol Δ (Bond & Hobson 2012). The DF is used to determine what a consumer is feeding on by calculating down the food chain. Knowledge of the DF

is essential for accurate dietary inferences especially when models are used (Bond & Jones 2009, Karnovsky et al. 2012). Unfortunately, there isn't one DF which fits all taxa and even where a DF is known for a tissue of a species there may be factors which can influence the DF (Hobson et al. 1993, Bearhop et al. 2000, Cherel et al. 2005b, Caut et al. 2009, Boecklen et al. 2011).

Through studies determining the DF, it has been found that not only does the DF differ among tissues within an organism, but also among organisms with varying physiological conditions. Several physiological processes affect the assimilation/excretion, and therefore the representation of SI in various tissues, influencing the DF. Boecklen et al. (2011), Caut et al. (2009) and Vanderklift & Ponsard (2003), amongst others, have compiled studies to determine which factors influence SI values the most and whether any patterns could be determined. This aids in the calculation of the DF for species where it is not known, as without DF, trophic levels and the location of prey cannot be determined accurately. One of the most straightforward ways of determining the DF for a species is to hold captive birds on a constant diet for which the SI values are known. Organisms need to remain on the known diet for the length of time it takes for the tested tissue to completely reflect the new diet, and then a comparison between predator and prey SI values can be made. This has been done for several seabirds amongst other species (Hobson & Clark 1992a, Cherel et al. 2005b, Craig et al. 2015). It is however not possible to test the DF of all species and so inferences about the DF are usually made based on the known DF of closely related species or by models (Cherel et al. 2005b, Robbins et al. 2005, Caut et al. 2009, Kurle et al. 2014, McMahon et al. 2015).

1.4 Aims of the study

Seabirds are important in the functioning of the ecosystems they are part of. They not only feed at the end of many food chains, but often utilise varying trophic levels. Without top predators in place to maintain ecosystems, trophic cascades could be a result (Heithaus et al. 2008). For example, a release of mesoconsumers from predation puts more pressure on the already exploited lower trophic levels, as seen in the often-cited example of declining numbers of sea otters (*Enhydra lutris* Linnaeus, 1758) resulting in sea urchins thriving, which in turn decimated kelp forests along the Alaskan coast (Estes & Palmisano 1974, Estes et al. 1998, Jackson et al. 2001). SIA is widely used in trophic ecology studies on seabirds. It allows for the assessment of diet over periods when birds are not accessible for sampling because of the varying turnover times of different tissues (Michener & Lajtha 2007). In studies using SIA to assess seabird diets, it is difficult to draw correct conclusions if there is a lack of information on the DFs and factors influencing the SI values (Bond & Jones 2009). Without this information, assumptions need to be made when using SIA for diet studies (Gannes et al. 1997).

The easiest way to increase knowledge and understanding of seabird SI values and DF, is to work with rehabilitation centres, aquariums or zoos, as organisms are kept in controlled environments and the SI value of the exact species and batch of food fed can be measured. It is important to utilise birds held in captivity to conduct studies that cannot be done as effectively in the wild due to the many unknown variables under field conditions. This study will focus on two areas of study: 1) whether the ontogenetic stage and breeding phase influence the $\delta^{13}C$ and $\delta^{15}N$ in the whole blood (WB), red blood cells (RBC) and PL of African penguins (*Spheniscus demersus* Linnaeus, 1758) residing in captivity. And 2) the DF of four seabird species namely: Cape cormorants (*Phalacrocorax capensis* Sparrman, 1789), kelp gulls (*Larus dominicanus* Lichtenstein, 1823), Hartlaub's gulls (*Larus hartlaubii* Bruch, 1853) and greater crested terns (*Thalasseus bergii* Lichtenstein, 1823), will be considered and added to the list of known DFs published from controlled studies. Chapter two will focus on the study species and methodology implemented, chapter three will consider how physiology affects the SI values, chapter four will consider the DF of various species, and this thesis will be closed with a brief synopsis of the study in chapter five.

Chapter 2 Study Species and Methods

2.1 Ethics Clearance

This work was conducted with the approval of the Rhodes University Animal Ethics Subcommittee (RU-LAD-16-03-0002). All blood sampling procedures followed, as well as the frequency of sampling permanently captive birds, were approved by Nicky Stander, the Bird Rehabilitation Manager of the Southern African Foundation for the Conservation of Coastal Birds (SANCCOB; Table View and Cape St Francis facilities). The species selected for this study were: African penguins (*Spheniscus demersus* Linnaeus, 1758), Cape cormorants (*Phalacrocorax capensis* Sparrman, 1789), kelp gulls (*Larus dominicanus* Lichtenstein, 1823), Hartlaub's gulls (*Larus hartlaubii* Bruch, 1853 also sometimes classified as *Chroicocephalus hartlaubii;* del Hoyo et al. 2014, Avibase 2003) and greater crested terns (*Thalasseus bergii* Lichtenstein, 1823). Selection of species was based on a few reasons. The first was the IUCN rating of some species such as the African penguin and the Cape cormorant, which are both classified as endangered, making them a conservation concern (BirdLife International 2016a, b). The second was that most species are experiencing population declines while a few, such as gulls and greater crested terns are not which may be linked to human activity. This captive study will provide parameters necessary for studies conducted in the wild to draw accurate conclusions. The handling time of each bird during sampling was kept to a minimum (most under 7 min, always under 15 min).

2.2 Study species

2.2.1 African penguins

African penguins are endemic to southern Africa and have been classified as endangered since 2010 due to the rapid decline of their populations (BirdLife International 2016a). They breed at 28 colonies from the south coast of South Africa up to the Namibian coast (Sherley et al. 2014). Breeding colonies are often on islands within 100 km of the coastline, and the eastern most colonies are found in Algoa bay on St Croix and Bird Islands. Though there are differences between colonies, generally two breeding peaks can be seen, a major one in the austral summer months and a minor one in the winter (Wolfaardt et al. 2008). Breeding is not entirely limited to these two times of year. Some pairs breed throughout the year, but this is more common in birds with failed clutches (Crawford et al. 1999). The average clutch size is two eggs and the eggs are incubated for 36-42 days though the fledgling success rate ranges from 0.32 – 0.77 per annum (Crawford et al. 1999, AZA Penguin Taxon Advisory Group 2014, BirdLife International 2016a, Sherley et al. 2018). Penguin pairs work together during incubation and the feeding of their chicks (De Roy et al. 2013). During

breeding, the parents' foraging distance shortens, as they need to return to the nest daily to feed their chicks, but foraging distance extends once the chicks can be left unattended for the day or have fledged (Davis & Darby 1990). Chicks take 60 – 100 days to reach fledgling age, by which time they have lost all their down and developed blue feathers which are waterproof (Seddon & van Heezik 1993). Due to the hue of their feather, these birds are often referred to as blues (Pichegru et al. 2013). They leave the colony and generally travel as far as the west coast of South Africa and Namibia to feed in the Benguela Current (Sherley et al. 2017). Their main diet is small pelagic fish, but they also feed on cephalopods (Randall & Randall 1986, Connan et al. 2016). After a year or two the birds, now called juveniles, return to land for their first moult, from browned fledgling feathers into the adult black and white plumage. The adults reach sexual maturity between ages three – five years and often return to their natal colony to find a mate, with which they often remain over multiple breeding seasons (Crawford et al. 2013). The average weight of an adult African penguin is 3-5 kg, with the females being slightly smaller than males (Sherley et al. 2018). Though there is a big overlap in female and male size range, within a breeding pair the male is almost always bigger than the female (Campbell et al. 2016).

2.2.2 Cape cormorants

Cape cormorants are also endemic to southern Africa but are found over a larger range than African penguins (Hockey et al. 2005). During the non-breeding season, they have been recorded up the east coast of Africa into Mozambican waters, while their eastern breeding limit is in Algoa Bay on the South African south coast and extends west around the African coast into southern Angola (Kemper & Simmons 2015). The species is not sexually dimorphic and the average adult weight is 1.1 kg (Sinclair et al. 2014a). Historically they have been important in guano harvesting, so much so that wooden platforms were erected along the Namibian coast for the birds to roost and nest, after which the guano could easily be collected (Crawford et al. 2007). Their main food sources are small pelagic fish such as anchovy (*Engraulis capensis* Gilchrist, 1913), sardine (*Sardinops sagax* Jenyns, 1842) and more recently the pelagic (bearded) goby (*Sufflogobius bibarbatus* von Bonde, 1923) (Kemper & Simmons 2015). With a decline in small pelagic fish stocks, a decline in the breeding population of Cape cormorants has followed (Crawford 1991, Crawford et al. 2007). These birds were classified as endangered in 2014 by IUCN and are therefore likely to be studied and protected further (van Zyl 2004, BirdLife International 2016b).

2.2.3 Greater crested terns

Greater crested terns are not endemic to southern Africa and are found along the coast of South Africa, up the east coast of Africa, along the Indian coast, throughout the Indonesian islands and throughout the coastal regions of Australia, though there is a subspecies in southern Africa often

referred to as the swift tern (*Thalasseus bergii bergii*; BirdLife International 2016c). Greater crested terns typically weigh between 300 – 400 g and feed by plunge diving (Sinclair et al. 2014b). They generally feed on small fish, but have been recorded as eating cephalopods, crustaceans and sometimes even insects (Gaglio et al. 2015). They are predominantly single prey loaders, making non-invasive observation of what they feed their chicks possible (Gaglio et al. 2017). Unlike the African penguin and the Cape cormorant, greater crested terns are not showing declining population trends and seem to be increasing (Crawford et al. 2009, Watermeyer et al. 2016). Different life history traits and/or feeding ecology may be the drivers of the population increase.

2.2.4 Kelp and Hartlaub's gulls

The last two species considered for this study are gull species with a substantial difference in their range, the kelp gull and the Hartlaub's gull (BirdLife International 2016d, e). The kelp gull is found along the coastline of most land masses around the world while the Hartlaub's gull is endemic to southern Africa, ranging from Terrace bay on the Namibian coast as far as Colchester in Algoa bay in the Eastern Cape province of South Africa (Hockey et al. 2005, Brooks 2018). They are both scavengers and generalist foragers, feeding on crustaceans, small fish and other shallow coastal water species, but also benefitting greatly from anthropogenic sources such as garbage dumps, being fed by people and scavenging discards from fishermen (Whittington et al. 2006, Crawford et al. 2009). Like the greater crested tern, both gull species are showing increasing population trends (BirdLife International 2016d, e). The Hartlaub's gull is much smaller than the kelp gull, weighing 180-300 g, limiting the threat they pose to other seabirds during nesting time (Sinclair et al. 2014c). Kelp gulls, weighing 1-1.5 kg, however, can pose a considerable threat to the breeding success of other seabirds such as Cape cormorants, Cape gannets (*Morus capensis* Lichtenstein, 1823) and African penguins as they are known to poach unguarded eggs or chicks (Branch et al. 2010, Sinclair et al. 2014c).

For this study, it was important that the stable isotope (SI) values of the food, as well as the physiological condition of the birds were known. The South African Foundation for the Conservation of Coastal Birds (SANCCOB), which runs three seabird rehabilitation centres in South Africa, allowed birds from their various centres to be used for the purposes of this study. Each centre has a permanent pen for all the birds that need to remain at the centre for an extended time or permanently. One centre for example, has three species of cormorant, two gull species, two penguin species and a tern species. The SANCCOB centre at Table View in Cape Town (referred to as SANCCOB TV from here onwards) is the biggest centre that SANCCOB runs and rehabilitates approximately 2000 birds annually (SANCCOB 2015, 2016). SANCCOB in Cape St Francis (SANCCOB

SF) is much smaller but houses a number of resident African penguins, which breed throughout the year.

2.2.5 Captive penguins used in this study

Half of the birds rehabilitated by SANCCOB TV are African penguins that are put into different areas of the centre depending on their age and the reason for admission. The chick rearing unit (CRU) houses penguin chicks under 1 kg. Once > 1 kg, chicks are moved to the nursery till they have lost all their secondary natal down and have a full fledgling plumage; they are then called blues because of their feather colour. Blues are then moved into the general pens where they start swimming. Once the fledglings have reached a weight of 2.6 kg, and their body down feathers are still dry after an hour of swimming and their blood shows no signs of infection, the penguins are released back into the wild. The general pens are where birds of all post-fledgling ages can be found, while juveniles and adults can be found in the permanent pen. This helped to ensure that birds of all ages were present in the centre at the time of this study. Young chicks and eggs can also be found at SANCCOB, as the rehabilitation centre takes in birds and eggs that have been collected from the colonies which are very close to human settlement. Eggs are removed when the nest is considered to be in a risky place because the adults have to cross traffic, or because they have been laid in someone's garden. To minimise the effect of feeding in the wild or having been admitted in a bad condition, only birds which hatched in the centre or had been in the centre for more than 3 months were used (Cherel et al. 2005b), though a month would normally have sufficed for a dietary signal to be reflected in the blood (Barquete et al. 2013). This ensured that the diet of the birds used for this study was known for more than a month prior to sampling. All African penguins in the centre are fed sardines (*Sardinops sagax* Jenyns 1842), but the younger birds are fed a formula (made up of fish, water and supplements as detailed below) as well. The amount of formula that each bird gets is dependent on its weight. This was taken into consideration when the SI values of the ingested prey were calculated (see below).

2.2.5.1 Bird selection for age effect

The effect of an animal's age on blood carbon and nitrogen SI was studied in captive African penguins. African penguins can be divided into six commonly recognised age classes (Barham et al. 2008, SANCCOB 2015): P0 chick (newly hatched, eyes closed), P1 chick (eyes have opened, small chick), P2 chick (medium to large chick, 100% covered in downy fluff, often with whitish cheeks), P3 chick (large chicks, more than 50% of body still covered in downy fluff), P4 chick (more than 50% of chick covered in fledging plumage – blue feathers), blue (bird in full fledging plumage), juvenile (birds with browning fledging feathers), and adult (black and white banded adult plumage). For this section of the study, four age classes were selected: P3 chicks (referred to as chicks; 42-54 days old),

blues (70-98 days old), juveniles (273-500 days old) and adults (365-1825 days old). Plumage was used as the primary means of classification into the four classes.

Birds were sampled on the 24th of June 2016, when adult penguins were not breeding and when penguins of all the selected age classes were present at SANCCOB TV. The numbers of individuals available for this study were: eleven chicks (five males and six females), eight blues (four males and females), four juveniles (two males and two females), and 13 adults (seven males and six females). The selection of adults and juveniles was limited to birds which had been in the centre for three or more months so that their blood tissue would reflect a controlled diet (Cherel et al. 2005b). At SANCCOB TV this diet consisted of sardines. The chicks and blues were all younger than 120 days and had been hatched in the centre to ensure that a diet of sardines was fed from hatching. It also ensured that all birds were living under the same conditions from hatching, eliminating the potential confounding effect of possible starvation or stress/disease experienced in the wild. The youngest bird was 42 days old, so it is unlikely that the effect of parent diet from the yolk was still reflected in the chick's blood due to RBC having a shorter turnover than 42 days (Fry & Arnold 1982, Barquete et al. 2013). The adults and juveniles were all resident birds at SANCCOB TV, and had not been released back into the wild for multiple reasons such as being too tame, being bred in captivity by resident parents, or being unable to move properly due to injury.

2.2.5.2 Bird selection for breeding stage effect

In seabirds, the annual cycle is generally split into a non-breeding period (when moulting often occurs; Warham 1996, Borboroglu & Boersma 2013), and the breeding period, which is separated into pre-laying, incubation and chick rearing. Chick-rearing in turn is split into guard-phase and crèche-phase for penguins (Borboroglu & Boersma 2013, De Roy et al. 2013). It is generally during the breeding stages that seabirds are accessible for study as they come to land to nest as well as to moult in some species like all penguins. To assess the effect of breeding physiology on C and N SI values, pre-laying was considered at a finer scale, namely egg production phase (EPP) as the period of diet before coming to nest on land overlaps with the period that a female is producing eggs (Burley & Vadehra 1989b, Ancel et al. 1997). This could influence the tissue SI values depending on whether a species is an income or a capital breeder or a mix of both. I.e. whether the nutrients deposited during egg production come from exogenous sources and/or endogenous reserves, with the latter becoming more important when food availability is low (Hobson et al. 2004). The males do not produce an egg and therefore should not show a change in SI values prior to breeding unless they change their diet to prepare for incubation duties (Garcia et al. 2010). For penguins, there is little information about oocyte maturation and yolk deposition during this time but in chickens, this can start as early as 60 days before laying (Burley & Vadehra 1989c). The next phase, rapid yolk

deposition, has been documented in Adélie (*Pygoscelis adeliae* Hombron & Jacquinot, 1841) and Fiordland crested penguins (*Eudyptes pachyrhynchus* Gray, 1845), and can take 14-18 days (Grau 1982, Astheimer & Grau 1985). After yolk deposition, the albumen is synthesised (species and breeder type dependent mostly from exogenous sources; Barzen & Serie 1990, Hobson et al. 2015), and just before laying, the shell is secreted mostly from endogenous reserves though this can vary with species (Grau 1982, Walsberg 1983, Astheimer & Grau 1985). The five categories into which birds were placed were thus: initial yolk deposition (IYD; 60-25 days before laying), rapid yolk deposition (RYD; 24-7 days before laying), albumen and shell production (AlbProd; 6-0 days before laying) and after laying. Ten pairs of African penguins residing at SANCCOB SF on the south coast were selected for sampling every 2-4 weeks from June to October 2016. Pairs were chosen if they had bred together in previous seasons, except two pairs which had not bred before. Birds at SANCCOB SF seem to lay quite regularly, possibly because eggs are pricked (as captive bred birds are not legally allowed to be released) and, therefore, may have been regarded as failed breeding attempts, causing the pairs to attempt breeding again. Of these ten pairs, one pair bred 3 times during April – October, and the two pairs which had never bred before did not lay any eggs throughout the sampling period and were not included in the data analysis.

2.2.6 Discrimination factors of multiple seabird species

The use of SI in food web studies has been growing exponentially since the mid-1990s (Boecklen et al. 2011, Bond & Hobson 2012). Accurate species- and tissue-specific discrimination factors (DFs - difference between predator tissue and its prey) are, however critical to the estimation of trophic level and, where possible the diet of predators (Bond & Jones 2009, Caut et al. 2009, Phillips et al. 2014, Ciancio et al. 2016). As direct observation of feeding is difficult (if not impossible) in seabirds (but see Gaglio et al. 2017) captive studies are currently the best option to measure these species- and tissues-specific DFs. The species which were sampled for this study were Cape cormorants (n=10), Hartlaub's gulls (n=4), kelp gulls (n=3), greater crested terns (n=2) and African penguins from SANCCOB TV (n=13). Penguins from SANCCOB SF were not included due to breeding. Sample sizes were dictated by the availability of birds. All birds housed at SANCCOB TV were sampled over two days (23 & 24 June 2016) during a non-breeding phase.

2.3 Blood sampling

The blood drawing procedure used in this study is the same as the one followed by SANCCOB for regular routine health checks. African penguins have a large vein along the top of the foot from which it is possible to draw blood using a 23G needle for venepuncture (Redrobe 2000). The other four bird species have a vein running along the inside of the leg from which blood is normally

collected. Needles (25G) were used for venepuncture in the flying birds and when blood could not be drawn from the leg, the brachial vein in the wing was used. This was the case for most Cape cormorants sampled.

It is generally accepted that 1% of the total body weight is equivalent to 10% of the blood volume (Oring et al. 1988, Fair et al. 2010). This is considered to be a safe amount of blood to draw from a healthy bird (Hoysak & Weatherhead 1991). SANCCOB regulations, however, only allow a maximum of 1% of the total blood volume or maximum 2 mL of blood (if 1% was more than 2 mL) to be collected from each bird into a 2 mL microcentrifuge tube. The amount of blood drawn, therefore, varied depending on the weight of each bird on the day of sampling (1.7 – 2 mL for African penguins, ~1 mL for Cape cormorants, ~0.8 mL for kelp gulls, ~0.2 mL for Hartlaub's gulls and greater crested terns). The microcentrifuge tubes used to collect the blood were rinsed with 1000 I.U./mL sodium-heparin and kept cool (once filled) till the blood was separated into different components where there was more than 0.8 mL of blood, < 5 hours after sampling.

Whole blood (WB) consists mainly of red blood cells (RBC) and plasma (PL) and each of these three tissues has a different turnover time (Hobson & Clark 1992b, Barquete et al. 2013). Where there was only 0.2 mL of blood as for the Hartlaub's gulls and greater crested terns, it was all set aside for stable isotope analysis (SIA) of WB. If there was more blood it was split as detailed. After setting aside 2 aliquots of 0.2 mL of WB for SIA and fatty acid signature analysis (FASA), the remaining blood was spun down for 5 min in a 6-tube mini-centrifuge (6 000 revolution per minute [rpm]) to separate the blood constituents. The total PL recovered was split for SIA and FASA, the intermediate layer, including white blood cells and PL which could not be removed without RBC contamination, was discarded, and the remaining RBC were split for SIA and FASA (Table 2-1). If less than 2 mL of blood was collected the order of priority for the possible subsequent analyses was: SIA of WB, FASA of WB, SIA of PL and RBC, and FASA of PL and RBC (

Table 2-1). All blood was handled with burnt glass pipettes, and samples for FASA were placed into glass test tubes with polytetrafluoroethylene (PTFE - teflon) caps to avoid contamination from plastic during lipid removal. Samples for FASA were transported in dry ice, while samples for SIA were kept cold. Fatty acid (FA) samples were kept in a Telsar -80°C freezer while the SI samples were stored in a -20 °C chest freezer (Gloutney & Hobson 1998). Unfortunately, due to the malfunction of the Telsar -80 °C freezer all samples collected for FASA were exposed to +43°C for several days, and were therefore not viable for analysis (Petersen & Klug 1994, Metherel & Stark 2016). The remainder of this thesis will thus focus on SIA. A small sample of WB was kept aside for molecular sexing of the birds that had not been previously sexed by the centres.

Table 2-1: Amount of blood (mL) separated for stable isotope and fatty acid signature analyses

Blood type	Amount for stable isotope analysis (mL)	Amount for fatty acid analysis (mL)
Whole blood (WB)	0.2	0.2
Red blood cells (RBC)	0.2-0.5	0.2-0.5
Plasma (PL)	0.2-0.5	0.2-0.5

2.4 Molecular sexing

2.4.1 Extraction of DNA

Birds, unlike mammals, have nucleated RBC, therefore it is possible to use this tissue to derive genetic information (Fridolfsson & Ellegren 1999). The DNeasy® Blood & Tissue kit (# 69504 QIAGEN) was used to extract deoxyribonucleic acid (DNA) from WB. The procedure developed for DNA extraction from animal tissue, provided with the extraction kit, was used as it yields higher DNA concentrations than the protocol for DNA extraction from nucleated blood (D Danckwerts pers. comm.).

Sub-samples of ca. 20-50 µL of blood from birds of unknown sex were placed into new, sterilised microcentrifuge tubes. To each tube, 180 µL of lysis Buffer ATL and 20 µL of enzyme proteinase K (> 600 mAU/mL) was added before thorough mixing using a Vortex mixer. Thereafter, samples where incubated at 56 °C in an Accublock Digital Dry Bath which was set at 450 rpm, for at least 12 h. This was done so that the proteinase K can digest all cell constituents and release the DNA into the stable lysis buffer. The buffer contains a high concentration of chaotropic salts such as: guanidine thiocyanate, urea, lithium perchlorate, and others, which destabilise the hydrogen bonds of proteins, including those of nucleases, by disrupting their association with water, enabling the transfer of DNA to the silica membrane at the base of each spin-column used in later steps (QIAGEN 2011).

The following day, 200 µL of Buffer AL was added and samples were kept at 56 °C for 10 min. Thereafter, 200 µL ethanol (96% concentration) was added into the microcentrifuge tube to enhance the binding of nucleic acids to silica found in spin columns. After mixing, each solution was transferred into a DNeasy Mini Spin-column, housed inside a 2 mL collection tube, and centrifuged at 8 000 rpm for 1 min. Most of the DNA remains bound to the silica membrane within each spin-column and the lysate containing proteins and polysaccharides, which passed through the spin-columns during centrifuging, was discarded. The spin-columns were each given new collection tubes

for the next step before 500 µL of the Buffer AW1 was added into each spin-column. All samples were then centrifuged at 8 000 rpm for another minute. The buffer, containing ethanol and a low concentration of chaotropic salts, washes the silica membrane of all unwanted proteins and salt. The removal of these residues is crucial to high DNA yields and purity (Powell et al. 1987, Integrated DNA Technologies 2011). A second wash was then performed, using 500 µL of the Buffer AW2, during which the samples were centrifuged at 14 000 rpm for 3 min. The higher speed of centrifuging ensured complete flow through and removal of all salt or protein residues whilst also drying the silica membrane so that DNA could be gleaned. After each washing step, the spin-columns were transferred into new collection tubes and the flow-through liquid was discarded. DNA was eluted from the spin-columns into new sterilised 2 mL microcentrifuge tubes by adding 150 µL of Buffer AE on top of the silica membrane. This was then centrifuged at 8000 rpm for another minute. All samples were incubated at room temperature for 5 min and this step was repeated using 50 µL of Buffer AE, ensuring maximum yield of pure DNA from the spin-columns, which were then discarded. At each step, centrifuging was repeated if necessary to ensure complete flow-through of all solutions and an effort was made to ensure that spin-columns never came into contact with the flow-through liquid.

DNA concentration and purity of each extracted sample were estimated using a Thermo Scientific NanoDrop 2000c UV-Vis Spectrophotometer. Nucleotide concentration varied greatly between 4.1 and 29.0 ng/µL. Extracted DNA was stored at -20 °C until further analysis. This was performed in the Zoology and Entomology Molecular lab at Rhodes University, South Africa.

2.4.2 DNA amplification

The molecular sexing of birds is based on Polymerase Chain Reaction (PCR) amplification of two sex-linked introns on the conserved chromo-helicase-DNA-binding (CHD) genes located on all non-ratite avian sex chromosomes (Fridolfsson & Ellegren 1999). The CHD-Z gene is located on the Z sex chromosome, common to both sexes, while the CHD-W gene is unique to the female W sex chromosome. Homologous portions of both genes are amplified using the primer pair 2550F (5'-GTT ACT GAT TCG TCT ACG AGA-3') and 2718R (5'-ATT GAA ATG ATC CAG TGC TTG-3'). The size of the amplified DNA intron varies with species, but females usually have two bands while males only have one (Fridolfsson & Ellegren 1999).

DNA amplification was performed in an 8 µL solution containing 6 µL of GoTaq Green Hotstar master mix applied 2x (Applied Biosystems), 0.4 µL of each primer (10 µM), and 1.2 µL of DNA free sterile deionised water. To this, 2 µL of genomic DNA (± 40 ng/µL) was added except when DNA concentrations were below 7.7 ng/µL, in which case 4 µL of genomic DNA was added to the mix.

Amplifications were performed using a BIO-RAD T100 Thermal cycler as follows: denaturation at 94 °C for 5 min, amplification with 35 cycles of 94 °C for 30 sec, 52 °C for 30 sec and 72 °C for 45 sec, and a final elongation step at 72 °C for 20 min. After the PCR process, 3 µL of 6x Gel Loading dye was then added to the PCR products, which were centrifuged at 6 000 rpm for 4 sec (to ensure that all of the solution could be pipetted), and 10 µL was placed into 1 % agarose TBE gel stained with ethidium bromide (10 µL EtBr/100 mL TBE) and exposed to electrolysis at 90 V for 50 min. Completed gels were photographed under ultra-violet light to visualise single or double amplified introns for males and females, respectively, using a BIO-RAD Molecular Imager Gel Doc XR+ machine.

2.5 Fish collection

All birds selected for the present study were fed fish for the entire experiment. The selected adult penguins were only fed whole sardine, while young penguins were also fed formula (made from sardine) when required to improve their growth (see below). Flying bird species were fed sardine as well as sardinella (*Sardinella aurita* Valenciennes 1847) (see below). For every new batch of fish brought into the centres, five randomly selected fish were removed and stored in a -20 °C freezer. All fish removed from the batch of fish fed to the birds were partly defrosted before being refrozen and kept aside for this study. Fish were collected at both SANCCOB centres prior to and up until the final blood samples were taken. At SANCCOB SF, fish from each batch coming into the centre were collected starting five weeks prior to the first blood samples taken (8 June 2016) and continuing through till the last blood samples taken (14 October 2016). At SANCCOB TV, fish were collected twice weekly from eight weeks prior to blood sampling, because batches came into the centre more frequently than at SANCCOB SF.

Hatchlings and growing penguins are fed formula along with fish to mimic the regurgitation they would normally get from their parents. The amount of formula that a bird received depended on the weight and age of the bird. Each of the three different areas in the SANCCOB TV centre – the Chick Rearing Unit, the nursery and the general pens - has its own kitchen where the necessary formula is made. The formula consists of 880 g fish mixed with multi-vitamins (5 Edelweis fisheater tablets (removed from gel capsule), 8 Portfolio Pharmaceuticals Brewer's Yeast tablets, 6 Edelweis Thiamine tablets, 1 tsp Aqua Marine cod-liver oil, 2 tsp Kyron protexin powder, 1 tsp Kyron Cani-Cal powder, 1 tsp Med Pet premoult powder) and 600 mL water all blended together. This blended mix is then sieved, thus removing hard parts which are not broken down by the blender, to prevent blocking the tubes used for feeding formula. The sardine in the formula was the same as that already being sampled so it was decided that sampling formula from all three kitchens twice a week would suffice, since it was not logistically feasible to sample formula every day. None of the birds sampled at

SANCCOB SF were given formula at any point during the sampling and therefore it was not necessary to sample formula from SANCCOB SF.

At SANCCOB TV, resident birds are not limited to penguins but also include flying seabirds such as the species sampled for this study. While the African penguins are fed whole sardine twice a day, the flying birds are offered chopped pieces of sardine and occasionally sardine tails as well as sardinella. From observation, it was noticed that each seabird species had its own preferences. Kelp gulls and Cape cormorants scavenged or were fed whole sardines along with the penguins. Kelp gulls and Hartlaub's gulls were quick to eat the eyes of any sardine fallen to the floor and sometimes ate the internal organs too, but often the whole fish would be too large for the Hartlaub's gulls to eat. It is possible that they were selective about which organs they ate, but special attention was not given to watching whether birds selected for gonads only or other organs too. Both gulls and the greater crested terns ate chopped sardine, sardine tails if they were cut small enough and small sardinella. While the Cape cormorants also helped themselves to the sardinella and sardine tails, they did not eat the chopped sardine. As much of this information as possible was taken into consideration for sample preparation and subsequent analysis of the results.

2.6 Sample preparation

2.6.1 Blood samples

Microcentrifuge tubes of frozen blood samples (WB, RBC, PL) were opened and covered with parafilm, which was then pricked 2-3 times, before being freeze-dried (while frozen) at -45°C for ~12 hours. If samples were not dry they were freeze-dried again for another ~12 hours. The samples were then crushed into a powder using a mortar and pestle. All equipment was carefully washed and dried between samples to avoid sample cross-contamination.

Where PL samples had enough crushed material (49 of 109 samples), 15-50 µg was removed and delipidated. This was done to reduce the artificial effect of transported lipids on the final $\delta^{13}C$ value of PL (Hobson & Clark 1992a, Bearhop et al. 2000, Cherel et al. 2005b, Matley et al. 2016). The process was conducted by rinsing each sample in 3 mL of 2:1 chloroform:methanol solution in glass test-tubes. Each sample was exposed to the rinsing solution for 70 min, and mixed at high speed, using a vortex, for 30 sec every 10 min. The solvent containing the lipids was then poured off and the remaining sample was left to air dry in a fume hood for 12-18 hours. The dried delipidated PL sample was then placed into a clean microcentrifuge tube (Figure 2-1).

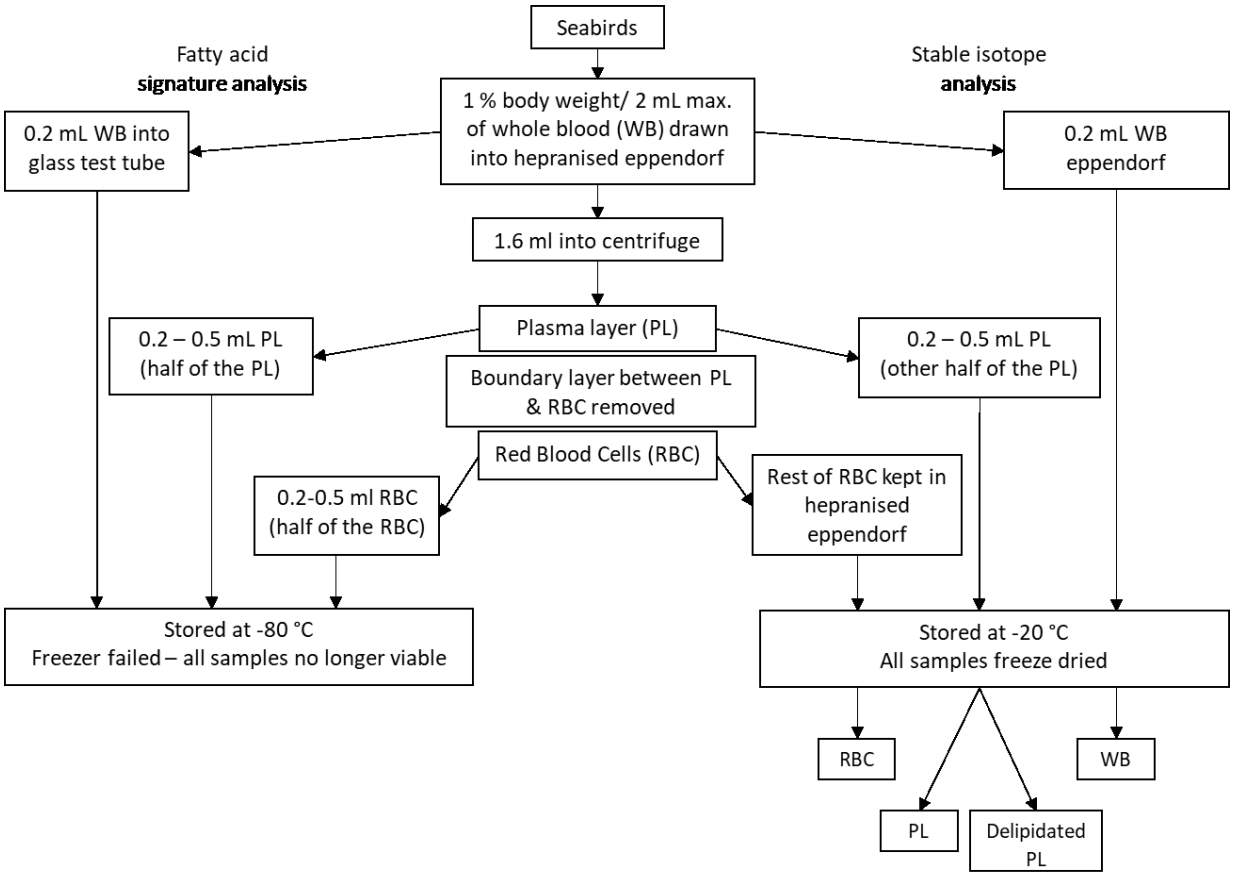

Figure 2-1: Flow chart of blood sampling of seabirds. Stable isotope analyses were prioritised if too little blood for all analyses was sampled.

2.6.2 Fish samples

Fish were defrosted in a fridge the night before preparation. A sample of flesh (FL; consisting mostly of epaxial muscle from the flexor dorsalis, but did include skin in some samples) from the upper posterior quarter of each fish was collected before being separately homogenised in a 500 W ice-crusher power drive blender (Kambrook, PIA3185). Thereafter, three sub-samples of each homogenised whole fish (WF) were taken. These samples were frozen before they were freeze-dried for ~24 hours, after which they were crushed and 30-100 µg were separated for delipidation. The same delipidation technique employed on the blood samples was used for fish samples. Thus, there were samples of whole fish (WF), delipidated WF, FL and delipidated FL samples.

Formula from SANCCOB TV was also subjected to freeze-drying before being crushed. Again 30-100 µg were separated out and delipidated. Because blood was sampled from various bird species feeding on different parts of sardine and on sardinella, all sardine from SANCCOB TV were divided into several tissues. These included: FL, "gut" consisting of a homogenous mix of all organs in the body cavity, one eye, and three sub-samples of homogenised fish (the remaining "gut" was placed

back with the fish to be homogenised). These tissues, except the eyes, were also separated and 30-100 µg were subjected to delipidation. This resulted in a total of 11 samples for each sardine being subject to SIA (Figure 2-2).

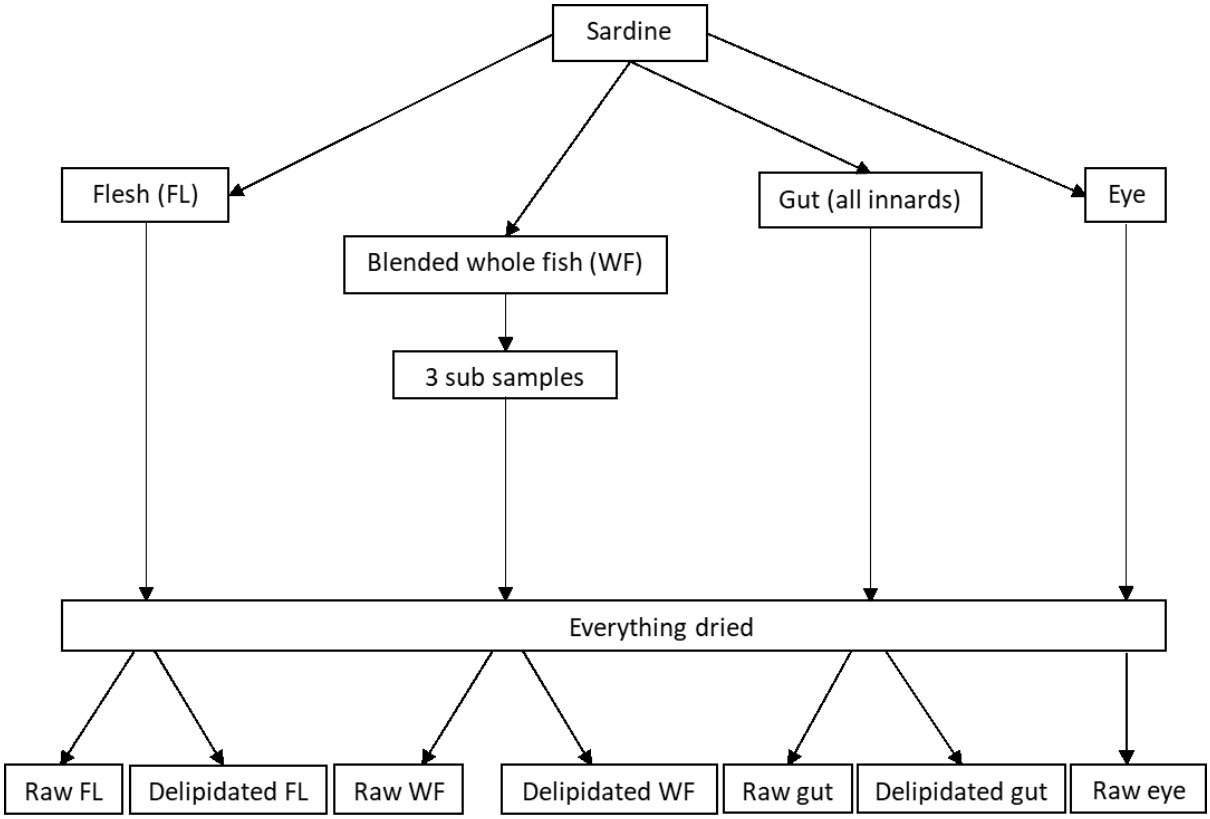

Figure 2-2: Flow chart of samples taken from sardine (Sardinops sagax).

Such detailed tissue sampling was not required for sardinella, as the birds were not selective about what part of the fish was eaten and normally ate the whole fish. The tissues sampled from sardinella included: FL and three sub-samples of homogenised WF. Again, the FL and WF were separated after freeze-drying and crushing, so that 30-100 µg could be delipidated. Thus, each sardinella provided a total of 8 samples for SIA (Figure 2-3).

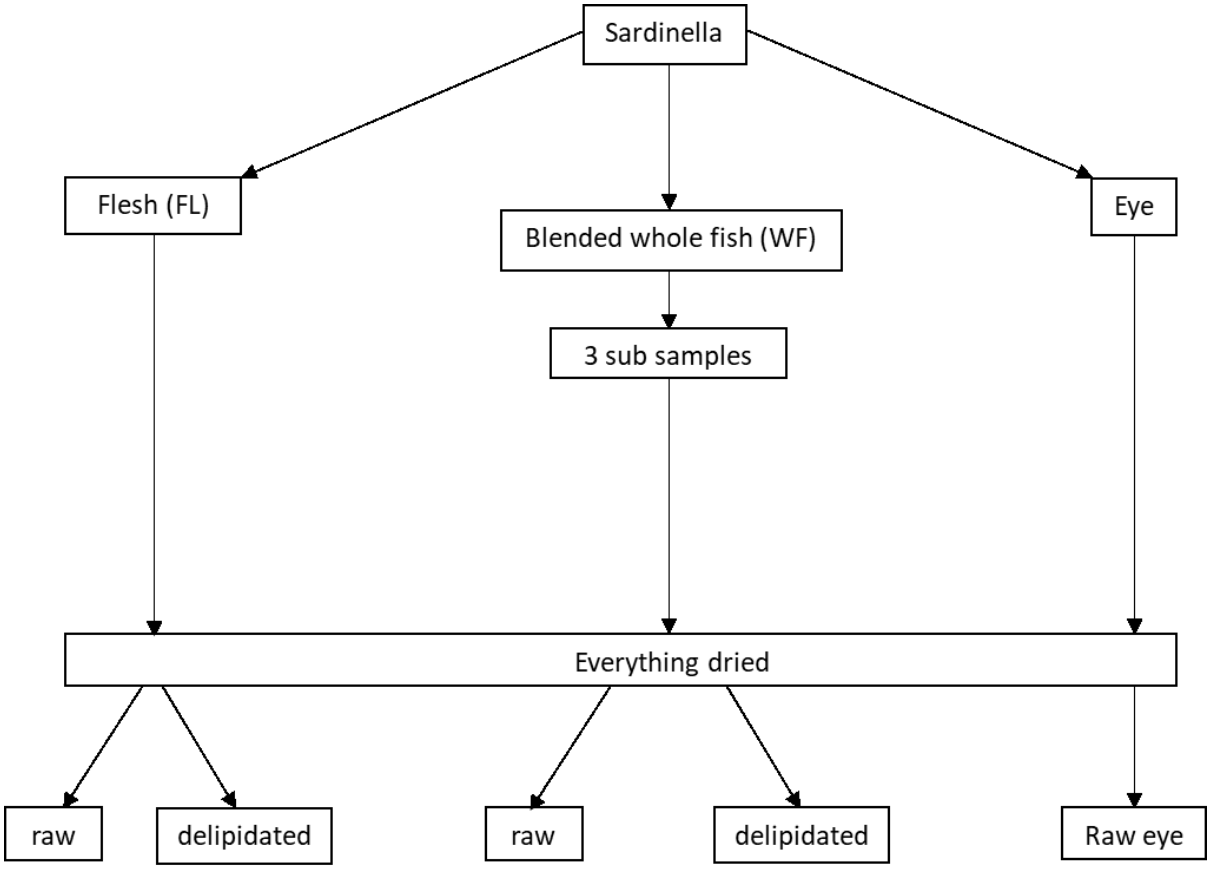

Figure 2-3: Flow chart of samples taken from sardinella (Sardinella auritus).

2.7 Stable Isotope Analysis (SIA)

SIA was conducted on all dried and crushed samples at the Archaeology Department of the University of Cape Town (UCT), South Africa. Between 0.40 mg and 0.50 mg of each sample were weighed into separate small tin cups using a Sartorius M2P micro balance, and then combusted at 1020°C in Flash 2000 organic elemental analyzer and the gases produced were passed to a Delta V Plus isotope ratio mass spectrometer (IRMS) via a Conflo IV gas control unit (all three items are made by Thermo Scientific, Bremen, Germany) to determine the isotope values for each sample. Relative abundances of carbon and nitrogen isotopes were analysed for each sample and expressed using the δX notation as parts per thousand (‰) per the following equation:

$$\delta X = \left[\left(R_{sample} - R_{reference}\right) - 1\right] \times 1000$$

where X= ^{15}N or ^{13}C, R is the heavy to light stable isotope ratio of an element ($^{15}N/^{14}N$; $^{13}C/^{12}C$) for the sample and the international references, air and Vienna Pee Dee Belemnite (V-PDB) for $\delta^{15}N$ and $\delta^{13}C$ respectively. Merck gelatine (a proteinaceous gel produced by Merck), seal bone (crushed, demineralized and dissolved in acid, and then reconstituted in gel form by UCT) and DL-valine (purchased from Sigma) were used as in-house standards and calibrated against the International

Atomic Energy Agency (IAEA) standards. The expected $\delta^{15}N$ and $\delta^{13}C$ values for the standards were 7.5 ‰ and -20.05 ‰ (Merck gelatine), 15.84 ‰ and -11.97 ‰ (seal bone), and 12.14 ‰ and -26.80 ‰ (DL-valine) respectively. The precision of the IRMS results was < 0.20 ‰ and < 0.15 ‰ for $\delta^{15}N$ and $\delta^{13}C$ respectively.

2.8 Additional calculations

2.8.1 Delipidated plasma

The C:N ratio of a substance can provide information on the amount of lipids in the substance. A C:N ratio greater than 4 and a correlation to the $\delta^{13}C$ value in the substance is often indicative of the influence of lipids on the $\delta^{13}C$ value. Only 49 of 109 PL samples were delipidated due to small sample volumes. To overcome this, the outstanding delipidated PL values were estimated using the difference between the known raw PL and delipidated PL values. The difference between the C:N ratio of PL and delipidated PL was plotted against the difference between the $\delta^{13}C$ of PL and delipidated PL. A correlation of the two yielded a R^2 value of 0.784 using an exponential equation of $y = 0.3455e^{-0.596x}$. This was then used to estimate a $\delta^{13}C$ value of delipidated PL from raw PL samples which were not delipidated, using the average delipidated C:N ratio of 3.39 calculated from the 49 delipidated PL. $\delta^{15}N$ is not significantly correlated to the C:N ratio and therefore not influenced by high C:N ratios. It may, however, be influenced by the delipidation process (Cherel et al. 2005b) so the $\delta^{15}N$ of the raw PL were thus used for analyses.

2.8.2 Whole fish

Fish from one batch sampled at SANCCOB SF on 3 June 2016 did not include WF samples but did include FL samples. The difference between the C:N ratio of FL and WF was plotted against the difference between the $\delta^{13}C$ of FL and WF to determine a correlation to calculate the $\delta^{13}C$ values for WF samples which could not be analysed. A linear correlation yielded a R^2 value of 0.880 using an equation of $y = -0.8426x - 0.0584$, was then used to estimate the $\delta^{13}C$ value for the missing WF samples. The WF $\delta^{15}N$ of the fish from batches collected before and after the missing batch were not significantly different from each other and were averaged to determine a value for the $\delta^{15}N$ of WF. For any analyses concerning WF, the SI values of the 3 subsamples of each fish were averaged, as data for each subsample were not available for all fish, before the fish of each group were averaged. An average of the whole group of fish, without first averaging the subsamples of each fish, would yield a slightly less accurate average SI value for the group of fish.

2.8.3 Delipidated whole fish

High lipid content in a tissue can artificially lower its $\delta^{13}C$ value (DeNiro & Epstein 1977). Lipids are therefore, often removed chemically or mathematically when the 'true' $\delta^{13}C$ value of a studied tissue is needed (e.g. Post et al. 2007, Logan et al. 2008). Of 96 samples of WF, 21 could not be delipidated as there was not enough sample after drying and crushing. The difference between the C:N ratio of raw WF and delipidated WF was plotted against the difference between the $\delta^{13}C$ of raw WF and delipidated WF. A correlation of the two yielded a R^2 value of 0.830 using a linear equation of $y = -0.7305x - 0.2833$. This was then used to 'back-calculate' a $\delta^{13}C$ value of delipidated WF from raw WF samples which could not be delipidated, using the average C:N ratio of 3.22 calculated from the 75 delipidated WF. As with the PL samples, the delipidated $\delta^{15}N$ values for FL and WF were not used for analyses as the $\delta^{15}N$ value may be influenced by the delipidation process (Cherel et al. 2005b).

2.8.4 Average prey

Additional calculations were needed to know the averaged SI value of food ingested by each bird, as not all ingested fish came from the same batch. There was a significant difference among SI values of fish from different batches fed to the penguins (ANOVA; $F_{(7,195)} = 20.8$, $p = 0.010$), and therefore each batch needed to be taken into consideration to determine an average prey SI value for nitrogen and carbon. To determine the proportion of each fish batch contributed to the blood values, the number of days the batch was fed to the birds (d) was multiplied by the average weight of fish in that batch (wght), the average number of fish consumed per meal (fish) and the number of meals per day (meals). This was then divided by the sum of these values for all batches fed to the bird over the relevant days as seen in the equation below. The diet of the past 15, 20 and 30 days was considered for PL, WB and RBC respectively (Barquete et al. 2013).

$$proportion\ of\ prey\ SI = \frac{\mathrm{d} * \mathrm{wght} * \mathrm{fish} * \mathrm{meals}}{\sum_{batches}^{7} \mathrm{d} * \mathrm{wght} * \mathrm{fish} * \mathrm{meals}}$$

The proportions of each fish batch incorporated into the tissue were then used to calculate a weighted average of SI values for prey carbon and nitrogen. All weighted averages of each fish batch fed, were added together to get a final ingested fish $\delta^{13}C$ and $\delta^{15}N$ value. Where formula was fed to young penguins, it was treated as fish in the equation, except that the average weight of the fish was replaced with the volume (mL) of formula the bird received per day.

Statistical analyses are detailed in the relevant chapters.

2.8.5 Discrimination factor

The discrimination factor is the difference between the SI value of tissue from a consumer and the tissue of its diet. For SIA to be useful, for example in mixing models, the DF needs to be known, but it is tissue-, species- and potentially even diet-specific (Caut et al. 2009, Phillips et al. 2014). Sometimes studies conducted in the wild have access to the FL of some potential prey items while other studies have access to whole prey to calculate the prey SI values. Since the DF can vary because of both diet and tissue, where opportunities arise to determine more DFs, it should be done. For this study the $\Delta^{15}N$ was calculated between WB, RBC and PL of predator and FL and WF of prey and the $\Delta^{13}C$ was calculated between WB, RBC, PL and delipidated PL of predator and FL, delipidated FL, WF and delipidated WF of prey, by subtracting the prey SI value from the predator SI value for all combinations.

Chapter 3 The effect of physiology on stable isotope values

Seabirds can be used as indicator species for ecosystem studies as their trophic ecology can give an indication of what is happening in the ecosystem (Mallory et al. 2010, Cunningham 2017). For studies conducted in the wild, seabirds are generally sampled during their time on land, when they are easily accessible. The dominant reason that seabirds come to land is to breed, as well as to moult in the case of penguins, which means that the majority of the sampled population are likely to be under the influence of particular physiological stresses such as fasting (moult), intense growth (chicks) or reproduction (breeding adults). When using a technique like stable isotope analysis (SIA) to assess a bird's trophic ecology, a technique that is nowadays often used to complement the traditional method of stomach content analysis (Barrett et al. 2007, Karnovsky et al. 2012), it is important to consider how these physiological processes may influence the stable isotope (SI) values of various tissues at the time of sampling.

A number of studies have looked into various aspects of the physiology of an animal and how it influences its SI values (e.g. Vanderklift & Ponsard 2003, Caut et al. 2009, Boecklen et al. 2011). Some studies, however, have rendered different results. For example, the influence of food reduction on $\delta^{15}N$ and $\delta^{13}C$ values is still under debate. On one side, the $\delta^{15}N$ value increased due to nutritional stress in various tissues (including muscle, liver, blood, feathers and bone collagen) of juvenile Japanese quail (*Coturnix japonica* Temminck & Schlegel, 1849) and Arctic-nesting female Ross' Geese (*Anser rossii* Cassin, 1861) (Hobson et al. 1993). On the other side, nutritional stress stunted growth and development in juvenile song sparrows (*Melospiza melodia* Wilson, 1810), but had no effect on the $\delta^{15}N$ or $\delta^{13}C$ values of several tissues (including whole blood (WB), liver, muscle and feathers; Kempster et al. 2007). In a fourth species, the tufted puffin (*Fratercula cirrhata* Pallas, 1769), food reduction induced small decreases in $\delta^{15}N$ and $\delta^{13}C$ values of blood cells and WB, but these would not hamper the use of SIA to determine ecological information (Williams et al. 2007).

There are several reasons why physiology (e.g. metabolism, somatic growth, reproduction) can affect the SI value of tissues, and not all tissues may be influenced in the same way (Boecklen et al. 2011). Some physiological conditions influence the chemical reactions relating to the uptake of nutrients, which directly influence the isotope pool in the body (Michener & Lajtha 2007), while others influence how nutrients are routed to different tissues and utilised by the body (Gannes et al. 1998). The mode of nitrogen excretion (e.g. through ammonium or uric acid) may also influence the body's SI pool (Vanderklift & Ponsard 2003).

Somatic metabolism and many other reactions utilise nitrogen-based proteins, which are preodominantly derived from dietary sources. During the catabolism of proteins, uric acid is formed

as a by-product, and then transported in the blood until excretion. Uric acid is depleted in ^{15}N, as lighter molecules are processed faster (Michener & Lajtha 2007), and can thus influence the $\delta^{15}N$ value of blood if it is being transported at high concentrations (Bearhop et al. 2000). During intense growth, uric acid may be found at higher levels in the blood because the somatic growth rate of a chick is much higher than in a fully grown, non-breeding adult (Bearhop et al. 2000). It is therefore possible that fast growing chicks will exhibit a lower $\delta^{15}N$ value during the steepest part of their growth curve due to the high catabolic rate and associated increase in circulating uric acid.

Uric acid is not the only molecule which may influence SI values in organisms. Lipids are constructed with lighter ^{12}C molecules than proteins (Sweeting et al. 2006), so a high lipid content in tissues lowers their $\delta^{13}C$ values (DeNiro & Epstein 1977). The lipid content of tissues varies depending on a number of factors but one important one to consider in birds is the influence of oogenesis in females as this increases the amount of circulating lipids in blood (Burley & Vadehra 1989b). This biased lowering of the tissue $\delta^{13}C$ value due to high lipid contents may then interfere with the question being asked. For example, in ecological studies where $\delta^{13}C$ values are often used to infer the source of carbon production at the base of the food chain, high lipid contents bias the data so that lipid removal is necessary (Taylor et al. 2017). An indirect way of estimating lipid contents in tissues, and deciding whether lipid removal is necessary, is to look at the C:N ratios (McConnaughey & McRoy 1979). The influence of lipids on tissue SI values can be removed either mathematically or chemically, each with its pros and cons. The use of species- and tissue-specific mathematical corrections is preferred but not always possible, so chemical removal is still in use (Post et al. 2007). Often the chemical removal of lipids can influence the $\delta^{15}N$ values because nitrogenous compounds rich in lipids, such as lipoproteins, are also removed in the delipidation process (Cherel et al. 2005b, Logan & Lutcavage 2008). Few lipids are utilised in the production of bird blood and the potential that this will alter the $\delta^{15}N$ value has led to whole blood (WB) and especially red blood cells (RBC) often not being delipidated before analysis (Bearhop et al. 2000, Cherel et al. 2005b).

Another physiological aspect which is used to glean more information on SI values, is the turnover time of tissue (Hobson & Clark 1992b). The turnover time of a tissue gives an indication of the period of diet incorporation that is reflected in the tissue, so the longer it takes a tissue to turnover, the longer the dietary window reflected in the tissue (Hobson & Clark 1992b). If the diet of the organism varies over time, a change in turnover could influence the SI value of tissues. Metabolic rate can influence the turnover of certain tissues, as seen in small mammals studied by MacAvoy et al. (2006). Although the interpretation is based on a simple correlation, MacAvoy and colleagues found that rats (*Rattus norvegicus* Berkenhout, 1769) showed a slower metabolic rate relative to size and a slower turnover of blood than smaller mice (*Mus musculus* Linnaeus, 1758), which had a higher

metabolic rate and turned over blood tissue in a shorter period. A change in metabolic rate did not, however, influence the turnover rate in house sparrows (*Passer domesticus* Linnaeus, 1758) studied by Carleton & Martínez del Rio (2005). A change in the turnover would result in a different period of the diet showing, which in turn could influence the SI value. However, in the study by Carleton & Martínez del Rio (2005), an induced increase in metabolic rate did not result in a significant change in the SI values of blood.

There are a number of conflicting studies and studies highlighting knowledge gaps, which indicate the importance of conducting more controlled studies to gain a better understanding of the effects of physiology on the SI values of various tissues (Gannes et al. 1998, Vanderklift & Ponsard 2003, Connan et al. 2016). It has been seen that using captive birds yields information which could not be gleaned from wild populations (Forero et al. 2002, Cherel et al. 2005a) as they allow for certain assumptions to be tested which are not testable in the field due to variables which cannot be controlled.

The South African Foundation for the Conservation of Coastal Birds (SANCCOB) is a rehabilitation organisation with centres at Table View in Cape Town and Cape St Francis. SANCCOB mostly admits African penguins (*Spheniscus demersus* Linnaeus, 1758; SANCCOB 2016). African penguins have undergone major population declines and have been classified as endangered since 2010 (BirdLife International 2016a). Many conservation strategies are being implemented to prevent further decline of this species (Crawford et al. 2013). These are often based on their trophic ecology and the extent to which the penguins interact with fisheries. A recent study called for an improved understanding of the potential impact of physiology on SI ratios to enhance their use in ecological studies in the wild (Connan et al. 2016). For this reason, captive African penguins were used to answer two questions in this chapter: 1) how does age influence the carbon and nitrogen SI values of the blood components, and 2) how does the egg production phase (EPP) affect the SI value of blood components?

The first question this chapter will address is whether carbon and nitrogen SI values of whole blood (WB), red blood cells (RBC) and plasma (PL) are influenced by age in African penguins. Chicks exhibit a steep growth curve early in life (Nel et al. 2003) and blues increase their protein requirements when they start developing muscles for swimming (Tipton & Wolfe 2001). Protein requirements during both of these physiological conditions are increased and can result in an increase in uric acid production, while the efficiency of protein use is lowered (Bearhop et al. 2000, Martínez del Rio et al. 2009b). I expect the $\delta^{15}N$ values measured in chicks and blues to be lower than in the older age classes (juveniles, adults). The effect of high uric acid concentration is likely to be seen most clearly

in the chick PL, as that is the serum in which molecules are transported. Because WB contains the PL and all that is found in the PL, including blood cells, it is possible that a slight decrease in $\delta^{15}N$ will be seen. The less efficient use of proteins is likely to result in ^{15}N depleted values compared to adults, as these can be more selective (or efficient) in their use of proteins. This is likely to result in a diminished $\delta^{15}N$ in chick and blue tissues. Once penguins have reached adult size, age is expected to have little effect on the SI values (Hobson & Clark 1992b, Mizutani et al. 1992).

The second question in this chapter is whether egg production (EP) influences the SI values of WB, PL and RBC of adult breeding African penguins. During EP, as opposed to non-breeding, the blood of a female bird transports more lipids (bound to proteins for transport and storage in the yolk) to produce the yolk and other components of the egg (Groscolas 1982). It is likely that the greatest effect of lipids will, therefore, be seen in early EP in the PL as this would be where the lipids would be suspended. An associated decrease in $\delta^{13}C$ is thus to be expected but can be eliminated by chemically removing the lipids. It is possible that an increase in proteins that are bound to the lipids (Burley & Vadehra 1989a) could influence the $\delta^{15}N$ value of the blood. The males do not produce eggs and the SI values of the blood components of males should, therefore, remain constant and provide a good control.

3.1 Methods

3.1.1 Experimental penguins

To study the effect of age on PL, RBC and WB carbon and nitrogen SIs, four age classes of penguins residing at SANCCOB Table View (SANCCOB TV) were chosen, namely: chicks of P3 age, blues (penguins in full fledging plumage), juvenile (penguins with browning fledging feathers between the ages of 9 months and 2 years) and adults (black and white banded adult plumage). Penguins were sampled at a time when penguins of all ages were found in the centre and during a period of non-breeding for the adults, though mostly non-paired individuals were chosen to mitigate the potential confounding effect of breeding. This fell on the 24th of June 2016 at SANCCOB TV. The chicks (n=10) and blues (n=8) which were hatched in the centre, and juveniles (n=4) and adults (n=18) which were resident were chosen for this study.

On the day of sampling each bird was weighed and up to 2 mL of blood was drawn by venepuncture of the tarsal vein. If the bird weighed less than 2 kg, as was the case with a few chicks, only 1% of the body weight (grams) was drawn in mL. The sardine fed to the penguins and the formula given to the chicks and blues were sampled twice weekly, from the chick rearing unit (CRU), nursery and general pens, from five weeks prior to sampling, to determine the SI values of the diet.

To assess the effect of EP on the blood components of African penguins, ten breeding pairs from SANCCOB Cape St Francis (SANCCOB SF) were selected. Two mL of blood were then sampled from each bird every two to four weeks for five months, from June to October 2016, to cover two breeding periods. Once the dates on which the eggs were laid were known, each pair was placed in an EPP category each time it was sampled. The four EPP categories were: initial yolk deposition (IYD), rapid yolk deposition (RYD), albumen production (AlbProd), and after laying (After). Sardine from each batch of fish fed to the penguins were sampled concurrently, starting eight weeks before the first blood sample was taken.

Samples of both fish (flesh – FL, and whole fish – WF) and blood components (WB, RBC and PL) were then dried and crushed, and both fish components and PL were subsampled and delipidated using a 2:1 chloroform:methanol solution. All samples were then sent to the Department of Archaeology, at University of Cape Town for nitrogen (N) and carbon (C) SIA. Calculations for average prey SI values and DFs were conducted where necessary. Refer to chapter 2 for details on methodology.

3.1.2 Data Analysis

3.1.2.1 Age effect

Prior to analyses, Shapiro-Wilks tests were used to test for normality in the data and Levene's tests were used to determine whether the data were homogeneous. Where assumptions of normality and homogeneity were met, an analysis of variance (ANOVA) was used, while a non-parametric Kruskal-Wallis test was conducted if either the normality or homogeneity assumptions were not met. Separate ANOVAs or Kruskal-Wallis tests were conducted to determine whether the dependent variables $\delta^{13}C$ and $\delta^{15}N$ values and the C:N ratios were significantly affected by penguin age (fixed factor, 4 levels: chicks, blues, juveniles and adults). Within each age class, Student's t-tests (or Wilcoxon signed rank test for non-parametric data) were conducted to determine if there was a difference between males and females. Blues are encouraged to swim for varying times to promote muscle development and preening for waterproofing. A third set of analyses was conducted to determine if the length of time for which blues swam influenced their $\delta^{13}C$ values, $\delta^{15}N$ values and C:N ratios. The three swimming times were: < 5 min – called no force swimmers from here onwards, 20 min and 1 h swims – three times a day. All analyses were run for three blood components – WB, RBC and PL and for $\delta^{13}C$ of delipidated PL. To determine if and how delipidation or prey tissue influences $\delta^{13}C$ and $\delta^{15}N$ values, an ANOVA was conducted on the $\delta^{13}C$ for four fish components (FL, delipidated FL, WF and delipidated WF) and on the $\delta^{15}N$ of the non-delipidated fish components (FL and WF).

To accurately determine whether the SI patterns seen across age classes of penguin WB, RBC and PL were influenced by age or diet, the discrimination factors (DFs – $\Delta^{13}C$ and $\Delta^{15}N$) were also analysed using ANOVA, as this would diminish the confounding effect of an age-specific diet. The reason that so many prey tissue types were analysed is so that more information is available for future studies. When considering DFs between a predator tissue and its prey, it is important to use the same tissue to determine the DF and the SI value of prey (e.g. Δ_{FL} = SI of predator tissue – SI of FL of prey, not WF of prey in DF was calculated with FL). This prevents incorrect conclusions being drawn about prey items for which matching DF and prey tissue information is not available (Cherel et al. 2005b). Upon detecting significant differences with the limit $\alpha = 0.05$, Tukey post hoc tests were conducted for parametric analyses, while significant non-parametric tests were followed by Conover's post hoc tests. These analyses were all conducted in R 3.4.1 with the following packages: tidyverse (Wickham 2017), PMCMR (Pohlert 2014), agricolae (De Mendiburu 2017), and lawstat (Gastwirth et al. 2017).

3.1.2.2 Physiology of breeding adults

A generalized linear model (GLM) was conducted in STATISTICA 13, to determine whether the physiological impact of egg production influenced $\delta^{13}C$ and $\delta^{15}N$ values in the blood components of breeding African penguins. The categorical variables included in the analyses were EPP, sex, the interaction of EPP and sex, all treated as fixed factors, while bird ID was nested in sex as a random factor. The continuous variables that were included in the analyses were the penguins' weight, the matching SI values of the fish components (FL, WF, delipidated FL and delipidated WF), and the complementary SI component of the blood tissue ($\delta^{13}C$ if $\delta^{15}N$ was being analysed and vice versa). For each analysis, only two prey components were included at a time. For example, for a GLM assessing the influence of EPP on the $\delta^{15}N$ of penguin WB, the following were included as continuous variables: $\delta^{13}C$ of WB, and $\delta^{15}N$ of FL and WF. In a second example for $\delta^{13}C$ of RBC, I included the $\delta^{15}N$ of RBC, the $\delta^{13}C$ of delipidated FL and delipidated WF. This resulted in 11 combinations namely: three blood components for $\delta^{15}N$, four for $\delta^{13}C$ with raw prey and four for $\delta^{13}C$ with delipidated prey. In the event of significant effects with $\alpha = 0.05$, Tukey post hoc tests were conducted to see where the differences lay and standardised residuals were assessed visually to ensure that the GLM was an appropriate model to apply to this data set. Where the residuals did not show homoscedasticity, the log transformed values of the continuous variables were used. GLM is considered robust for unbalanced design and an analysis of covariance (ANCOVA) is considered robust against failed assumptions making less homoscedastic analyses acceptable (Olejnik & Algina 1984, Carey 2013).

The captive penguins housed at SANCCOB are not given the opportunity of raising their chicks as the centres have reached carrying capacity. This led to some of the penguins laying three times during

the course of the experiment. This meant that individual penguins may have been sampled multiple times in the same EPP. These duplicate samples were removed to ensure independence of samples within EPPs. The samples that were chosen for the analysis were selected based on the date of sampling. An attempt was made to ensure that as many samples per EPP as possible, were sampled on the same day. A random effect of individual penguin was included in the analyses, as the same penguins were followed throughout the sampling period and may appear in multiple EPPs.

3.2 Results

3.2.1 Age effect

3.2.1.1 Age class comparison

There were significant differences in $\delta^{13}C$ and $\delta^{15}N$ in the food fed to the penguins of different age classes (all $p < 0.0001$ for $\delta^{15}N_{FL}$ and $\delta^{15}N_{WF}$ and for $\delta^{13}C_{FL}$, $\delta^{13}C_{WF}$, $\delta^{13}C_{delipidated\ FL}$ and $\delta^{13}C_{delipidated\ WF}$, incorporated into PL over 15, into WB over 20 and into RBC over 30 days; Sup. Table 1). Food fed to chicks (i.e. roughly half/half formula and fish) consistently had significantly lower $\delta^{13}C$ and $\delta^{15}N$ values than that fed to the blues (i.e. more fish and less formula than chicks). Food fed to blues, in turn, had significantly lower SI values than that fed to juveniles and adults, which fed on the same batches of fish as blues but were not given formula. The $\delta^{13}C_{FL}$ ranged from -18.0 ‰ to -17.5 ‰ and the $\delta^{15}N_{FL}$ ranged from 10.9 ‰ to 11.3 ‰. The $\delta^{13}C_{WF}$ ranged from -17.8 ‰ to -18.2 ‰ and the $\delta^{15}N_{WF}$ ranged from 10.5 ‰ to 10.7 ‰. Adding formula to the diet decreased the values of the estimated $\delta^{15}N$ and $\delta^{13}C$ of diet of birds, as did increasing the number of days the diet was incorporated into tissue (as different tissues have different turnover times, giving a range of 15 – 30 days incorporation time; Sup. Table 2). The C:N ratio was not averaged as the SI values for prey were estimated by calculation (see chapter 2). Instead the C:N ratio of the raw data was compared across food components namely: FL, WF, and formula from three areas in the centre (chick rearing unit - CRU, nursery and general). There was a significant difference in C:N ratio between formula and sardine ($W_{(4)} = 65.42$, $p < 0.0001$), with C:N ratio of CRU (4.96), nursery (4.98) and general (5.05) being significantly higher than C:N ratio of FL (4.16) and WF (4.37).

Significant differences in $\delta^{13}C$ and $\delta^{15}N$ were highlighted for WB and RBC among the four age classes ($\delta^{15}N_{WB}$: $F_{(3,32)} = 33.65$, $p < 0.0001$; $\delta^{15}N_{RBC}$ $W_{(3)} = 24.31$, $p < 0.0001$; $\delta^{13}C_{WB}$: $F_{(3,32)} = 15.55$, $p < 0.0001$; $\delta^{13}C_{RBC}$: $F_{(3,32)} = 9.93$, $p < 0.0001$). In all analyses of both $\delta^{13}C$ and $\delta^{15}N$ of WB and RBC, chicks and blues were not significantly different from each other, neither were the juveniles and adults, but chicks and blues had significantly lower SI values than the juveniles and adults by an average c. 0.2 ‰ for $\delta^{13}C$ and 0.5 ‰ for $\delta^{15}N$ (Figure 3-1; Figure 3-2; Table 3-1). The $\delta^{13}C$ values of PL ranged from -17.1 ‰ to -16.9 ‰, and a significant difference existed among the four age classes ($F_{(3,31)}$ =

4.63, $p = 0.009$) with chicks having significantly lower $\delta^{13}C$ values compared to adults but not compared to blues and juveniles, which, in turn, were not different from adults (Table 3-1). There were no significant differences in $\delta^{15}N_{PL}$ amongst the four age classes ($F_{(3,31)} = 1.57$, $p = 0.220$; Figure 3-2) but there was in the $\delta^{13}C_{delipidated\ PL}$ ($W_{(3)} = 8.17$, $p = 0.040$; Figure 3-1), though a post-hoc analysis could not determine where the differences lay (Sup. Table 2).

Table 3-1: Summary of results of comparisons of blood components (whole blood – WB, red blood cells – RBC, plasma – PL, and delipidated PL) and fish components (flesh – FL, whole fish – WF, delipidated FL and delipidated WF) among four age classes - adults, juveniles, blues and chicks.

		Significantly	Age class – homogenous groups			
Component	Tissue	different	Adults	Juveniles	Blues	Chicks
Stable isotope values & C:N ratios						
$\delta^{13}C$	WB and RBC	yes	a	a	b	b
$\delta^{15}N$	WB and RBC	yes	a	a	b	b
C:N	WB	yes	a	ab	bc	c
C:N	RBC	no				
$\delta^{13}C$	PL	yes	a	ab	ab	b
$\delta^{15}N$	PL	no				
C:N	PL	yes	a	ab	b	b
$\delta^{13}C$	Delipidated PL	yes	a	a	a	a
$\delta^{13}C$	FL, WF, delipidated FL and delipidated WF	yes	a	a	b	c
$\delta^{15}N$	FL and WF	yes	a	a	b	c
Discrimination factors						
$\Delta^{13}C$	WB-FL and WB-WF	yes	a	ab	b	a
$\Delta^{13}C$	WB-delipidated FL and RBC-delipidated FL	yes	a	a	b	b
$\Delta^{13}C$	RBC-delipidated WF	yes	a	a	a	a
$\Delta^{13}C$	WB-delipidated WF	yes	a	a	b	ab
$\Delta^{13}C$	delipidated PL-FL	yes	a	a	ab	b
$\Delta^{13}C$	delipidated PL-delipidated FL and delipidated PL-delipidated WF	yes	a	ab	b	b
$\Delta^{13}C$	delipidated PL-WF	yes	a	ab	ab	b
$\Delta^{13}C$	PL-FL, PL-WF, PL-delipidated FL, PL-delipidated WF, RBC-FL and RBC-WF	no				
$\Delta^{15}N$	WB-FL and RBC-FL	yes	a	a	b	a
$\Delta^{15}N$	WB-WF and RBC-WF	yes	a	a	b	b
$\Delta^{15}N$	PL-FL and PL-WF	no				

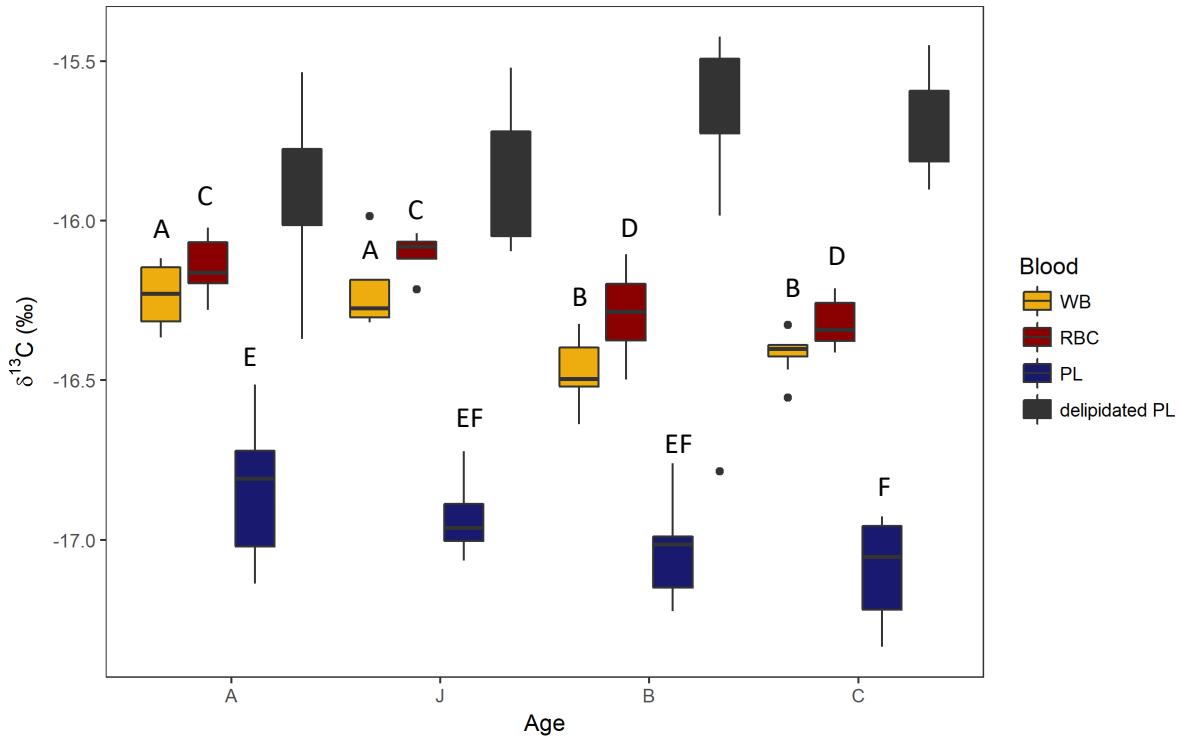

*Figure 3-1: A boxplot showing the mean and interquartile range (IQR) of $\delta^{13}C$ of blood components (whole blood - WB, red blood cells - RBC, plasma – PL and delipidated PL) across penguins in four age classes (A- adults, J- juveniles, B- blues and C- chicks). Whiskers indicate the range of values (< 1.5*IQR). Points indicate outliers. Homogenous groups for $\delta^{13}C_{WB}$ denoted by A-B, for $\delta^{13}C_{RBC}$ denoted by C-D and for $\delta^{13}C_{PL}$ denoted by E-F.*

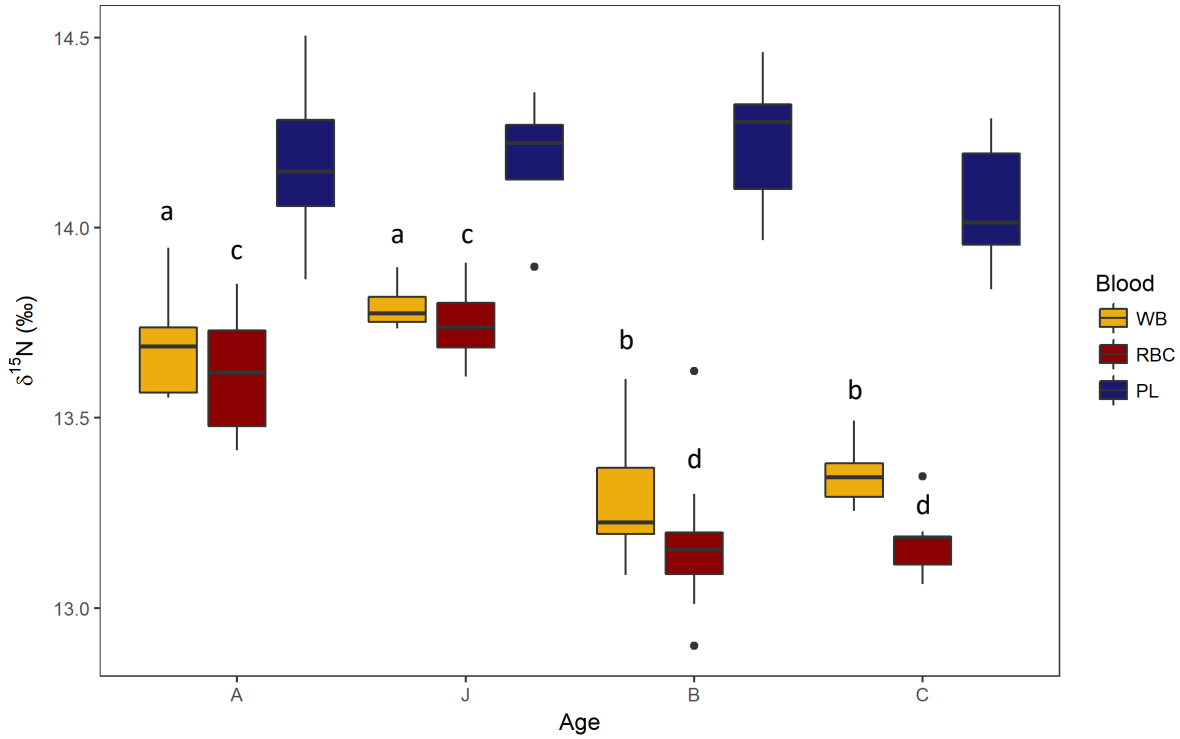

*Figure 3-2: A boxplot showing the mean and interquartile range (IQR) of $\delta^{15}N$ of blood components (whole blood - WB, red blood cells – RBC and plasma - PL) across penguins in four age classes (A- adults, J- juveniles, B- blues and C- chicks). Whiskers indicate the range of values (< 1.5*IQR). Points indicate outliers. Homogenous groups for $\delta^{15}N_{WB}$ denoted by a-b and for $\delta^{15}N_{RBC}$ denoted by c-d.*

Blues swimming for an hour (n=3) had a significantly higher $\delta^{15}N$ (average = 13.4 ‰) than blues swimming for < 5 minutes (n=4; average = 13.2 ‰), while the blue (13.2 ‰) swimming for 20 minutes (n=1) had a $\delta^{15}N$ value similar to both 1 hour swimmers and no force swimmers ($F_{(2,5)} = 7.15$, $p = 0.034$).

There were significant differences among the C:N ratios of the four penguin age classes for WB, which on average ranged from 3.32 to 3.40 ($F_{(3,32)} = 12.02$, $p < 0.0001$), and for PL, which on average ranged from 4.01 to 4.22 ($F_{(3,31)} = 8.80$, $p = 0.0002$) but not for RBC ($F_{(3,31)} = 0.89$, $p = 0.456$). The chicks and blues had significantly higher C:N ratios than the adults, but were similar to one another. Blues and the juveniles were similar, and the juveniles and adults had similar C:N ratios for WB (Sup. Table 3). Regarding the C:N ratios of PL, the chicks and blues again had a higher ratio than adults but juveniles had a similar C:N ratio to all other age classes. Since most of the $\delta^{13}C_{\text{delipidated PL}}$ was calculated, as opposed to measured, because there wasn't enough PL to delipidate some, the C:N ratio was also calculated. It, therefore, could not be compared across age classes.

There were significant differences among the $\Delta^{13}C$ of age classes for $\Delta^{13}C_{\text{WB – FL}}$, $\Delta^{13}C_{\text{WB – WF}}$, $\Delta^{13}C_{\text{WB – delipidated FL}}$, $\Delta^{13}C_{\text{WB – delipidated WF}}$, as well as for $\Delta^{13}C_{\text{delipidated PL – FL}}$, $\Delta^{13}C_{\text{delipidated PL – WF}}$, $\Delta^{13}C_{\text{delipidated PL – delipidated FL}}$, $\Delta^{13}C_{\text{delipidated PL – delipidated WF}}$, (all $p < 0.01$ for WB and $p \leq 0.01$ for delipidated PL minus each of the four fish components respectively; Sup. Table 4). The greatest difference among WB DFs of different age classes was between $\Delta^{13}C_{\text{WB – delipidated FL}}$ of chicks and blues (which had the same $\Delta^{13}C$), and juveniles and was 0.2 ‰. For $\Delta^{13}C_{\text{WB – FL}}$ and $\Delta^{13}C_{\text{WB – WF}}$, blues had values that were significantly smaller than chicks and adults while juveniles did not differ from any other age class (Figure 3-3). The chicks and the blues were not significantly different from each other but had significantly lower $\Delta^{13}C$s than juveniles and adults for the $\Delta^{13}C_{\text{WB – delipidated FL}}$. For the $\Delta^{13}C_{\text{WB – delipidated WF}}$, chicks were not significantly different from blues, juveniles or adults, but blues had significantly lower $\Delta^{13}C$ than juveniles and adults (Figure 3-3). The greatest difference among delipidated PL DFs of different age classes was between $\Delta^{13}C_{\text{delipidated PL – FL}}$ of chicks and adults and was 0.5 ‰. Chicks had significantly higher $\Delta^{13}C$ than adults for both $\Delta^{13}C_{\text{delipidated PL – FL}}$ and $\Delta^{13}C_{\text{delipidated PL – WF}}$, but not than blues in either, and not than juveniles for $\Delta^{13}C_{\text{PL – WF}}$. Blues, juveniles and adults were not significantly different from each other. The $\Delta^{13}C_{\text{delipidated PL – delipidated FL}}$ and $\Delta^{13}C_{\text{delipidated PL – delipidated WF}}$ showed the same pattern of chicks and blues having significantly greater $\Delta^{13}C$ than adults, while juveniles were not significantly different from any other age classes (all results for DFs listed in Sup. Table 4). The $\Delta^{13}C$ were significantly different among age classes for RBC when considering the delipidated fish components ($\Delta^{13}C_{\text{RBC – delipidated FL}}$: $F_{(3,32)} = 14.23$, $p < 0.0001$; $\Delta^{13}C_{\text{RBC – delipidated WF}}$: $F_{(3,32)} = 3.33$, $p = 0.032$) but not for the FL and WF ($\Delta^{13}C_{\text{RBC –FL}}$: $W_{(3)} = 3.20$, $p = 0.362$; $\Delta^{13}C_{\text{RBC – WF}}$: $F_{(3,32)} = 1.65$, $p = 0.197$). The chicks and blues were not significantly different from each other but had significantly lower $\Delta^{13}C$s than juveniles

and adults for the $\Delta^{13}C_{RBC\ -\ delipidated\ FL}$. A similar pattern was seen in the average $\Delta^{13}C_{RBC\ -\ delipidated\ WF}$, although a post hoc analysis could not determine where the significant differences lay. In contrast, no significant differences were found among the $\Delta^{13}C$ of age classes for $\Delta^{13}C_{PL\ -\ FL}$, $\Delta^{13}C_{PL\ -\ WF}$, $\Delta^{13}C_{PL\ -\ delipidated\ FL}$, and $\Delta^{13}C_{PL\ -\ delipidated\ WF}$ (Sup. Table 4).

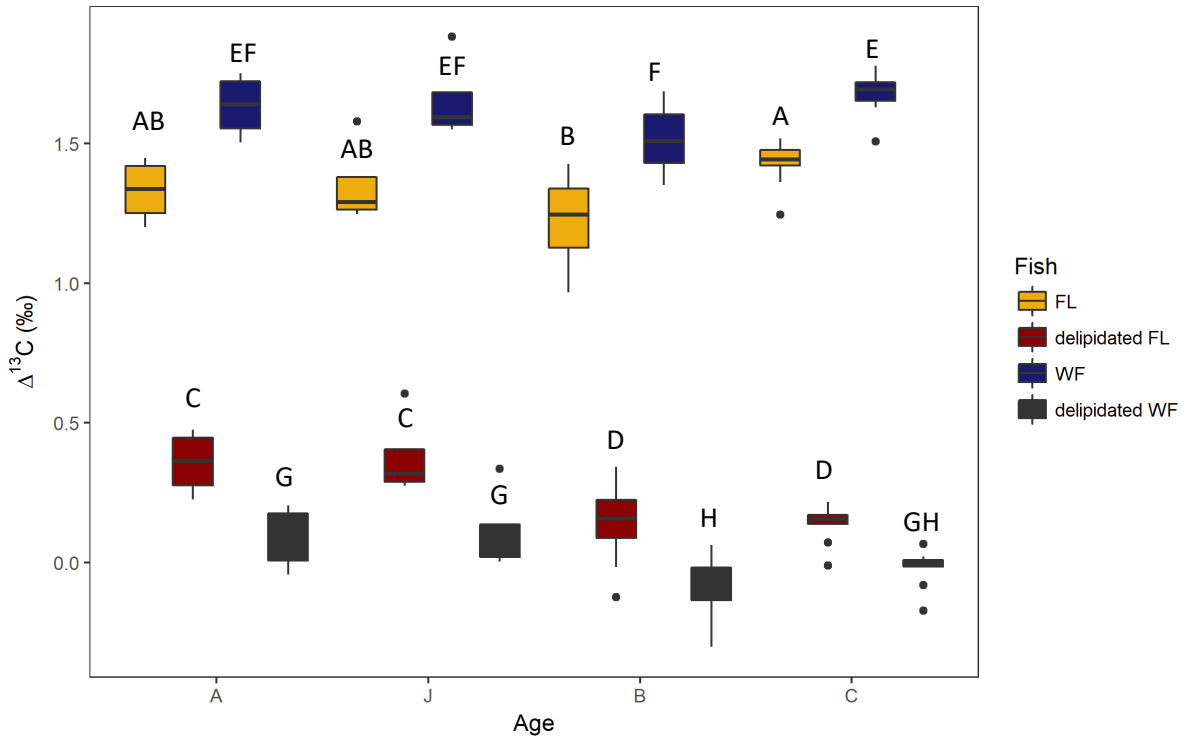

*Figure 3-3: A boxplot showing the mean and interquartile range (IQR) of $\Delta^{13}C_{WB\ -\ fish}$ of fish components (flesh – FL, delipidated FL, whole fish – WF and delipidated WF) across penguins in four age classes (A- adults, J- juveniles, B- blues and C- chicks) with whiskers indicating the range of values below 1.5*IQR. Points indicate outliers. Homogenous groups for $\Delta^{13}C_{WB\ -\ FL}$ denoted by A-B, for $\Delta^{13}C_{WB\ -\ delipidated\ FL}$ denoted by C-D, for $\Delta^{13}C_{WB\ -\ WF}$ denoted by E-F and $\Delta^{13}C_{WB\ -\ delipidated\ WF}$ denoted by G-H.*

When considering the $\Delta^{15}N_{PL\ -\ FL}$ and $\Delta^{15}N_{PL\ -\ WF}$, there was no significant difference amongst the age classes ($\Delta^{15}N_{PL\ -\ FL}$: $F_{(3,32)} = 12.31$, $p = 0.096$; $\Delta^{15}N_{PL\ -\ WF}$: $F_{(3,32)} = 0.75$, $p = 0.534$), though blues always had the highest average $\Delta^{15}N$ (Sup. Table 4). However, a comparison among $\Delta^{15}N_{WB\ -\ FL}$, $\Delta^{15}N_{WB\ -\ WF}$, $\Delta^{15}N_{RBC\ -\ FL}$ and $\Delta^{15}N_{RBC\ -\ WF}$ showed significant differences among the age classes ($F_{(3,32)} = 17.28$ and 23.65, $p < 0.0001$ for $\Delta^{15}N_{WB\ -\ FL}$ and $\Delta^{15}N_{WB\ -\ WF}$ respectively [Figure 3-4], $F_{(3,32)} = 12.32$ and 21.83, $p < 0.0001$ for $\Delta^{15}N_{RBC\ -\ FL}$ and $\Delta^{15}N_{RBC\ -\ WF}$ respectively; Sup. Table 4). Blues had significantly lower $\Delta^{15}N$ ratios than juveniles and adults, the juveniles always exhibiting the highest $\Delta^{15}N$. For both the $\Delta^{15}N_{WB\ -\ FL}$ and $\Delta^{15}N_{RBC\ -\ FL}$, which ranged from 2.2 ‰ to 2.6 ‰ and 2.1 ‰ to 2.6 ‰ respectively, the chicks were not significantly different from the juveniles and adults. For the $\Delta^{15}N_{WB\ -\ WF}$ and $\Delta^{15}N_{RBC\ -\ WF}$, which ranged from 2.6 ‰ to 3.1 ‰ and 2.5 ‰ to 3.1 ‰ respectively, the chicks and blues were not significantly different from one another, but both had significantly lower $\Delta^{15}N$ ratios than juveniles and adults (Figure 3-4; Sup. Table 4).

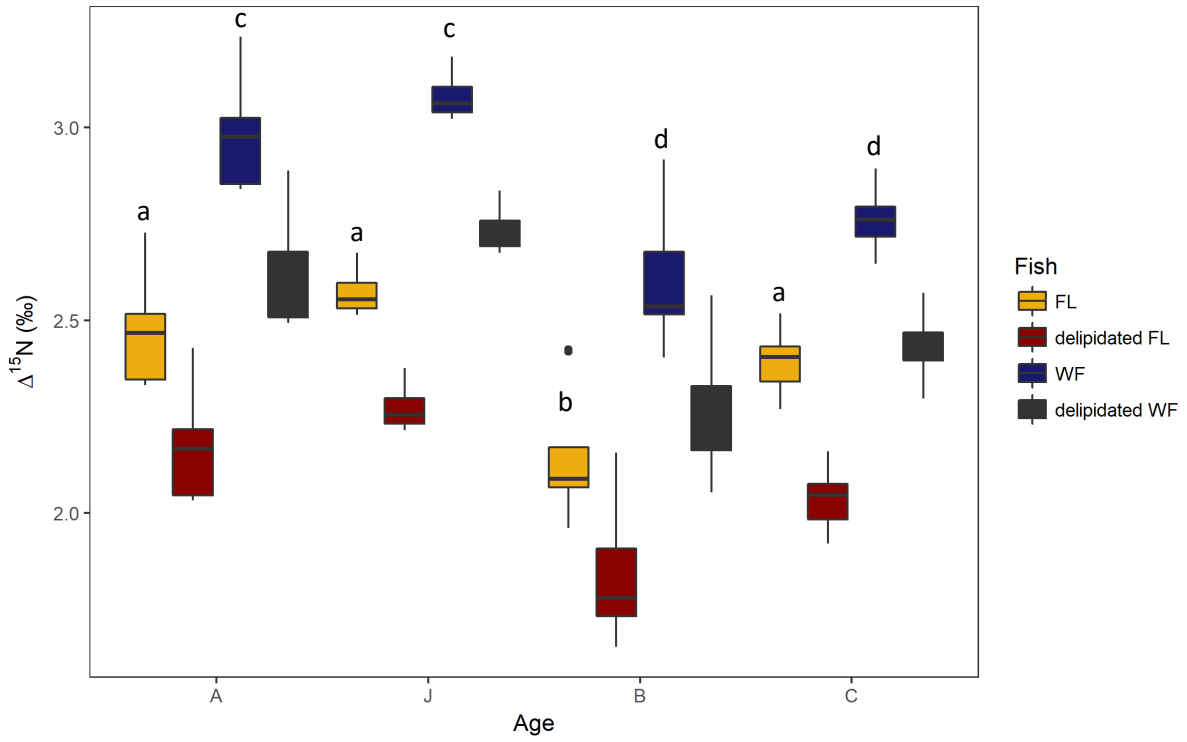

*Figure 3-4: A boxplot showing the mean and interquartile range (IQR) of $\Delta^{15}N_{WB\ -\ fish}$ of fish components (flesh – FL, delipidated FL, whole fish – WF and delipidated WF) across penguins in four age classes (A- adults, J- juveniles, B- blues and C- chicks). Whiskers indicate the range of values (< 1.5*IQR). Points indicate outliers. Homogenous groups for $\Delta^{15}N_{WB\ -\ FL}$ denoted by a-b and for $\Delta^{15}N_{WB\ -\ WF}$ denoted by c-d.*

3.2.1.2 Effects of sex within age class

Adult males (n=7) were significantly heavier than adult females (n=6) by an average of 437 g ($t_{(11)} = 2.63$, $p = 0.024$). Female chicks (n=5) were significantly younger than male chicks (n=4) by 3.2 days ($t_{(9)} = -3.04$, $p = 0.029$). There was no significant difference between male and female $\delta^{13}C$ values for any blood component or age class, but sex did affect some $\delta^{15}N$ values (Table 3-2). The $\delta^{15}N_{WB}$ of the male chicks was enriched in ^{15}N compared to female chicks (13.4 ‰ vs 13.3 ‰, respectively; $t_{(9)} = -3.44$, $p = 0.018$), while male adults, had significantly lower $\delta^{15}N_{WB}$ values than female adults (13.6 ‰ vs 13.8 ‰, respectively; $t_{(11)} = 2.34$, $p = 0.040$) as seen in Sup. Table 5. Since there was a difference between males and females in chicks and adults, the difference among age classes was compared separately for males and females. The $\delta^{15}N_{WB}$ of males and females of the adult and the juvenile were significantly higher than those of the chicks and the blues for both males and females ($F_{(3;14)} = 16.81$, $p < 0.0001$ and $W_{(3)} = 12.90$, $p = 0.005$ respectively), but neither male nor female adults were significantly different from the respective juveniles, and neither male nor female blues were significantly different from the respective chicks (Sup. Table 5). There were no significant differences between males and females $\delta^{15}N_{WB}$ in blues or juveniles, or between male and female $\delta^{13}C_{WB}$ values, nor between male and female $\delta^{13}C$ or $\delta^{15}N$ of PL, RBC, or delipidated PL for all four age classes (all $p > 0.058$).

Though they ate the same things (Sup. Table 6), the $\Delta^{15}N_{WB-FL}$ and $\Delta^{15}N_{WB-WF}$ of the male chicks were significantly greater than those of the female chicks, by c. 0.3 ‰ for both ($t_{(9)} = -3.58$, $p = 0.009$ and $t_{(9)} = -3.46$, $p = 0.014$ respectively; Sup. Table 7). The male adults also exhibited significantly higher $\Delta^{15}N_{WB-FL}$ and $\Delta^{15}N_{WB-WF}$ than females, by 0.2 ‰ for both ($t_{(10.81)} = 2.34$, $p = 0.040$ for both; Sup. Table 7). The $\Delta^{15}N_{WB}$ values of males were significantly different across age classes for both fish components (FL: $F_{(3,14)} = 8.78$, $p = 0.002$; WF: $F_{(3,14)} = 11.80$, $p = 0.0004$) and followed the same pattern regardless of which fish component was considered. For females, the $\Delta^{15}N_{WB-FL}$ and $\Delta^{15}N_{WB-WF}$ were also significantly different across age classes ($\Delta^{15}N_{WB-FL}$: $F_{(3,14)} = 13.40$, $p = 0.0002$; $\Delta^{15}N_{WB-WF}$: $F_{(3,14)} = 17.53$, $p < 0.0001$); blues had a significantly lower $\Delta^{15}N$ than adults and juveniles, though they had a similar $\Delta^{15}N$ to that of the chicks. For the $\Delta^{15}N_{WB-WF}$ of females, the blues and chicks had a significantly lower DF than adults and juveniles (Sup. Table 7), while the juveniles and adults did not differ significantly from one another.

Table 3-2: Summary of results of comparisons of blood components (whole blood – WB, red blood cells – RBC, plasma – PL, and delipidated PL) and fish components (flesh – FL, whole fish – WF, delipidated FL and delipidated WF) between sexes within four age classes.

Component	Tissue	Age class – significantly different between sexes			
		Adults	Juveniles	Blues	Chicks
Stable isotope values & C:N ratios					
$\delta^{13}C$	WB, RBC, PL and delipidated PL	no	no	no	no
$\delta^{15}N$	WB	yes	no	no	yes
$\delta^{15}N$	RBC and PL	no	no	no	no
C:N	WB	yes	yes	yes	yes
C:N	RBC	yes	yes	yes	yes
C:N	PL	no	no	no	no
Discrimination factors					
$\Delta^{13}C$	WB-FL and WB-WF	no	no	no	no
$\Delta^{13}C$	WB-delipidated FL and WB-delipidated WF	no	no	no	no
$\Delta^{15}N$	WB-FL and WB-WF	yes	no	no	yes

3.2.2 Physiology of breeding adults

There was no significant difference in $\delta^{13}C$ across EPPs for WB, RBC, PL or delipidated PL, but there was a significant effect of the interaction of sex and EPP on the $\delta^{13}C_{WB}$ ($F_{(3,20)}$ = 3.98, p = 0.023, summarised in Table 3-3), when controlling for the $\delta^{13}C$ of the two raw fish components, the $\delta^{15}N$ of the respective blood samples and weight (Figure 3-5; Sup. Table 8). The only covariate which was significant was FL for both $\delta^{13}C_{WB}$ and $\delta^{13}C_{RBC}$ ($\delta^{13}C_{WB}$: $F_{(1,16)}$ = 6.85, p = 0.017; $\delta^{13}C_{RBC}$: $F_{(1,16)}$ = 15.17, p = 0.001). Sex also significantly influenced the $\delta^{13}C_{PL}$ ($F_{(1,16)}$ = 15.71, p = 0.0004), but not RBC or delipidated PL. Weight had a significant effect on the $\delta^{13}C$ of PL ($F_{(1,16)}$ = 11.91, p = 0.003). When using the delipidated fish components instead of the raw components as covariates, the same pattern was seen (Figure 3-5; Sup. Table 9).

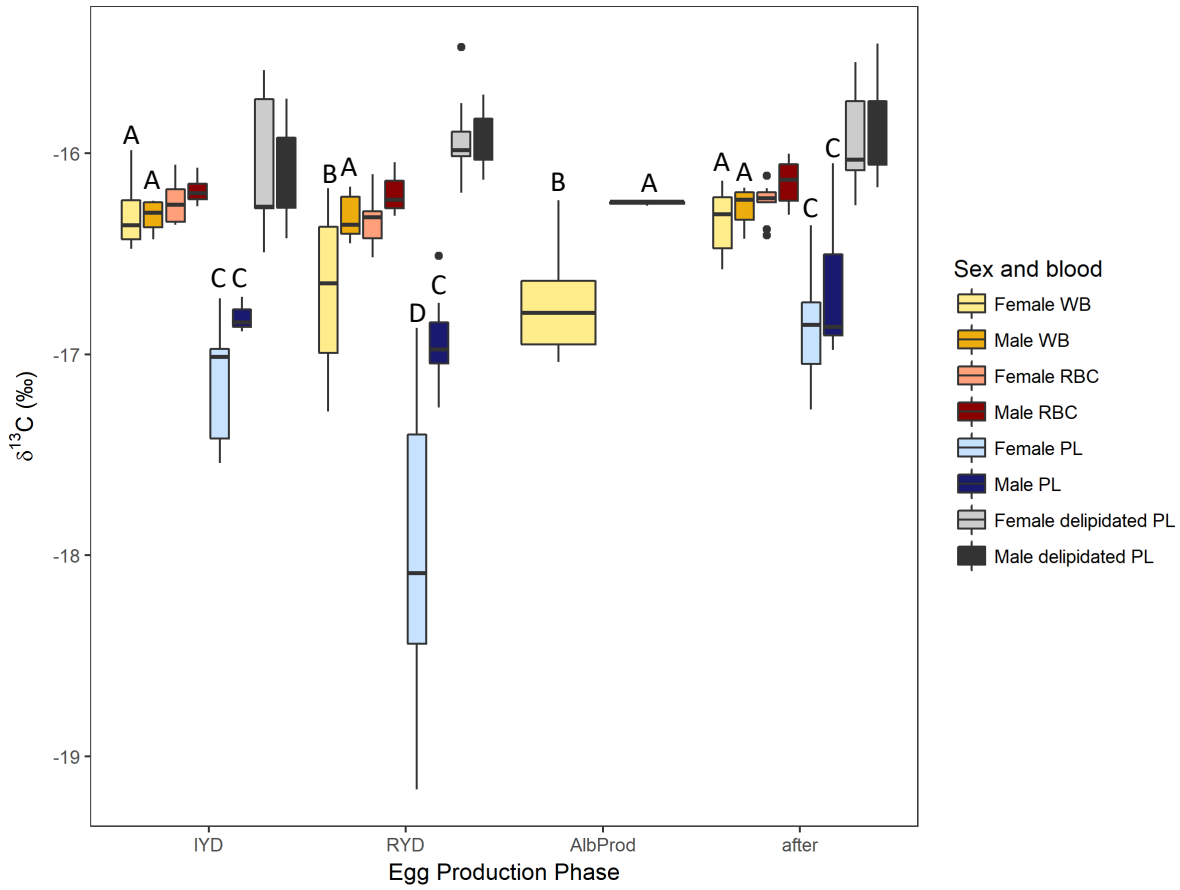

*Figure 3-5: A boxplot showing the mean and interquartile range (IQR) of change in $\delta^{13}C$ of males and females over four egg production phases (initial yolk deposition -IYD, rapid yolk deposition - RYD, albumen production - AlbProd, and after laying - after) for three blood components (whole blood - WB, red blood cells – RBC, plasma – PL and delipidated PL) with whiskers indicating the range of values below 1.5*IQR. Points indicate outliers. Homogenous groups for WB are denoted by A-B and by C-D for PL.*

Neither sex nor EPP, nor the interaction of the two had a significant influence on the $\delta^{15}N_{WB}$, $\delta^{15}N_{RBC}$, or $\delta^{15}N_{PL}$, when controlling for the $\delta^{15}N$ of the two raw fish components, weight and $\delta^{13}C$ of the corresponding blood samples (Figure 3-6; Sup. Table 10). Penguin ID had a significant influence on the $\delta^{15}N_{WB}$ and $\delta^{15}N_{RBC}$ ($F_{(14,16)}$ = 9.07 and 7.71, $p < 0.0001$ for WB and RBC, summarised in Table 3-3). DFs were not considered as the effect of food was included into the GLM analyses.

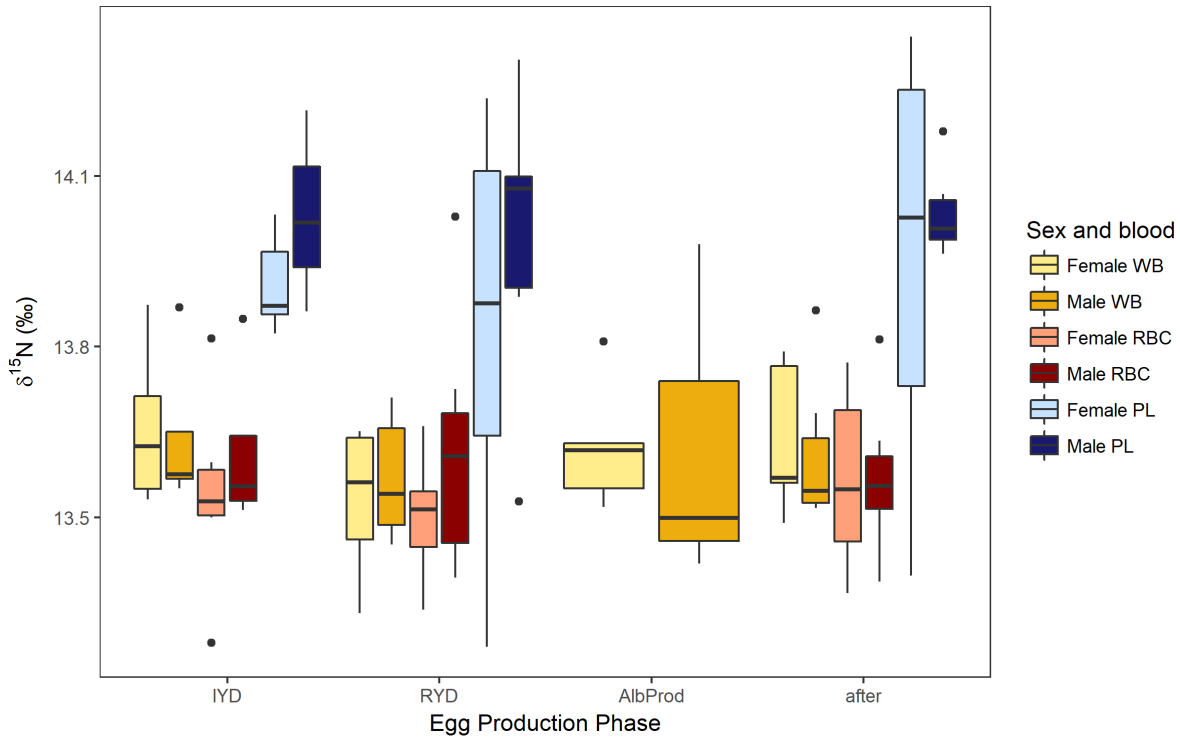

*Figure 3-6: A boxplot showing the mean and interquartile range (IQR) of change in $\delta^{15}N$ of males and females over four egg production phases (initial yolk deposition -IYD, rapid yolk deposition - RYD, albumen production - AlbProd, and after laying - after) for three blood components (whole blood - WB, red blood cells - RBC and plasma - PL) with whiskers indicating the range of values below 1.5*IQR. The points indicate outliers.*

Table 3-3: Summary of significant effects on the stable isotope (SI) values of blood components (whole blood – WB, red blood cells – RBC and plasma – PL) across egg production phases (EPPs). NA *is used when analysis not conducted.*

Component	Tissue	Categorical variable	Continuous variable using raw FL and WF	Continuous variable using delipidated FL and WF
$\delta^{13}C$	WB	Sex*EPP and sex	FL	delipidated FL
$\delta^{13}C$	RBC	none	FL	delipidated FL
$\delta^{13}C$	PL	Sex and individual	Weight	Weight
$\delta^{13}C$	delipidated PL	none	none	None
$\delta^{15}N$	WB	Individual	none	NA
$\delta^{15}N$	RBC	Individual	none	NA
$\delta^{15}N$	PL	none	none	NA

3.3 Discussion

As SIA is used routinely in wild studies nowadays, calls for more laboratory work particularly investigating the influence an individual's physiology on its stable isotope pool continue to be made (Gannes et al. 1997, Martínez del Rio et al. 2009a, Bastos et al. 2017). Areas of knowledge that are fundamental for the effective use of SIA for diet studies of wild birds include assimilation and routing of isotopes, tissue turnover times, accurate DFs, and a sound understanding of how different conditions may influence these values (Bond & Jones 2009, Martínez del Rio et al. 2009b). Several studies have been conducted to determine whether physiology influences the SI values of various tissues, and have concluded that it does (reviewed in Boecklen et al. 2011). The results from the present study are in accord with this conclusion, but various conditions which birds may experience influence the SI values of different tissues in multiple ways.

In this chapter, the questions considered were whether the age and the EPP of seabirds influenced the $\delta^{15}N$ and $\delta^{13}C$ of three blood components – WB, RBC and PL, using African penguins as a case study. This was done to enable studies conducted in the wild to draw better conclusions about their findings. Both age and EPP influenced the $\delta^{13}C_{WB}$, while only age influenced the $\delta^{15}N_{WB}$. Neither the $\delta^{13}C_{RBC}$ or $\delta^{15}N_{RBC}$ was affected by EPP but age affected both if the delipidated FL component of prey was assessed to reduce the influence of food on the $\delta^{13}C_{RBC}$. If the FL was not delipidated, no effect of age on $\delta^{13}C_{RBC}$ was seen. The $\delta^{13}C_{PL}$ was influenced by EPP but not age, while the $\delta^{15}N_{PL}$ was not influenced by either age or EPP.

All penguins were in the centre over the same period, therefore limiting the variation in conditions under which the penguins were kept. It was, however, unavoidable that chicks and blues were fed sardine and formula to ensure healthy growth. Therefore, the DFs between blood types and fish components were assessed to remove as much as possible of the confounding effect that a diet, which varied with age, would add to the analyses. Different SI values associated with similar DFs across age classes were assumed to indicate little to no influence of age on the SI patterns seen in the blood components, which rather solely reflect the effects of diet. Conversely, significantly different DFs were more likely to signify that the SI pattern observed in the blood components was influenced by age.

3.3.1 Effect of ontogeny on carbon stable isotopes and discrimination factors

There are gaps in our understanding of why $\delta^{13}C$ values behave as they do (Williams et al. 2007), but as in Williams et al. (2007) for tuffted puffins, the $\delta^{13}C$ of WB and RBC were significantly lower in chicks. The pattern was not as clear in the $\delta^{13}C$ of PL but the chicks still had significantly lower $\delta^{13}C$ values than adults (Figure 3-1). The $\Delta^{13}C_{RBC\ –\ FL}$ and $\Delta^{13}C_{RBC\ -\ WF}$ and the $\Delta^{13}C_{PL\ –\ FL}$ and $\Delta^{13}C_{\ PL\ -\ WF}$ were

homogenous across ages, signifying that the chicks and blues had lower $\delta^{13}C$ in RBC and PL than the two other age classes due to their different diet. Their diet of sardine was supplemented with formula, which was richer in lipids compared to sardines (as indicated by the C:N ratios), and translated in more lipids circulating in the body. The $\Delta^{13}C_{WB\ -\ FL}$ and $\Delta^{13}C_{WB\ -\ WF}$ were not all homogenous, suggesting that the difference seen in $\delta^{13}C_{WB}$ among age classes was not solely influenced by diet but also by age. The significantly lower $\delta^{13}C_{WB}$ values of chicks and blues may be indicative of more lipids being transported and the significantly higher C:N ratios seen in the WB of chicks and blues corroborated that. The C:N ratios of PL of chicks and blues was also significantly higher than that of adult birds, though the $\Delta^{13}C$ attributed the pattern seen in $\delta^{13}C_{PL}$ to be more likely due to food. A limitation of C:N ratio in growing birds to keep in mind though is that both the $\delta^{13}C$ and $\delta^{15}N$ values were influenced by age and therefore may influence the C:N ratio in an unknown way.

The blues are the age group at which the penguins start swimming for the first time. They may, therefore, rely more on the lipids in their diet or even utilise some of their endogenous reserves (Lemon 1991), both of which would cause a decrease in the $\delta^{13}C$ value, as lipids are depleted in ^{13}C compared to other tissues (Budge et al. 2008). This lower $\delta^{13}C$ was seen in WB, RBC and PL, but the $\Delta^{13}C$ attribute the pattern in RBC and PL almost solely to diet. It is possible that, as with proteins, increased carbohydrate assimilation from the food decreases the DF, as the assimilation process becomes less efficient and takes up more ^{13}C depleted nutrients to satisfy the body's carbon needs (MacAvoy et al. 2005, Williams et al. 2007). Though there was a difference between the younger and older penguins, the greatest difference between $\Delta^{13}C$ of any two age classes, was between $\Delta^{13}C_{WB\ -\ delipidated\ FL}$ of the younger penguins and juveniles. The observed difference was 0.2 ‰, which is not likely to have a significant influence on the ecological interpretation of the data, as it is not great enough to be considered a difference in feeding source or trophic level (Hobson et al. 1994, Hill & McQuaid 2008, Kurle & McWhorter 2017).

The C:N ratios of PL were significantly higher in chicks and blues compared to adults which encouraged me to use delipidation to reduce the influence of lipids on the $\delta^{13}C_{PL}$. Delipidating the PL removed lipids and reduced the variation between the $\delta^{13}C$ values among the four age classes. The C:N ratios of delipidated PL were c. 0.6 units smaller than those of PL for juveniles and adults and 0.8 units smaller for chicks and blues indicating that more lipids were removed by delipidation in chicks. The $\Delta^{13}C_{delipidated\ PL}$ were significantly different across age classes, which indicated that the $\delta^{13}C_{delipidated\ PL}$ is influenced by diet and age. There was no significant difference in the $\delta^{13}C_{delipidated\ PL}$ among age classes, but since it was influenced by both diet and age, this should not be misinterpreted for penguins eating the same diet, as in this case they definitely were not.

Becker et al. (2007) suggested that, to minimise unexplained variation in $\delta^{13}C$ results, it is better to use delipidated prey SI values, especially when looking at $\Delta^{13}C$. The $\Delta^{13}C_{PL\ -\ delipidated\ WF}$ and $\Delta^{13}C_{PL\ -\ delipidated\ FL}$, indicated that the $\delta^{13}C_{PL}$ was predominantly influenced by the diet of the penguins as there was no difference in DFs among the four different age classes. This is no different from the conclusion drawn from the $\Delta^{13}C_{PL\ -\ WF}$ and $\Delta^{13}C_{PL\ -\ FL}$. The same was seen for the $\Delta^{13}C_{RBC\ -\ delipidated\ WF}$, but the $\Delta^{13}C_{RBC\ -\ delipidated\ FL}$ showed something slightly different. The chicks and blues exhibited significantly smaller DFs than the juveniles and adults. This indicates that the chicks and blues had a significantly smaller $\delta^{13}C_{RBC}$ values than the juveniles and adults, not solely due to diet but also because they were younger. Although this was not seen for the other three DFs, it may indicate that younger birds rely more on lipids, despite the lack of a significant difference in C:N ratios of RBC between the four age classes. The $\Delta^{13}C_{WB\ -\ delipidated\ FL}$, also showed that chicks and blues had similar DFs, which were significantly smaller than those of the juveniles and adults by 0.2 ‰. The $\Delta^{13}C_{WB\ -\ delipidated\ WF}$ showed a similar pattern but chicks were not different from juveniles and adults (Figure 3-3). As both DFs showed differences across ages, the simpler explanation is that age did have an influence on the $\delta^{13}C_{WB}$, though the range of DFs was very small (0.2 ‰). The small difference between chicks and juveniles $\Delta^{13}C_{WB\ -\ delipidated\ FL}$ (0.2 ‰), is probably not small enough to undermine assumptions about diet source (Hill & McQuaid 2008, Kurle & McWhorter 2017).

The idea of removing the lipids of the prey and considering the DFs between tissue and delipidated prey components may give a more accurate estimate of SI values was suggested by Becker et al. (2007). In this case, however, this did not seem to change the main conclusions, except in the case of $\Delta^{13}C_{RBC\text{-}delipidated\ FL}$. Polito et al. (2009) conducted a similar study on captive gentoo penguins (*Pygoscelis papua* Forster, 1781), but looked at the SI values of egg components as well as the DFs between the egg components and the FL and WF components of the prey. They found a difference in DF depending on which delipidated prey tissue was used. It seems that it is more important to know which value is used for wild studies as there can be a 0.2 ‰ difference between DF_{FL} and DF_{WF}, while using delipidated prey DFs decreases the DF by c. 2 ‰, as seen in this study and in Cherel et al. (2005a). My results showed that it is important to take physiology into consideration when interpreting SI values of penguins of a young or still growing age, as suggested by Williams et al. (2007). It is important to know the range of DFs between younger and older birds as chicks are easier to sample from colonies in the wild as they do not leave until they have fledged (e.g. Forero et al. 2002, Yohannes et al. 2014).

3.3.2 Effect of ontogeny on nitrogen stable isotopes and discrimination factors

Due to the fractionation at each chemical reaction, the lighter molecules would be excreted first through a catabolic reaction. Uric acid is the end product of protein catabolism and, if transported in

high quantities could artificially decrease the $\delta^{15}N$ of blood (Steele & Daniel 1978, Peterson & Fry 1987, Bearhop et al. 2000, Williams et al. 2007). An organism may produce higher levels of uric acid and urea during its growth period (Skadhauge 1983), and a decrease in the $\delta^{15}N$ value would therefore be expected in growing individuals. The hypothesis that $\delta^{15}N$ would be lower in the younger birds compared to the older penguins was confirmed in this study. Accordingly, lower $\delta^{15}N_{WB}$ and $\delta^{15}N_{RBC}$ values in the younger penguins were measured in this study; the $\delta^{15}N$ values of chicks and blues were low compared to juveniles and adults.

Ponsard & Averbuch's (1999) prediction that growing animals should reflect the same $\delta^{15}N$ as their adult counterparts on the same diet, may be upheld as the chicks' food shows the same pattern of being lower in $\delta^{15}N$ than what was fed to the adults. The $\Delta^{15}N_{WB-FL}$ and $\Delta^{15}N_{RBC-FL}$, however, showed that the younger penguins did not have lower $\delta^{15}N$ values solely because of food but that there was also an effect of age, going against the prediction made by Ponsard and Averbuch (1999). The blues had significantly smaller $\Delta^{15}N_{WB-FL}$, $\Delta^{15}N_{WB-WF}$, $\Delta^{15}N_{RBC-FL}$, and $\Delta^{15}N_{RBC-WF}$ than adults and juveniles, which may be because at this stage of growth they were swimming, for five minutes, 20 minutes or an hour, three times a day, which would encourage muscle growth. An increase in exercise increases the body's need for proteins, which are catabolised for energy and muscle growth (Lemon 1991, Campbell et al. 2007). Similarly to intense growth in chicks, this increased catabolic activity would result in an increase in uric acid (Lumeij 1987), which could lead to a decrease in the $\delta^{15}N$ of blood. Blues were made to swim for different lengths of time ranging from no-force swim of less than five minutes to an hour swim. Only a significant difference in $\Delta^{15}N_{WB-WF}$ among blues swimming in different regimes was observed, with the penguins swimming for the least time having the smallest average $\Delta^{15}N$. This may be because the penguins swimming for less than five minutes were just starting to develop their muscles which may require more proteins than the penguins swimming for an hour using well developed muscles. If that is the case, there should be a clear difference in the $\Delta^{15}N_{PL-FL}$ or $\Delta^{15}N_{PL-WF}$ between the no force swimmers and the birds swimming for 1 hour, as PL turns over faster and would reflect a change faster than WB (Barquete et al. 2013).

There were no significant differences for $\Delta^{15}N_{PL-FL}$ or $\Delta^{15}N_{PL-WF}$ for blues swimming in different regimes, suggesting that all the blues utilised similar amounts of proteins, as a significant difference in protein catabolism would have resulted in a marked increase in uric acid, which in turn should have decreased the $\delta^{15}N$ value significantly. A smaller DF was also not seen in either $\Delta^{15}N_{PL-FL}$ or $\Delta^{15}N_{PL-WF}$ of blues compared to the other age classes. The $\delta^{15}N$ of PL is more sensitive to uric acid than WB (Bearhop et al. 2000) but the absence of a lower DF in blues compared to other penguins could indicate that a high uric acid concentration is unlikely to have caused the smaller $\Delta^{15}N$ of blues seen between WB and fish and RBC and fish. The chicks didn't have the same lowered $\Delta^{15}N_{WB-FL}$ and

$\Delta^{15}N_{RBC\ -\ FL}$ which may indicate that the lower $\Delta^{15}N$ seen in blues is not carried over from chick physiology. The $\Delta^{15}N_{WB-WF}$ and $\Delta^{15}N_{RBC-WF}$ of chicks were, however, lowered which may indicate that the lowered $\Delta^{15}N$ in blues was a carry-over effect of the lower protein-use efficiency of chicks (Wolf et al. 1985, Bearhop et al. 2000), especially since the $\Delta^{15}N_{PL}$ of blues was not lower. The lack of difference between the $\Delta^{15}N_{PL\ -\ WF}$ across four penguin age classes, may indicate that the driving force for the difference seen in the $\delta^{15}N_{PL}$ of penguins of different age classes was mainly related to their diet. It is possible that the formula given to the younger birds was higher in protein due to the supplements. An increase in protein content has been seen to increase the $\delta^{15}N$ (Kelly & Martínez del Rio 2010). As the chicks receive more formula, it may mask a decrease in $\delta^{15}N$ due to growth. As the blues receive less formula the effect of a higher protein diet may no longer be seen in the $\delta^{15}N$ explaining the lower values. The shorter turnover of PL may limit the effects of physiology and could potentially offer more reliable information in wild studies, though it reflects the diet of a smaller time window compared to WB or RBC (Barquete et al. 2013, Lourenço et al. 2015).

Both the chicks and blues had a significantly lower $\Delta^{15}N_{WB-WF}$ and $\Delta^{15}N_{RBC-WF}$ than the juveniles and adults but only blues had a significantly smaller $\Delta^{15}N_{WB-FL}$ and $\Delta^{15}N_{RBC-FL}$ than juveniles and adults. When the average $\delta^{15}N_{WF}$ is examined, the range and maximum variation between food fed to penguins of different ages, though still significantly different across ages, was smaller than the range and variation seen in the $\delta^{15}N_{FL}$ of diet fed across age classes (Figure 3-2; Sup. Table 4). The smaller variation in $\delta^{15}N_{WF}$ than in $\delta^{15}N_{FL}$, is in contrast to what has been found by others such as Becker et al. (2007). After studying the DFs between the feathers of the common murre (*Uria aalge* Pontoppidan, 1763) and a diet of capelin (*Mallotus villosus* Müller, 1776), and calculating the SI values for capelin WF, FL and delipidated FL and delipidated WF, they recommended that FL of prey be used to determine DFs as there was less variation in the $\delta^{15}N_{FL}$ values. As I found the reverse, it is unclear which tissue (FL or WF) would be better to use to calculate DFs and give a clearer understanding of what influences the SI values of penguin blood across four age classes. So both the FL and WF were used to calculate $\Delta^{15}N$, and the delipidated fish components were not considered as the delipidated process affects the $\delta^{15}N$ value (Matley et al. 2016).

A marked diminution in $\Delta^{15}N_{WB-FL}$, $\Delta^{15}N_{RBC-FL}$, $\Delta^{15}N_{WB-WF}$ and $\Delta^{15}N_{RBC-WF}$, was observed in blues, the greatest difference between $\Delta^{15}N$ of any two groups, was between $\Delta^{15}N_{RBC-WF}$ of juveniles and blues and amounted to 0.5 ‰, well below the overall average DF between penguins and their food of which the smallest was for $\Delta^{15}N_{WB-FL}$ (2.2 ± 0.2 ‰: Sup. Table 4). These results therefore suggest that, while it is important to take physiology into account, it is unlikely to create trophic level distortions of diet analyses. This is in accordance with Williams et al. (2007) who tested the effect of food restrictions on growing puffins.

3.3.3 Influence of sex on stable isotopes and discrimination factors during non-breeding

There is a slight sexual dimorphism between male and female African penguins (Pichegru et al. 2013). This may influence the physiology in early development so for this reason, this study considered the SI values of males and females within each age class and whether the DFs were influenced by sex. Only the $\delta^{15}N_{WB}$ of adults and chicks differed between males and females, though the sample size for juveniles was very small (2 males and 2 females). Adult males had lower $\delta^{15}N_{WB}$ than females while chick males had a higher $\delta^{15}N_{WB}$ than females. It is unlikely that 3 days difference in average age would result in the difference seen between male and female chick $\delta^{15}N_{WB}$, but it may indicate that the females experienced their peak growth rate more recently. This, however, would possibly be clearer in the PL, which has a faster turnover (Barquete et al. 2013), but there was no difference in $\delta^{15}N_{PL}$ in this study. The heavier adult males may utilise more nutrients to maintain their body mass and therefore have a lower $\delta^{15}N_{WB}$ values, though there was no effect of weight as a co-variate on $\delta^{15}N_{WB}$ values. Studies conducted in the wild have shown a difference in foraging strategies or hunting grounds between sexes (e.g. Clarke et al. 1998, Danckwerts et al. 2016) though, this was not the case in a rehabilitation setting where birds were fed the same diet. Another possible reason for a difference between adult male and female SI values is a difference in nutritional requirements because of egg production (Maklakov et al. 2009). In my case however, unpaired penguins and pairs not breeding at the time were sampled. It is possible that there was a carry-over effect of breeding on the females, while male chicks may grow faster than female chicks therefore resulting in a shift of $\delta^{15}N_{WB}$. Overall, it is unclear why a difference between males and females was observed in non-breeding birds. The effect of physiology during non-breeding adults should be studied to a greater extent.

3.3.4 Influence of sex on stable isotopes and discrimination factors during breeding

Males and females are often considered separately (e.g. Forero et al. 2002, Forero & Hobson 2003, Connan et al. 2018) as they may feed in different locations or target different prey because their nutritional requirements or their diving capabilities differ, for example the production of eggs by females can raise their energy requirements (O'Brien et al. 2016).

The main constituent of the egg yolk is water, but a third of the yolk is made of lipids while proteins make up almost a fifth (Burley & Vadehra 1989c). These are all deposited during the maturing of the oocyte which can start up to 60 days before laying in the case of chickens (*Gallus gallus*; Burley & Vadehra 1989a). During the deposition of lipids and proteins (of which some are lipoproteins; Burley & Vadehra 1989b) into the yolk, the $\delta^{13}C$ of the blood is likely to be influenced, as lipids are transported by the blood for deposition (Burley & Vadehra 1989c). This could affect the SI value of blood components, especially PL (Groscolas 1982) but by delipidating the PL, the effect of lipid

transportation should then be removed (McConnaughey & McRoy 1979, Cherel et al. 2005b). There may be a small effect on the $\delta^{15}N$ values of tissue especially when the proteinaceous component of the egg, the albumen, is deposited 6-0 days before laying (Grau 1982). In this study, $\delta^{15}N$ of WB, RBC and PL were not affected by the EPP. The $\delta^{13}C_{WB}$ was, however, affected by an interaction of sex and EPP. This was mainly an effect on the $\delta^{13}C$ for females in the RYD phase which exhibited significantly lower $\delta^{13}C$ values than females in other phases and lower values than all males (Figure 3-5). There were significant differences in the C:N ratios of blood components among females in different EPPs, with the C:N ratio of females in RYD phase always (except in the case of delipidated PL) being higher than C:N ratio of females in other EPPs. This, together with the significantly lower $\delta^{13}C$ values during RYD, is probably explained by an increased amount of lipids being transported by the blood to be deposited in the yolk up until the female lays the clutch (Groscolas 1982). The PL showed a similar pattern to WB, with a decrease in $\delta^{13}C$ for females during RYD, though the difference was not significant, resulting in only sex having a significant effect on the $\delta^{13}C_{PL}$ value. This surprising result may be explained by the relatively small sample sizes ($n \leq 6$). As expected, the RBC and delipidated PL did not show the same pattern. It is likely that because the lipids and RBC are held in suspension in the PL, when the RBC are separated out of the PL, the signature of the lipids does not influence the cellular blood component as much, though there was still a difference in C:N ratio with females in RYD having significantly higher values. Connan et al. (2016) assessed the WB SI values of male and female African penguins during the breeding and non-breeding seasons. They found that $\delta^{13}C$ of females sampled while not breeding were lower than the values for females sampled while breeding and males in both seasons. These lower $\delta^{13}C$ values were associated with higher C:N ratios suggesting higher lipid content in female blood when they were not breeding. It may be that these females were starting to deposit lipids and preparing egg yolks for the next breeding season. I found the same in this study with the only difference in $\delta^{13}C$ during EPP being driven by the high lipid content during RYD which occurs 7 – 24 days before laying and is not seen in other EPPs. While it is not always possible to know how close to laying the birds are when they are sampled, this clearly influences the results and needs to be considered.

The delipidation of PL was done to remove the potential influence of any lipids being transported that could compromise the interpretation of $\delta^{13}C$ in ecological studies. The homogeneity of delipidated $\delta^{13}C_{PL}$ across EPPs, and the decrease in C:N ratio by 0.8 units, indicates that lipids should be removed from PL in order to get a clearer signal from the PL, as suggested by Bearhop et al. (2000) and Cherel et al. (2005b).

3.4 Conclusions

This study indicated that age does influence the SI values of blood components, but the scale of the change was not enough to give the appearance of a trophic shift or a foraging shift. This study also indicated that sex can influence SI values, with differences observed between non-breeding males and females on the same diet and living under the same conditions. There is a need to further assess the effect of size and sex on SI values, using a larger sample size. The area in which males and females differ is in their energy requirements during reproduction, as the female produces eggs. In breeding birds, this was shown to influence their $\delta^{13}C$ ratios and supports the argument to delipidate PL, as well as to carefully consider C:N ratios of tissue sampled in the wild particularly at the onset of breeding.

For the best outcomes of captive studies, the SI values of prey should be constant over time, and all the organisms included in the experiment need to be on the same diet. This was unfortunately not possible in this study as younger penguins were fed a supplementary formula to ensure their health and quick progress through the rehabilitation centre and back into the wild. The fish used to feed the penguins also exhibited much more variation among batches than expected, but this was overcome by carefully calculating exactly what each bird was fed, taking into account the proportions of each batch of fish in their diet. Some of the other limitations of this study include the fact that the samples sizes were not more than ten, and were as low as two in some cases. Despite this, important information that will be available for wild studies was gathered during this study.

The importance of conducting studies in controlled environments is to be able to test assumptions and gain better understanding of techniques used in conditions where fewer variables can be tested or controlled. The results of the DF determined in the study will be available, so that studies conducted in the wild have more information to work with. Unfortunately, the FA composition of the various blood components could not be studied due to freezer malfunction. This would have helped provide a better understanding of the variations observed in SI as well as establishing bases for the use of FA as trophic markers in the wild. Further studies which could be done include considering the difference in SI values of tissues between males and females, and an attempt to determine what would cause a difference between the two apart from egg production. Other questions concern the influence of prey types on SI values.

Chapter 4 The effect of diet and species on discrimination factors

The use of biomarkers such as stable isotopes (SI), metals or fatty acids has become increasingly popular in the last twenty years for a range of purposes such as the study of migration (e.g. Hobson 1999), food web structure (e.g. Iverson 2009), and pollution (e.g. Boening 1999) to cite a few. Stable isotope analysis (SIA) in particular has become a technique of choice in the ecologist's toolkit, due to the wide range of applications and reasonable costs (Fry 2006, Caut et al. 2009, Bouillon et al. 2012). The suitability of SIA for ecological studies depends on whether reliable information, about diet for example, can be gleaned. What an organism eats and its physiology, are reflected in its tissue isotope signature, subject to a certain discrimination factor (DF) denoted by Δ (DeNiro & Epstein 1978, 1981, Boecklen et al. 2011). If DFs were constant under all circumstances and not influenced by diet, physiology, tissue or species, determining what a species feeds on would be straightforward (Cherel et al. 2005b). Early studies had already shown that DFs were not constant in every situation (DeNiro & Epstein 1978, Hobson & Clark 1992b). More recent works have highlighted the influence of factors such as the metabolic rate, growth rate and breeding of an organism on DFs (Fuller et al. 2004, Williams et al. 2007, Boecklen et al. 2011, chapter 3).

Not only does the DF vary within a species, but also among species, meaning that one DF doesn't fit all taxa (Vanderklift & Ponsard 2003, Cherel et al. 2005b, Bond & Jones 2009, Martínez del Rio et al. 2009b). This was first demonstrated by Hobson and Clark (1992a, b) who showed that domestic chickens (*Gallus gallus* Linnaeus, 1758) and Japanese quails (*Coturnix japonica* Temminck & Schlegel, 1849) fed on the same diet exhibited different DFs. With the development of new mathematical tools to analyse stable isotopes, the accuracy of parameters such as DFs in the models has become crucial (Bond & Diamond 2011, Phillips et al. 2014). At present, several options are used to work around the absence of species- and tissue-specific DFs for use in mixing models to determine the diets of predators. These options include the use of averaged DFs measured in other species (e.g. Forero et al. 2002, Connan et al. 2016, 2017), the SI analysis of prey recovered in the stomach contents of sampled birds (Cherel et al. 2005c) and the mathematical estimation of DFs which includes a wide range of parameters from diet to genetic relatedness to species with known DFs (Healy et al. in press). While such studies are valuable and bring new insights into the ecology of species of interest, the use of accurate tissue- and species-specific DFs may allow for still finer investigations (Bond & Diamond 2011). One way of determining DFs as accurately as possible is to conduct controlled captive studies. Their benefit is that more variables, such as the diet can be controlled, and more tissues and $DF_{consumer\ -\ prey}$ combinations can be assessed. A handful of

controlled studies have been conducted to determine the DFs for $\delta^{13}C$ and $\delta^{15}N$ for seabirds, including penguins (Cherel et al. 2005b, Barquete et al. 2013, Chiaradia et al. 2014, Ciancio et al. 2016), gulls (Hobson & Clark 1992a, b), cormorants (Craig et al. 2015), skuas (Bearhop et al. 2002), eiders (Federer et al. 2010), auklets (Sears et al. 2009) and dunlins though they were fed a terrestrial diet (Evans Ogden et al. 2004). The DFs determined are highly variable within and between species and range from -2.6 ‰ to 7.1 ‰ for $\Delta^{13}C$ and 1.2 ‰ and 4.9 ‰ for $\Delta^{15}N$.

When conducting captive studies to determine DFs, the easiest and most robust way is to collect tissue samples after the predator has been fed a constant diet (Wolf et al. 2009). The necessary duration of a constant diet depends on the turn-over time of the tissue of interest which, for the components of blood, can vary from a week for plasma (PL) to several weeks for red blood cells (RBC) (Hobson & Clark 1992a, b, Bond & Jones 2009). The food fed to animals in zoos and aquariums is managed, providing an opportunity to conduct controlled studies (AZA Penguin Taxon Advisory Group 2014). To determine the DF in a controlled study, it is ideal when the study organism only gets one prey species. If this is not possible, a strict protocol of recording every single feed should be kept; this is, however, not always possible, as was the case with the flying birds at SANCCOB. The present chapter aimed at determining the DFs of several seabird species using captive individuals housed at SANCCOB Table View (SANCCOB TV). These DFs would then be available for use in wild studies. Sardine (*Sardinops sagax* Jenyns, 1842) represented the bulk of their diet, but sardinella (*Sardinella aurita* Valenciennes, 1847) was also sometimes part of their diet though in much lower proportions. The following questions were posed: 1) How do three diets with different proportions of sardine and sardinella influence $\Delta^{13}C_{WB\ -\ prey\ components}$ and $\Delta^{15}N_{WB\ -\ prey\ components}$ in four seabird species?, 2) Are there significant differences among $\Delta^{13}C_{WB\ -\ prey\ components}$ and $\Delta^{15}N_{WB\ -\ prey\ components}$ of seabird species when fed the same diets?, and 3) Are there significant differences among the $\Delta^{13}C_{WB\ -\ prey\ components}$ and $\Delta^{15}N_{WB\ -\ prey\ components}$ calculated with the mostly likely proportions of prey, five seabird species would have fed on?

Assuming a similar assimilation routine for sardine and sardinella nutrients within seabird species, differences in DFs among three different diets tested will be mainly influenced by the diet SI values. Different seabird species may exhibit differences in nutritional status, isotopic routing, or metabolism. Therefore, differences between their DFs are expected and such differences are expected to be greater between seabird foraging guilds (penguins, gulls) than within guilds.

4.1 Methods

4.1.1 Study site

SANCCOB rehabilitates seabirds and occasionally does not release individuals because they have a permanent injury or have become too tame. This means that several species are kept as resident birds providing the opportunity to determine the DFs of multiple species. The species studied here were all kept at SANCCOB TV: kelp gulls (*Larus dominicanus* Lichtenstein, 1823), Hartlaub's gulls (*Larus hartlaubii* Bruch, 1853), Cape cormorants (*Phalacrocorax capensis* Sparrman, 1789), greater crested terns (*Thalasseus bergii* Lichtenstein, 1823) and African penguins (*Spheniscus demersus* Linnaeus, 1758).

Blood samples were collected on the 23rd and 24th June 2016, by venepuncture (as detailed in chapter 2). At the time, none of the sampled individuals were breeding. The flying birds were not over 2 kg and according to SANCCOB the limit to the amount of blood volume that can be drawn is 0.1 % of their body weight. The maximum volume of blood attainable from the Hartlaub's gulls and greater crested terns was thus 0.3 mL, limiting the investigation for these species to whole blood (WB) samples only. Considering the limited number of birds at the centre for some species, the potential influence of sex on DFs could not be considered.

The flying birds were fed two species of fish, namely sardine and sardinella but unfortunately it was logistically not possible to record the proportions of each accurately. The most likely proportions of fish eaten by the various seabirds were determined by general observation of the behaviour and habits of the birds in the rehabilitation centre, over multiple years that I worked in the centre. The most-likely percentage of sardine in the diet was estimated to be 90-95 % sardine for Cape cormorants, 85-90 % sardine for kelp gulls, 75-80 % sardine for Hartlaub's gulls and 80-85 % sardine for greater crested terns. Sardinella were on offer one meal out of four, which was taken into consideration when estimating the most likely proportions of each prey species eaten by each bird species. For this study, all diets used to calculate DFs were estimates and were labelled as diet 1, diet 2 or diet 3. The diet proportions are represented either as sardine:sardinella (e.g. 50:50), or as a percentage of sardine in the diet (e.g. 50 %). The three sardine:sardinella proportions used for the first two objectives of this section of the study were 50:50 – diet 1, 75:25 – diet 2, and 100:0 – diet 3. For the third objective, the following proportions of sardine in the diet were used for further analyses: Cape cormorants 90 %, kelp gulls 85 %, greater crested terns 80 % and Hartlaub's gulls 75 %. All procedures and calculations are described in Chapter 2.

4.1.2 Statistical comparisons

The mean $\delta^{13}C$, $\delta^{15}N$ and C:N ratios of the two prey species (estimated for three batches of sardine and two batches of sardinella) were compared using ANOVA to determine whether there was a significant difference between the two species. Normality and homoscedasticity were tested for analyses using Shapiro Wilks and Levene's test respectively, and were assumptions were not met a Kruskal Wallis test was conducted.

Data analyses were only conducted with the DFs (not SI values) as these integrate prey and predator values and are of use in ecological studies conducted in the wild. An analysis of variance (ANOVA) was used to determine if there were significant differences among the DFs between WB and the three diets listed above. This was done separately for kelp gulls, Cape cormorants and Hartlaub's gulls using both $\delta^{15}C$ and $\delta^{13}N$ of flesh (FL) and whole fish (WF), and only $\delta^{13}C$ of delipidated FL and delipidated WF to determine the respective DFs. Greater crested terns were only represented by two sub-adult individuals, one male and one female. The female WB exhibited a very high C:N ratio, indicating lipid rich blood, which was probably what caused the SI values of the two individuals to be very different. The greater crested terns were, therefore, not deemed viable for analyses and were not discussed any further.

An ANOVA was used to determine if the DFs for the various diets were different within and among species. For diets 1 and 2, three seabird species were compared. Because African penguins ate 100 % sardine diets, they could not be included in the analysis of diets 1 and 2, but raised the number of species tested to four in the case of diet 3. An ANOVA was also used to determine if the DFs were different among species on a diet of the most likely prey proportions for each species. Where normality and/or homoscedasticity did not hold, a non-parametric Kruskal Wallis test was used. Where significant effects were identified, Tukey post hoc tests were used to assess where the differences lay. All analyses were conducted in R v3.4.1 (2017) with packages tidyverse (Pohlert 2014), lawstat (De Mendiburu 2017), agricolae (Gastwirth et al. 2017) and PMCMR (Wickham 2017).

4.2 Results

4.2.1 Between-fish species comparison

The birds were fed a combination of two fish species (sardine and sardinella) which were significantly different in all but one analysis. Sardine always had higher $\delta^{13}C$ values and C:N ratios, and lower $\delta^{15}N$ values than sardinella. For $\delta^{13}C_{FL}$, the values were significantly different between sardine (-17.8 ± 0.6 ‰) and sardinella (-18.4 ± 0.5 ‰) ($F_{(4,20)} = 6.18$, $p = 0.002$), while $\delta^{15}N_{FL}$ values were not significantly different ($W_{(4)} = 5.64$, $p = 0.228$). The C:N ratio of FL of sardine (4.02 ± 0.35) was significantly greater than that of sardinella (3.44 ± 0.22) ($F_{(4,20)} = 9.90$, $p = 0.0001$).

The WF SI values were significantly different for $\delta^{13}C_{WF}$ (sardine -18.0 ± 0.5 ‰ vs sardinella -19.0 ± 0.8 ‰; $W_{(4)} = 52.05$, $p < 0.0001$) and $\delta^{15}N_{WF}$ (sardine 10.7 ± 0.3 ‰ vs sardinella 11.1 ± 0.3 ‰; $F_{(4,70)} = -13.55$, $p < 0.0001$). Sardine WF had a significantly higher C:N ratio (4.30 ± 0.36) than sardinella WF (3.88 ± 0.44; $F_{(4,70)} = 27.19$, $p < 0.0001$).

The fish components were delipidated and both delipidated FL and delipidated WF $\delta^{13}C$ of sardine (-16.9 ± 1.0 ‰ and -16.5 ± 0.4 ‰ respectively) were significantly greater than delipidated FL and delipidated WF $\delta^{13}C$ of sardinella (-17.7 ± 0.4 ‰ and -17.6 ± 0.4 ‰) ($W_{(4)} = 18.07$, $p = 0.001$ for FL and $W_{(4)} = 57.17$, $p < 0.0001$ for WF). The C:N ratios were significantly greater in sardine for both delipidated FL and delipidated WF (3.27 ± 0.15 and 3.28 ± 0.06 respectively) than in sardinella (3.11 ± 0.03 and 3.14 ± 0.04 respectively) (delipidated FL: $F_{(4,20)} = 2.98$, $p = 0.044$; delipidated WF $W_{(4)} = 52.83$, $p < 0.0001$).

Within seabird species comparison of range of sardine intake, there were no significant differences among the three diet $\Delta^{13}C_{WB-FL}$ for Hartlaub's gulls and Cape cormorants with ranges of mean values of 1.3 ‰ to 1.6 ‰ and 0.9 ‰ to 1.3 ‰ respectively ($F_{(2,9)} = 4.17$, $p = 0.052$ and $F_{(2,9)} = 1.13$, $p = 0.337$ respectively; Figure 4-1). In contrast, significant differences were observed among $\Delta^{13}C_{WB-FL}$ of the three diets for kelp gulls, with diet 1 > diet 2 > diet 3 and an average range of 1.4 ‰ to 1.7 ‰ ($F_{(2,6)} = 24.25$, $p = 0.001$; Figure 4-1; summarised in Table 4-1). There was a significant difference among the $\Delta^{13}C_{WB-delipidated\ FL}$ for Hartlaub's gulls, diet 2 had intermediate values and diet 1 > diet 3, with an average range of 0.1 ‰ to 0.6 ‰ ($F_{(2,9)} = 10.05$, $p = 0.001$), and kelp gulls with an average range of 0.2 ‰ to 0.7 ‰ ($F_{(2,9)} = 58.48$, $p = 0.0001$; Figure 4-22). There were no significant differences in $\Delta^{13}C_{WB-delipidated\ FL}$ in the case of Cape cormorants feeding on different diets ($F_{(2,27)} = 2.73$, $p = 0.083$).

There were significant differences among the three $\Delta^{13}C_{WB-WF}$ and $\Delta^{13}C_{WB-delipidated\ WF}$ for Hartlaub's gulls (Figure 4-32 & below Figure 4-2), with an average range of 2.1 ‰ to 1.4 ‰ ($F_{(2,9)} = 16.34$, $p = 0.001$) and 0.4 ‰ to -0.2 ‰ ($F_{(2,9)} = 17.69$, $p = 0.001$), respectively. A similar result was observed for Cape cormorants with an average range of 1.7 ‰ to 1.1 ‰ for $\Delta^{13}C_{WB-WF}$ ($F_{(2,27)} = 4.44$, $p = 0.022$) and 0.1 ‰ to -0.6 ‰ for $\Delta^{13}C_{WB-delipidated\ WF}$ ($F_{(2,27)} = 4.81$, $p = 0.016$), and kelp gulls with an average range of 2.2 ‰ to 1.5 ‰ for $\Delta^{13}C_{WB-WF}$ ($F_{(2,6)} = 95.08$, $p < 0.0001$) and 0.5 ‰ to -0.7 ‰ for $\Delta^{13}C_{WB-delipidated\ WF}$ ($F_{(2,6)} = 102.94$, $p < 0.0001$). In all species but the Cape cormorant, the $\Delta^{13}C$ for diet 1 was greater than $\Delta^{13}C$ for diet 2 which was higher than $\Delta^{13}C$ for diet 3 as determined by post hoc tests. For Cape cormorant $\Delta^{13}C_{WB-delipidated\ WF}$ diet 2 was intermediate to diet 1 and 3, and diet 1 was greater than diet 3 $\Delta^{13}C_{WB-delipidated\ WF}$ (summarised in Table 4-1).

No significant differences were highlighted among the three $\Delta^{15}N_{WB\ -\ FL}$ for Hartlaub's gulls ($F_{(2,9)}$ = 2.69, p = 0.122) or kelp gulls ($F_{(2,9)}$ = 4.27, p = 0.070; Figure 4-1; summarised in Table 4-1). In Cape cormorants however the $\Delta^{15}N$ for diet 3 was higher than for diet 1, while diet 2 was intermediate to the two diets ($F_{(2,27)}$ = 10.96, p = 0.001; homogenous groups denoted in Figure 4-1). The Cape cormorant was the only species for which there was a significant difference among the three $\Delta^{15}N_{WB-WF}$ ($F_{(2,27)}$ = 14.89, p < 0.0001; Figure 4-33; summarised in Table 4-1) with diet 1 < diet 2 < diet 3.

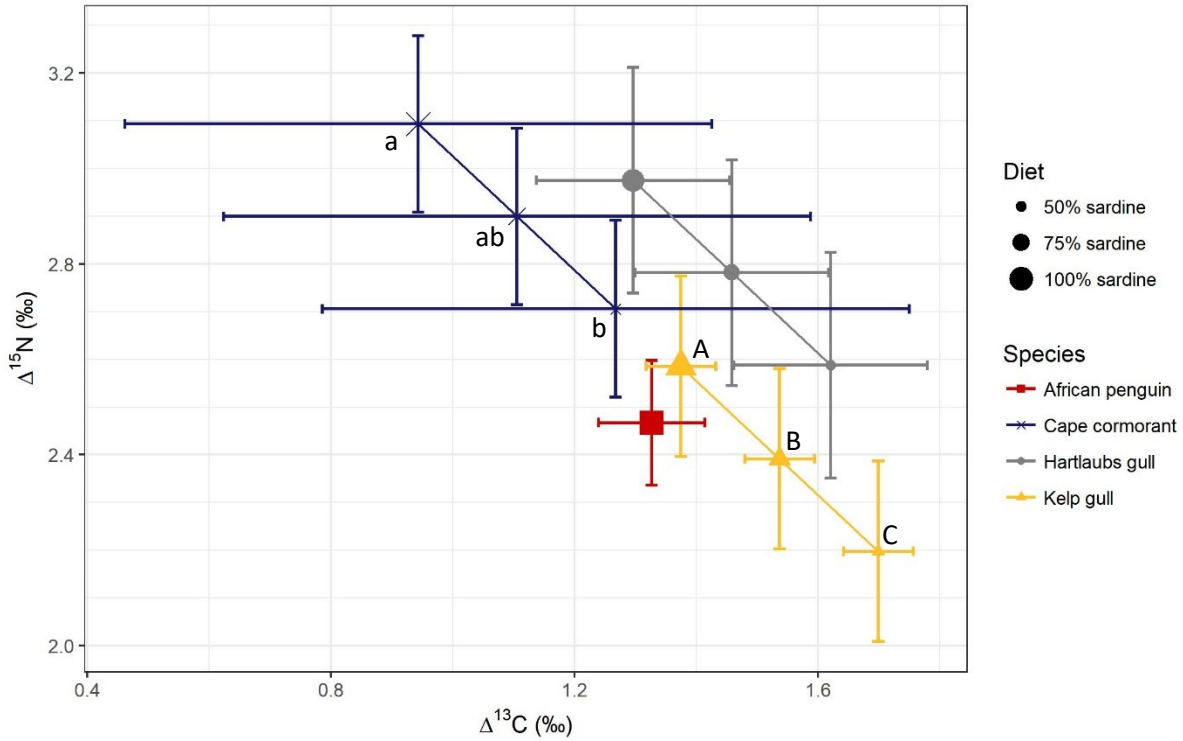

Figure 4-1: Average discrimination factor (± SD) for whole blood – flesh for each species (African penguins n=13, Cape cormorants n=10, Hartlaub's gulls n=4, kelp gulls n=3) calculated based on three proportions of sardine in mixed diet of sardine and sardinella. The homogenous groups are denoted using a-b for $\Delta^{15}N$ and A-C for $\Delta^{13}C$.

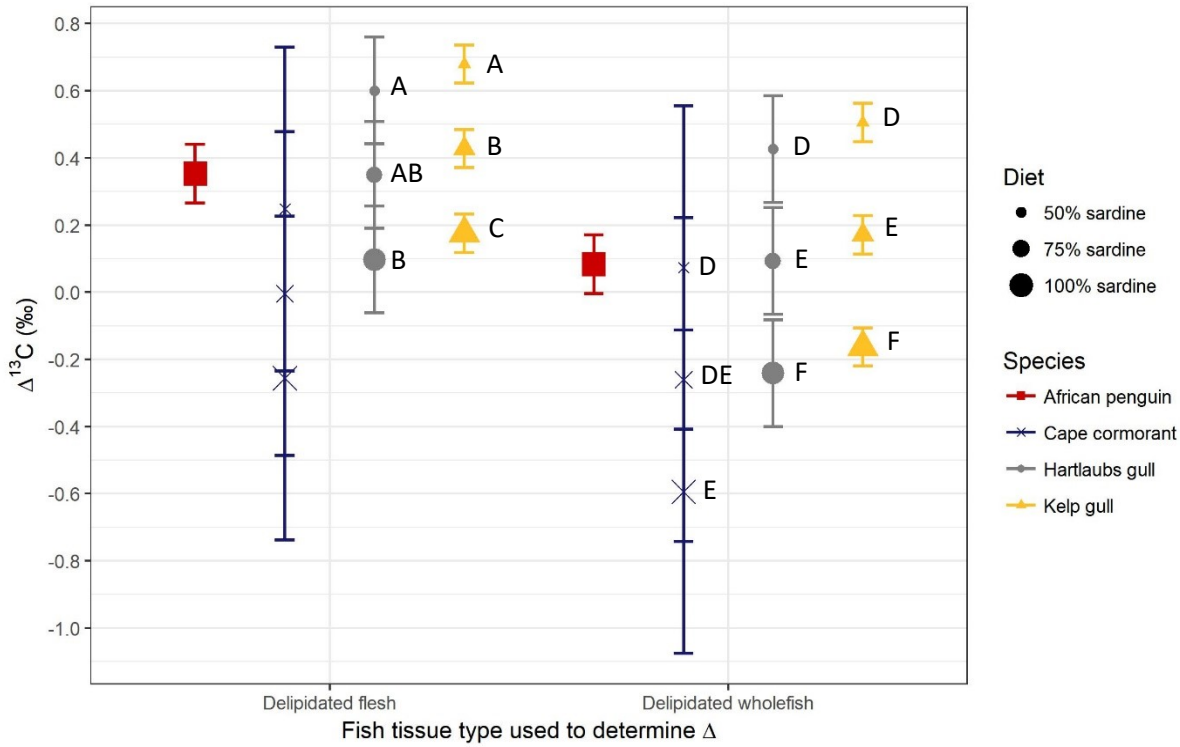

Figure 4-2: Average discrimination factor (± SD) for whole blood – delipidated flesh and whole blood – delipidated whole fish for each species (African penguins n=13, Cape cormorants n=10, Hartlaub's gulls n=4, kelp gulls n=3) using three proportions of sardine in mixed diets of sardine and sardinella. The homogenous groups within species are denoted using A-C for delipidated FL and D-F for delipidated WF.

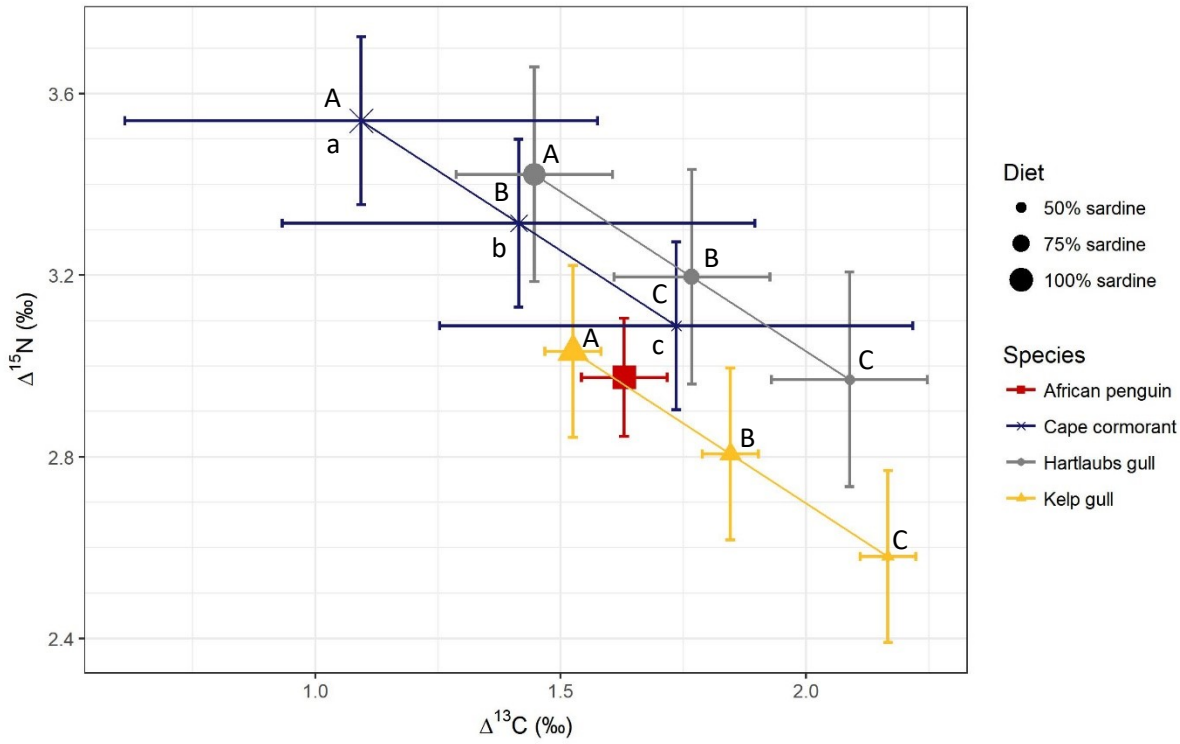

Figure 4-3: Average discrimination factor (± SD) for whole blood – whole fish for each species (African penguins n=13, Cape cormorants n=10, Hartlaub's gulls n=4, kelp gulls n=3) using three proportions of sardine in mixed diets of sardine and sardinella. The homogenous groups are denoted using a-c for $\Delta^{15}N$ and A-C for $\Delta^{13}C$.

Table 4-1: Summary of whether comparisons of three discrimination factors determined from 50 %, 75 % and 100 % sardine in a sardine:sardinella mixed diet, were significantly different from one another within species.

		Significantly different	
Tissues	Species	$\Delta^{13}C$	$\Delta^{15}N$
WB-FL	Hartlaub's gulls	no	no
WB-FL	Kelp gulls	yes	no
WB-FL	Cape cormorants	no	yes
WB-WF	Hartlaub's gulls	yes	no
WB-WF	Kelp gulls	yes	no
WB-WF	Cape cormorants	yes	yes
WB-delipidated FL	Hartlaub's gulls	yes	
WB-delipidated FL	Kelp gulls	yes	
WB-delipidated FL	Cape cormorants	no	
WB-delipidated WF	Hartlaub's gulls	yes	
WB-delipidated WF	Kelp gulls	yes	
WB-delipidated WF	Cape cormorants	yes	

4.2.2 Inter-specific comparison of discrimination factors

4.2.2.1 Comparison within diets

For the comparison of diet 3, African penguins were included in the analysis. The $\Delta^{13}C_{WB-FL}$, $\Delta^{13}C_{WB-WF}$, $\Delta^{13}C_{WB-delipidated\ FL}$ and $\Delta^{13}C_{WB-delipidated\ WF}$ were significantly different among species ($\Delta^{13}C_{WB-FL}$: $W_{(3)} = 11.52$, $p = 0.009$; $\Delta^{13}C_{WB-WF}$: $W_{(3)} = 14.43$, $p = 0.002$; $\Delta^{13}C_{WB-delipidated\ FL}$ $W_{(3)} = 16.50$, $p = 0.001$; $\Delta^{13}C_{WB-delipidated\ WF}$: $W_{(3)} = 18.24$, $p < 0.001$). Cape cormorants < African penguins = kelp gulls = Hartlaub's gulls, which were both = Cape cormorants, for $\Delta^{13}C_{WB-WF}$, $\Delta^{13}C_{WB-delipidated\ FL}$ and $\Delta^{13}C_{WB-delipidated\ WF}$ of diet 3. A post hoc could not determine where the differences were for $\Delta^{13}C_{WB-FL}$. There were significant differences in $\Delta^{15}N_{WB-FL}$ and $\Delta^{15}N_{WB-WF}$ among species on diet 3 ($W_{(3)} = 21.34$, $p < 0.0001$ and $W_{(3)} = 20.43$, $p = 0.0001$ respectively) and $\Delta^{15}N$ of both the Cape cormorants and Hartlaub's gulls were higher than those of African penguins and kelp gulls (summarised in Table 4-2).

There was no significant difference among the $\Delta^{13}C_{WB-FL}$, $\Delta^{13}C_{WB-WF}$, $\Delta^{13}C_{WB-delipidated\ FL}$ or $\Delta^{13}C_{WB-delipidated\ WF}$ among the three species, for diets 1 and 2. When looking at nitrogen however, there were significant differences in the $\Delta^{15}N_{WB-FL}$ and $\Delta^{15}N_{WB-WF}$ among kelp gulls, Hartlaub's gulls and Cape cormorants on diets 1 and 2 ($F_{(2,14)} = 7.62$, $p = 0.006$ for both). $\Delta^{15}N$ of Cape cormorants was higher than that of kelp gulls, while Hartlaub's gulls $\Delta^{15}N_{WB-FL}$ and $\Delta^{15}N_{WB-WF}$ values did not differ from those of Cape cormorants or kelp gulls (summarised in Table 4-2).

Table 4-2: Summary of analyses comparing seabird species (CC – Cape cormorant, HG – Hartlaub's gull, KG – kelp gull, AP – African penguin) on the same diet with different fish components flesh (FL), whole fish (WF), delipidated FL and delipidated WF.

Diet	Prey tissue	Discrimination factor	Seabird species – homogenous groups			
			CC	HG	KG	AP
Diet 1	FL	$\Delta^{13}C$				NA
Diet 1	FL	$\Delta^{15}N$	a	ab	b	NA
Diet 1	WF	$\Delta^{13}C$				NA
Diet 1	WF	$\Delta^{15}N$	a	ab	b	NA
Diet 1	Delipidated FL	$\Delta^{13}C$				NA
Diet 1	Delipidated WF	$\Delta^{13}C$				NA
Diet 2	FL	$\Delta^{13}C$				NA
Diet 2	FL	$\Delta^{15}N$	a	ab	b	NA
Diet 2	WF	$\Delta^{13}C$				NA
Diet 2	WF	$\Delta^{15}N$	a	ab	b	NA
Diet 2	Delipidated FL	$\Delta^{13}C$				NA
Diet 2	Delipidated WF	$\Delta^{13}C$				NA
Diet 3	FL	$\Delta^{13}C$	ab	ab	a	a
Diet 3	FL	$\Delta^{15}N$	a	a	b	b
Diet 3	WF	$\Delta^{13}C$	a	ab	ab	b
Diet 3	WF	$\Delta^{15}N$	a	a	b	b
Diet 3	Delipidated FL	$\Delta^{13}C$	a	ab	ab	b
Diet 3	Delipidated WF	$\Delta^{13}C$	a	ab	ab	b

4.2.2.2 Comparison for most likely proportions of sardine

The proportions of sardine in the diet used for each species were: African penguin 100 %, Cape cormorants 90 %, Hartlaub's gulls 75 % and kelp gulls 85 %.

$\Delta^{13}C_{WB-FL}$ and $\Delta^{13}C_{WB-delipidated\ FL}$ were both significantly different among the four species ($\Delta^{13}C_{WB-FL}$: $F_{(3,26)} = 3.76$, $p = 0.023$, Figure 4-2; $\Delta^{13}C_{WB-delipidated\ FL}$: $F_{(3,267} = 6.47$, $p = 0.002$, Figure 4-5; summarised in Table 4-3). Though a post hoc test could not determine where the significant differences lay for $\Delta^{13}C_{WB-FL}$, $\Delta^{13}C_{WB-delipidated\ FL}$ of Cape cormorants was less than that of Hartlaub's gulls by 0.5 ‰. $\Delta^{15}N_{WB-FL}$ was significantly different among species ($F_{(3,26)} = 21.79$, $p < 0.0001$;

Figure 4-2). Post-hoc testing showed that the $\Delta^{15}N_{WB-FL}$ of Cape cormorants and Hartlaub's gulls were similar and were greater than that of penguins. Cape cormorants had greater $\Delta^{15}N_{WB-FL}$ than that of kelp gulls and African penguins by 0.6 ‰.

The $\Delta^{13}C_{WB-WF}$ and $\Delta^{13}C_{WB-delipidated\ WF}$ among the four species were both significantly different ($F_{(3,26)} = 5.39$, $p = 0.005$ for $\Delta^{13}C_{WB-WF}$ [Figure 4-6] and $F_{(3,267} = 7.44$, $p = 0.001$ for $\Delta^{13}C_{WB-delipidated\ WF}$ [Figure 4-5]). Cape cormorants exhibited higher $\Delta^{13}C_{WB-WF}$ and $\Delta^{13}C_{WB-delipidated\ WF}$ than Hartlaub's gulls by 0.6 ‰ for both and $\Delta^{13}C_{WB-delipidated\ WF}$ of Cape cormorants was lower than the African penguin values by 0.5 ‰.

Similarly, values of $\Delta^{15}N_{WB-WF}$ were significantly different among species ($F_{(3,26)} = 17.28$, $p < 0.0001$; Figure 4-6; summarised in Table 4-3). Cape cormorants had significantly higher values than African

penguins, by 0.5 ‰, and than kelp gulls, by 0.6 ‰, while Hartlaub's gulls had intermediate values that were not significantly different from the other two groups (Figure 4-6).

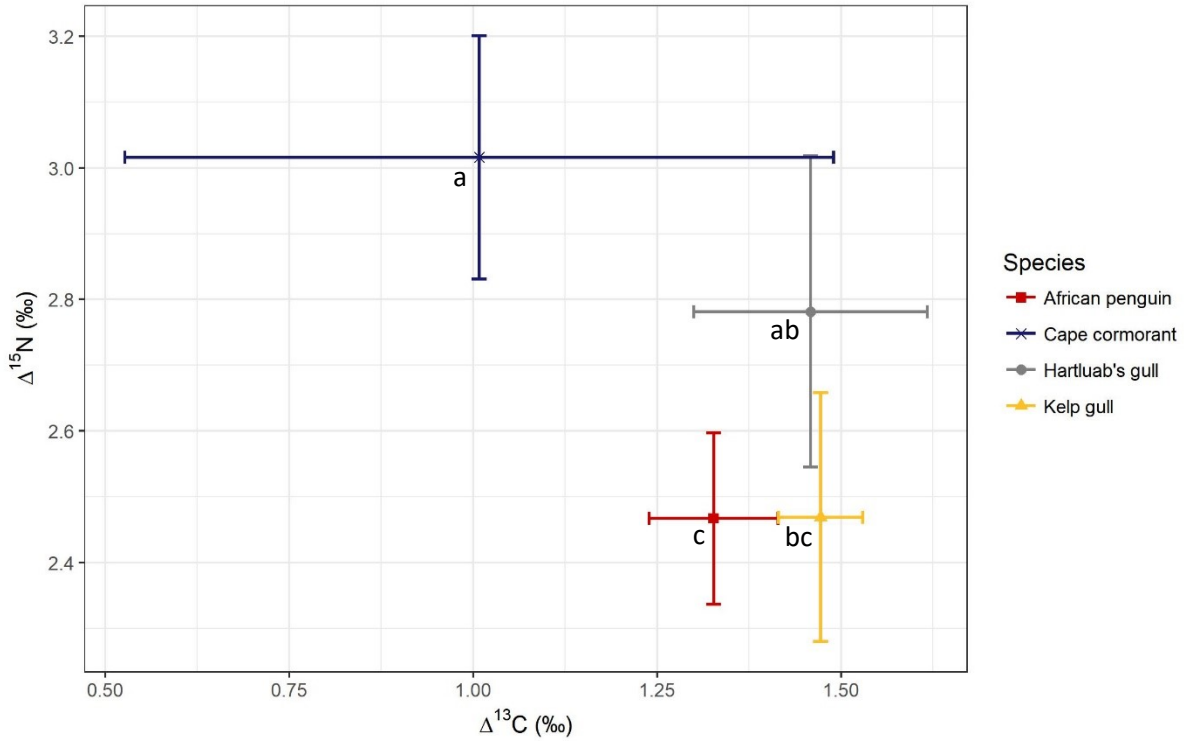

Figure 4-2: Average discrimination factors (± SD) of whole blood – flesh for four seabird species eating a sardine:sardinella diet of different proportions. African penguins (n=13) 100 % sardine, Cape cormorants (n=10) 90 % sardine, Hartlaub's gulls (n=4) 75 % sardine, and kelp gulls (n=3) 85 % sardine. Homogenous groups denoted by a-c for $\Delta^{15}N$.

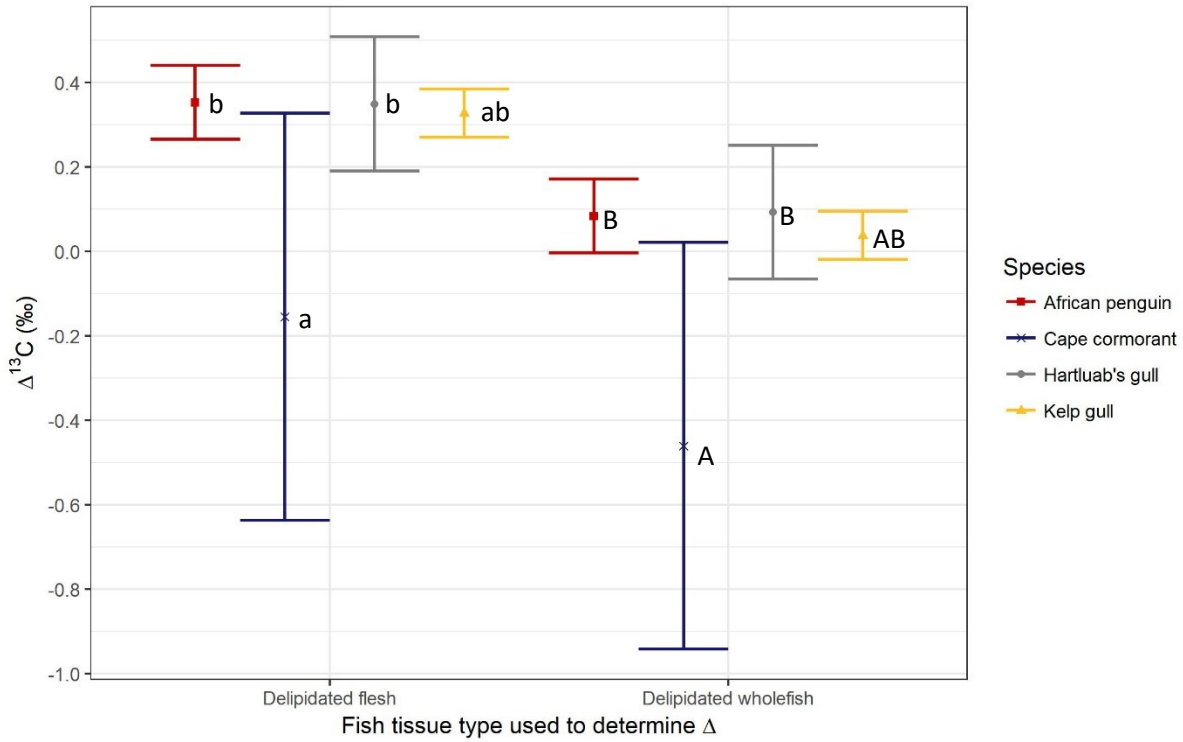

Figure 4-5: Average ^{13}C discrimination factor (± SD) of whole blood – delipidated flesh and whole blood – delipidated whole fish for four seabird species. African penguins (n=13) 100 % sardine, Cape cormorants (n=10) 90 % sardine, Hartlaub's gulls (n=4) 75 % sardine, and kelp gulls (n=3) 85 % sardine. Homogenous groups denoted by and a-c for delipidated flesh and A-B for delipidated whole fish.

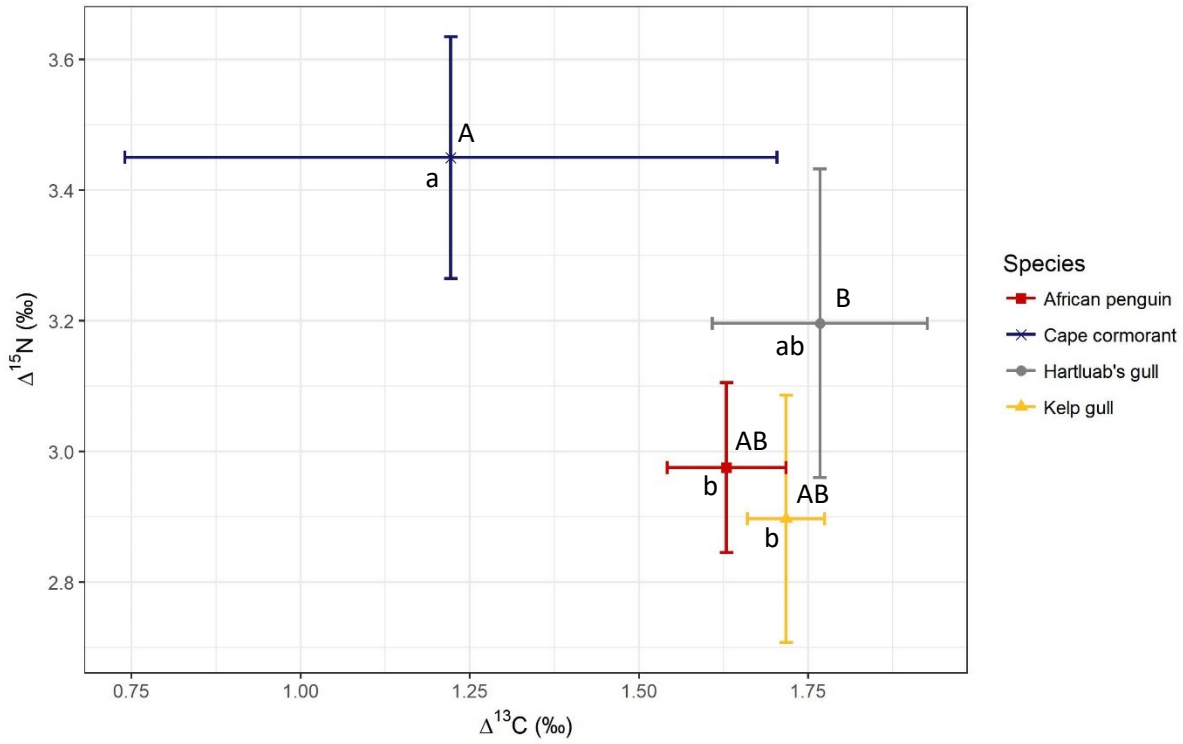

Figure 4-6: Average discrimination factors (± SD) of whole blood – whole fish for four seabird species eating a sardine:sardinella diet of different proportions. African penguins (n=13) 100 % sardine, Cape cormorants (n=10) 90 % sardine, Hartlaub's gulls (n=4) 75 % sardine, and kelp gulls (n=3) 85 % sardine. Homogenous groups denoted by a-c for $\Delta^{15}N$ and A-B for $\Delta^{13}C$.

Table 4-3: Homogenous groups of the comparisons of discrimination factors across four species on their preferred diets, namely: Cape cormorants (n=10) 90 % sardine, Hartlaub's gulls (n=4) 75 % sardine, kelp gulls (n=3) 85 % sardine and African penguins (n=13) 100 % sardine. Different components of the diet were assessed namely: flesh (FL), whole fish (WF), delipidated FL and delipidated WF. All analyses were significant.

		Seabird species			
Prey tissue	Discrimination factor	Cape cormorant	Hartlaub's gull	Kelp gull	African penguin
FL	$\Delta^{13}C$	A	a	a	a
	$\Delta\ ^{15}N$	A	ab	bc	c
delipidated FL	$\Delta^{13}C$	A	b	ab	b
WF	$\Delta^{13}C$	A	b	ab	ab
	$\Delta^{15}N$	A	ab	b	b
delipidated WF	$\Delta^{13}C$	A	b	ab	b

4.3 Discussion

As SIA is more widely used for trophic ecology studies, the need for accurate species-specific DFs increases. A better understanding of what influences these DFs is also important for accurate inferences to be made. What the birds ingest not only influences the SI values of their tissues but can also influence the DF, referred to as diet-dependent DF (DDDF; Caut et al. 2009). My study provided a platform where the SI values of many blood and diet combinations could be assessed, as the FL was sampled from fish before the WF was blended. Both components of the fish were also delipidated resulting in four values for fish from which the DF could be calculated. This information makes it possible for wild studies to use the same DF associated with the prey SI values that may be available (e.g. if a study has access only to FL SI values, the appropriate DF for a model to determine diet would be the $\Delta_{consumer - FL}$ value).

4.3.1 Influence of diet on discrimination factors

In my study, when comparing C:N ratios of the two fish species, the higher ratios measured in sardine (FL and WF) suggested that this species was richer in lipids than sardinella. When FL and WF were delipidated, their C:N ratios decreased, and a higher correction factor was observed for sardine $\delta^{13}C$ than sardinella $\delta^{13}C$ as expected due to the higher lipid content (Post et al. 2007). When comparing the range of DFs, within seabird species, calculated for diets 1, 2 and 3 of an estimated sardine:sardinella mixed diet, there were significant differences in most of the analyses. This is most likely because of the significant differences in SI values of the two fish species being large enough to cause a significant difference in DF calculated with three potential sardine:sardinella proportions. The analyses which were not significant had high variation among individuals which could have masked differences between the three DFs. The variation seen among individuals could be

attributed to small samples size (gulls) or the behaviour of individuals. Some birds are more active and fly around the centre or swim more regularly while other individuals suffer from neurological damage or have amputations preventing them from flying, though two distinct groups cannot be seen in the data so it is unlikely that only this factor would have influenced the results. Regarding the Cape cormorants, the high inter-individual variation was mainly due to two individuals with very high C:N ratios. This in turn would have strongly influenced their $\delta^{13}C$. Why the blood of these two cormorants (one male, one female) was richer in lipids than the other eight birds, is difficult to explain. All birds were sampled in the morning before being fed to exclude the influence of any very short-term impact of digestion. It is, however, possible that those two cormorants may have found fish/pieces of fish lying in the pool that morning; as very recent feeding has been shown to influence blood composition a few hours after ingestion (Bearhop et al. 2002).

The high level of inter-individual variation seen in Hartlaub's gulls is likely explained by their feeding behaviour. Many seabird species forage on small pelagic fish but individuals as well as species may have their own preferences (Cherel et al. 2005b, Martínez del Rio et al. 2009a, Mallory et al. 2010). This preference may also change if the prey availability changes (Schreiber & Burger 2001). Therefore, it is unlikely that kelp gulls, Cape cormorants and Hartlaub's gulls ate the sardine and sardinella in the same proportions. Observations showed that some individuals preferentially picked out the eyes and the internal organs of sardine, which both have a high C:N ratio (4.68 and 5.23). Though WF samples included the eyes and gut, there was no way to quantify how many eyes or how much of the sardine internal organs were eaten along with the fish diet or whether the birds were selective about which organs they ate in the 3-5 weeks prior to sampling. This variation was surprisingly not seen in the kelp gulls which, along with the Hartlaub's gulls, exhibited preferential feeding behaviour.

Though only 11 of 18 analyses of the range of DFs across a diet per species were significant, a general trend was seen. There was a consistent increase in $\Delta^{15}N$ as more sardine were added to the calculated $\delta^{15}N$ for prey, while $\Delta^{13}C$ consistently decreased as more sardine were added to the calculated prey $\delta^{13}C$. The maximum difference between the average $\Delta^{15}N$ of diet 1 and diet 3 was 0.45 ‰, which is much lower than the usual 3-5 ‰ $\Delta^{15}N$ indicative of trophic level shifts (DeNiro & Epstein 1981, Post 2002). It is a small enough range that, though there may be significant differences in the DDDF, it is unlikely to change the interpretation of trophic level. The maximum range in $\Delta^{13}C$ was 0.7 ‰ is also less than the 1.3-3 ‰ attributed to a trophic and/or forage location shift (DeNiro & Epstein 1978, Bond & Jones 2009) again not preventing good interpretation of results from wild studies.

4.3.2 Influence of seabird species on discrimination factors

Since diet affects the DF, the differences in DFs seen among species may be confounded by DDDF (Bond & Jones 2009). Hobson and Clark (1992b) were the first to show that DFs were predator species specific when they observed that two bird species on the same diet exhibited DFs that were significantly different. A study conducted by (Barquete et al. 2013) also calculated two $\Delta^{15}N_{WB-WF}$ values, differing by 0.3 ‰, for African penguins eating sardine, even though the SI values for the two sardine batches were the same. The comparison of four species on the same estimated diet in this chapter produced similar findings for some species but not others, indicating that some, but not all, species differed (Table 4-2). The $\Delta^{13}C$ was not different among any species when comparing just the three flying birds on the same estimated diet. When African penguins were added for estimated diet 3, then there was a significant difference among the $\Delta^{13}C$ of species. There was always a significant difference among $\Delta^{15}N$ among species, which was in accordance with Hobson et al. (1992a). There were, however, still species which formed homogenous groups which were not similar in feeding guild or genetic phylogeny. More studies with multiple species on the same estimated diet are definitely needed to understand the effect of species better, without the confounding effect of a variable diet.

When considering the most likely composition of their diet in terms of the proportions of sardine and sardinella, African penguins were included. Significant differences were found among the seabird species in all six analyses ($\Delta^{15}N_{WB-FL}$ and $\Delta^{15}N_{WB-WF}$, and $\Delta^{13}C_{WB-FL}$, $\Delta^{13}C_{WB-WF}$, $\Delta^{13}C_{WB-delipidated\ FL}$ and $\Delta^{13}C_{WB-delipidated\ WF}$; Table 4-3). Different tissues were used to provide more DFs for use in wild studies. Despite the different tissues used to calculate DF there was still a significant difference among species, which followed roughly the same pattern. Variations in homogenous groups among the four species for different DFs are potentially driven by the variation in food, or lack thereof, depending on which tissue is used (Cherel et al. 2005b).

Each species is likely to have had a different proportion of sardine in the diet and yet the DFs did not follow a distinct pattern that reflected the most-likely proportion of sardine in diet. The Cape cormorants and kelp gulls are the closest in weight among the four species, and had the most similar likely diets, with cormorants DFs calculated to be eating 90 % sardine and kelp gulls DFs calculated to be eating 85 % sardine diets, but the $\Delta^{15}N$ of Cape cormorants was more like that of the Hartlaub's gulls, which were calculated to have eaten a 75 % sardine diet. The $\Delta^{13}C$ of Cape cormorants was closer to both the African penguins on 100 % sardine diet and the Hartlaub's gulls, than the kelp gull with the most similar likely diet.

Some studies use the DFs of species genetically similar to the species being studied, if no DF information is available (Healy et al. in press). It is seen here that the two gull species which are both in the genus *Larus* (though Hartlaub's gulls are identified as *Chroicocephalus* in some bird classifications), did not always have the most similar DFs. The $\Delta^{15}N$ was generally the most similar between kelp gulls and African penguins and the $\Delta^{13}C$ was generally closer between Hartlaub's gulls and African penguins.

When considering other species which have experimentally determined DFs, there is also a great range of DFs (Table 4-4). The $\Delta^{13}C$ calculated with delipidated prey was often smaller than that calculated with raw prey, as is the case with $\Delta^{15}N$, as the $\delta^{15}N$ was affected by delipidatation. The variation within feeding guilds of species was similar to, and in some cases more than, the variation among all the species. This makes it risky to average the DFs from birds in the same guild to determine a DF that can be used for models. Ciancio et al. (2016) added DFs for Magellanic penguins (*Spheniscus magellanicus* Forster, 1781) and compared the variation on known DFs of seabirds. Ciancio et al. (2016) found that $\Delta^{13}C$ was much more variable than $\Delta^{15}N$, and concluded that a blanket value, especially for $\Delta^{13}C$ should be used with caution. The variation seems to be great and random. This again highlights that an understanding of what influences the DFs is missing and there should be a larger focus placed on utilising the opportunities of captive animals to expand our knowledge of DFs.

Table 4-4: Discrimination factors between blood components (WB – whole blood, RBC – red blood cells, PL – plasma, BC – cellular blood and del PL – delipidated PL) and diet components (WF – whole fish/food and FL – muscle/flesh) from various controlled studies of adult seabirds (m: males; f: females) (updated from Cherel et al. 2005b).

Species	Scientific name	Consumer tissue	Prey	Prey tissue	raw prey		delipidated prey		Reference
					$\Delta^{13}C$	$\Delta^{15}N$	$\Delta^{13}C$	$\Delta^{15}N$	
African penguin	*Spheniscus demersus*	WB	Sardine	FL	1.3	2.5	0.4	2.2	This study, chapter 3
				WF	1.6	3	0.1	2.6	This study, chapter 3
		WB	Sardine	WF		2.5			Barquete et al. 2013
				WF		2.8			Barquete et al. 2013
		WB	Hake	WF		1.8			Barquete et al. 2013
		RBC	Sardine	FL	1.6	2.4	0.6	2.2	This study, chapter 3
				WF	1.8	2.9	0.2	2.6	This study, chapter 3
		RBC	Sardine	WF		2.5			Barquete et al. 2013
		RBC	Hake	WF		1.9			Barquete et al. 2013
		PL	Sardine	FL	0.6	2.9	-0.4	2.6	This study, chapter 3
				WF	1	3.4	-0.6	3.1	This study, chapter 3
		PL	Sardine	WF		2.5	-0.1		Barquete et al. 2013
		PL	Hake	WF		2			Barquete et al. 2013
		del PL	Sardine	FL	1.5		0.5		This study, chapter 3
				WF	1.9		0.3		This study, chapter 3
Magellanic penguin	*Spheniscus magellanicus*	WB	Anchovy	FL	0.9	2.8	0.4	2.3	Ciancio et al. 2016
Little penguin	*Eudyptes minor*						0.1	3.3	Chiaradia et al. 2014
King penguin	*Aptenodytes patagonicus*	WB	Herring	FL			-0.6	1.2	Cherel et al. 2005b
				WF			-0.8	2.1	Cherel et al. 2005b
Southern Rockhopper penguin	*Eudyptes chrysocome*	WB	Capelin	FL			0.46	1.9	Cherel et al. 2005b
				WF			0	2.7	Cherel et al. 2005b
Cape cormorant	*Phalacrocorax*	WB	90 % sardine,	FL	1.1	3	-0.2	2.7	This study, chapter 4

	capensis		10 % sardinella**					
			90 % sardine, 10 % sardinella**	WF	1.2	3.5	-0.5	3.1	This study, chapter 4
		RBC	90 % sardine, 10 % sardinella**	FL	1.6	2.5	0.5	2.2	This study, chapter 4
			90 % sardine, 10 % sardinella**	WF	1.8	2.9	0.2	2.6	This study, chapter 4
		PL	90 % sardine, 10 % sardinella**	FL	1.8	2.4	0.6	2.1	This study, chapter 4
			90 % sardine, 10 % sardinella**	WF	2	2.9	0.3	2.5	This study, chapter 4
Double crested cormorant	*Phalacrocorax auritus*	WB	Catfish	WF		2.9	-1.1		Craig et al. 2015
		RBC	Catfish	WF		3	-0.8		Craig et al. 2015
		PL	Catfish	WF		3.9	-0.6		Craig et al. 2015
Kelp gull	*Larus dominicanus*	WB	85 % sardine, 15 % sardinella**	FL	1.5	2.5	0.3	2.1	This study, chapter 4
			85 % sardine, 15 % sardinella**	WF	1.7	2.9	0	2.6	This study, chapter 4
		RBC	85 % sardine, 15 % sardinella**	FL	1.7	2.3	0.6	2	This study, chapter 4
			85 % sardine, 15 % sardinella**	WF	1.9	2.8	0.3	2.4	This study, chapter 4
		PL	85 % sardine,	FL	0.3	3.1	-0.9	2.7	This study, chapter 4

			15 % sardinella**						
			85 % sardine, 15 % sardinella**	WF	0.5	3.5	-1.2	3.1	This study, chapter 4
Hartlaub's gull	*Larus hartlaubii*	WB	75 % sardine, 25 % sardinella**	FL	1.5	2.8	0.3	2.4	This study, chapter 4
			75 % sardine, 25 % sardinella**	WF	1.8	3.2	0.1	2.9	This study, chapter 4
Ring-billed gull	*Larus delawarensis*	WB	Perch	WF			-0.3	3.1	Hobson and Clark 1992b
Greater crested tern*	*Thalasseus bergii*	WB	80 % sardine, 20 % sardinella**	FL	-0.6 (f) 1.0 (m)	3.2 (f) 2.0 (m)	-1.6 (f) 0.0 (m)	2.9 (f) 1.6 (m)	This study, chapter 4
			80 % sardine, 20 % sardinella**	WF	-0.1 (f) 1.5 (m)	3.6 (f) 2.4 (m)	-2.2 (f) -0.6 (m)	3.6 (f) 2.3 (m)	This study, chapter 4
Great skua	*Catharacta skua*	WB	Sprat	WF	4.3	2.6	1.1	2.8	Bearhop et al. 2002
		WB	Beef	Mince	7.1	4.2	2.3	4.2	Bearhop et al. 2002
Spectacled eider	*Somateria fischeri*	BC	95 % commercial diet, 5 % Atlantic silverside		2	4			Federer et al. 2010
		PL	95 % commercial diet, 5 % Atlantic silverside		0	4.9			Federer et al. 2010
Rhinoceros auklet (subadult)	*Cerorhinca monocerata*	RBC	Silverside	WF				3.5	Sears et al. 2009

* there were only one male and one female available to sample. The SI values were very different, and the female had a high C:N ratio.
** these are estimated diets that were considered the most-likely combination fish species eaten by each bird species

4.4 Conclusions

Although DF for species that were not yet studied have been proposed in this study, there are limitations. One of the greatest limitations of this study was that it was unfortunately not possible for the birds to be fed a single species of prey. This resulted in the need to calculate a range of DFs, this can, however, be useful for certain models. This study showed the $\Delta^{15}N_{WB-WF}$ to be higher than either value determined by Barquete et al. (2013) while the $\Delta^{15}N_{WB-FL}$ was the same as the first $\Delta^{15}N_{WB-WF}$ calculated by Barquete et al. (2013) for African penguins eating sardine. This shows that even when the same food source is used, there are other factors which can influence the results.

Another limitation of this study is the small sample size for some species, namely the greater crested terns, the kelp gulls and the Hartlaub's gulls, which all had less than 5 birds. This small sample size also prevented any comparisons between the sexes, though the effect of sex is likely to have been minor as all birds were sampled outside their breeding season. One option to increase the number of individuals per species could be to capture wild birds and house them in captivity to conduct a similar study as has been done previously (Craig et al. 2015). Seabird species differ in their energy and nutrient requirements, in some of their nutrient assimilation pathways, in their gut biota, which influences the assimilation of nutrients, and in many other regards not considered or studied yet (Carey 1996, Schreiber & Burger 2001). This line of research is definitely needed to improve the accurate use of SIs in ecological wild studies.

Chapter 5 General synthesis

Carbon and nitrogen stable isotope analysis (SIA) is nowadays widely used in diet analysis studies to gain information about forage location and the trophic level that an organism may be feeding at. Like most techniques, SIA is not without its limitations. One of the limitations is that there is only a partial understanding of the factors which influence the stable isotope (SI) values (Martínez del Rio et al. 2009b), and even where there is an understanding there is sometimes a lack of agreement across studies (e.g. Hobson & Clark 1993 vs Kempster et al. 2007). While Hobson and Clark (1993) found that there was an influence of fasting on $\delta^{15}N$, Kempster et al. (2007) did not. Another limitation is that a discrimination factor (DF) between consumer and prey tissues is needed to use SI values to gain understanding of the foraging location and trophic level of an organism. There is not one DF which fits all taxa, as DFs can even vary within an individual depending on its physiological state, and there is a constant call for more experiments to be done, as not many controlled studies have been conducted on seabirds (Bond & Jones 2009).

Captive birds are ideal for testing assumptions of diet analysis techniques as variables which cannot be controlled in the wild can be controlled in captive facilities, allowing confounding factors to be eliminated. In this study, captive birds, housed at the South African Foundation for the Conservation of Coastal Birds (SANCCOB), were used to assess two aspects which make the interpretation of SIA difficult. The first is the effects of physiological factors which, in some studies, have influenced carbon- and nitrogen-SI values (Hobson & Clark 1993, Fuller et al. 2004). The physiological factors considered in this study include ontogeny of African penguins (*Spheniscus demersus* Linnaeus, 1758) and egg production. Different tissues give information of diet over different time periods as turnover of tissue restricts the diet "history". The tissues that are more frequently sampled in wild studies are those that can be sampled non-destructively. For these two reasons three blood components, whole blood (WB), red blood cells (RBC) and plasma (PL) were assessed to determine the effect of the two afore mentioned physiological factors. The second part to this study addressed the limited number of studies conducted to determine species- and tissue-specific DFs of seabirds on controlled diets by assessing whether DFs differ among species on the same diet and adding more DFs to the database. The seabirds used for this include Cape cormorants (*Phalacrocorax capensis* Sparrman, 1789), kelp gulls (*Larus dominicanus* Lichtenstein, 1823), Hartlaub's gulls (*Larus hartlaubii* Bruch, 1853) and greater crested terns (*Thalasseus bergii* Lichtenstein, 1823).

5.1 Physiological effect on stable isotope values

From this study it is seen that the age of African penguins does influence the SI values of their blood components. Since diets varied across age, as the younger penguins received formula supplements,

the DFs were used as a proxy to determine whether diet or age affected the SI values. Both $\delta^{13}C$ and $\delta^{15}N$ if various tissues responded to the influence of age. The $\delta^{13}C$ of WB and the delipidated PL was influenced by age while the RBC showed an influence of age if the delipidated prey $\delta^{13}C$ values were used to calculate the DF. There was no influence of age on RBC (when raw prey $\delta^{13}C$ values were used to calculate the DF) nor was there an influence of age on PL. The $\delta^{15}N$ showed similar patterns of WB and RBC being influenced by age while PL was not influenced by age and didn't vary across ages. From these results it seems that PL may be the best tissue to use if sampling chicks to avoid the influence of age on the SI values.

Lipids are generally depleted in ^{13}C, so lipid rich tissues have lower $\delta^{13}C$ values than lipid poor tissues. The production of eggs in birds increases the amount of lipids produced in the liver. Lipids are then transported in the blood to the ovaries where an oocyte is matured to form the yolk of an egg (Burley & Vadehra 1989a). The effect of egg production was clearly seen in the female $\delta^{13}C$ values of WB and PL during the rapid yolk deposition phase (from c. 20 days before laying) and the albumen production phase. Males effectively acted as a control and showed no difference from females outside of those two egg production phases.

These two studies clearly show that the physiology of an organism can influence the SI values of blood tissue. Not all physiological conditions affect all blood components in the same way, making different components more or less sensitive to physiological influences. Changes in SI values may also relate to variation in DF. This study also shows that depending on the combination of consumer and prey tissue used to calculate the DF, a different conclusion may be reached. The differences in DFs when using different tissues may not be large enough to change ecological interpretation, but it is still very important to consider and specify which tissues are chosen for study.

5.2 Discrimination factors of multiple species

To use SIAs in Wild studies, the DF between consumer and prey tissues needs to be known. This controlled study provided more information about DF for species not yet studied. As the proportion of sardine in the diet increased, so did the $\Delta^{15}N$ while the $\Delta^{13}C$ decreased. This agreed with Caut et al. (2009), who concluded that there was a diet dependent DF (DDDF). Unlike Hobson & Clark (1992a), who showed a significant difference between the DFs of species on the same diet, my study showed that not all species had significantly different DFs when fed the same diet. The $\Delta^{13}C$ of flying birds on diet 1 and 2 were not significantly different from one another, while the $\Delta^{15}N$ was similar between the two gull species and between Hartlaub's gulls and Cape cormorants. When African penguins were added for diet 3 the $\Delta^{13}C$ was almost always significantly different between Cape cormorants and African penguins while both gulls were similar to all other species. The $\Delta^{15}N$ showed

two homogenous groups, one with Cape cormorants and Hartlaub's gulls and another with kelp gulls and African penguins. There seemed to be no homogeny within feeding guild or potential phylogenetic groups (as there seems to be a disagreement as the whether Hartlaub's gulls belong to the genus *Larus* or the genus *Chroicocephalus*). The lack of difference between DFs of species on the same diet may indicate how strongly DF is dependent on diet.

There are frequent calls to add more experimentally calculated DF to the available dataset (Gannes et al. 1997, Cherel et al. 2005b, Martínez del Rio et al. 2009b, Healy et al. in press). These enable studies conducted in the wild to be more accurate when mixing models are used, improving the conclusions made about diet. Through this study and the one by Ciancio et al. (2016), it can be seen that there is high variability among DFs of species within and across feeding guilds. $\Delta^{13}C$ exhibit more variations than $\Delta^{15}N$, so more caution should be applied when averaging $\Delta^{13}C$ values to use in studies where DFs have not yet been determined for the study species. A new model which incorporates genetic phylogeny enables the determination of DF where no experiments have been conducted (Healy et al. in press). In ecological studies, indicator species are often used to monitor environmental conditions and, therefore, it is important to gain more knowledge on species specifically studied in the wild.

5.3 The greater picture

The move from destructive diet analysis techniques to biochemical techniques to investigate trophic relationships has been encouraged because they generally pose a low risk to organisms, with small samples or non-intrusive sampling methods as well as providing information of diet over a longer time scale. Technology has also improved over the past few decades, making these techniques faster and more affordable (Gannes et al. 1997, Michener & Lajtha 2007, Boecklen et al. 2011). The increase in use of SIA has led to a call for more controlled studies to test the assumptions underlying SIA and refine our understanding of the effects of intrinsic factors.

Just as technology has improved to allow the use of SIA to assess substances, so too the technique has been expanded. SIA of specific compounds has been increasing and more research is being conducted on amino acid-specific stable isotope analysis (Bradley et al. 2014, Mompeán et al. 2016). As nutrients are assimilated, certain amino acids may be kept and used as they are while others may be stored until needed, at which point they may be manipulated according to the body's needs. Contrarily, other amino acids may be altered for storage and then altered again for use (Michener & Lajtha 2007). By looking at specific amino acids and their SI ratios, more accurate deductions about diet and feeding grounds may be drawn (Larsen et al. 2013). These techniques are currently at their

infancy and it is likely that as they become more and more used, limitations will emerge as it was the case for SIA.

We are more and more aware of the need for caution when using SIA as a diet analysis technique, and the results of this study corroborate the need to incorporate the effects of a wide array of factors. More studies where variables can be controlled are highly recommended. Despite all the warnings of applying caution when using SIA and associated DFs, if the limitations of SIA are known, and studies do not use the technique beyond its limits, it provides useful information.

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Supplementary tables

Sup. Table 1: Stable-carbon and nitrogen isotope values (mean ± SD) of prey tissue incorporated into penguin blood over turnover time for each blood tissue component

			$\delta^{13}C$		$\delta^{15}N$		
Prey tissue (days incorporated into tissue)	Age class	n ♀: n♂	W value (‰)	p homogenous groups	W value (‰)		p homogenous groups
Flesh (20)			**33.57**	**<0.0001**	**28.13**	**<0.0001**	
	A	6: 7	-17.6 ± 0	a	11.2 ± 0		a
	J	2: 2	-17.6 ± 0	a	11.2 ± 0		a
	B	4: 4	-17.7 ± 0.1	b	11.1 ± 0.1		b
	C	5: 4	-17.8 ± 0.03	c	11.0 ± 0.02		c
Delipidated flesh (20)					25.09	<0.0001	
	A	6: 7	-16.6 ± 0	a			
	J	2: 2	-16.6 ± 0	a			
	B	4: 4	-16.6 ± 0.1	b			
	C	5: 4	-16.6 ± 0.02	c			
Whole fish (20)			**33.57**	**<0.0001**	**33.38**	**<0.0001**	
	A	6: 7	-17.9 ± 0	a	10.7 ± 0		a
	J	2: 2	-17.9 ± 0	a	10.7 ± 0		a
	B	4: 4	-18.0 ± 0.1	b	10.7 ± 0.02		b
	C	5: 4	-18.1 ± 0.02	c	10.6 ± 0.01		c
Delipidated whole fish (20)					25.89	<00001	
	A	6: 7	-16.3 ± 0	a			
	J	2: 2	-16.3 ± 0	a			
	B	4: 4	-16.4 ± 0.04	b			
	C	5: 4	-16.4 ± 0.01	c			
Flesh (30)			**33.57**	**<0.0001**	**26.84**	**<0.0001**	
	A	6: 7	-17.7 ± 0	a	11.2 ± 0		a
	J	2: 2	-17.7 ± 0	a	11.2 ± 0		a
	B	4: 4	-17.8 ± 0.11	b	11.1 ± 0.1		b
	C	5: 4	-18.0 ± 0.15	c	10.9 ± 0.1		c
Delipidated flesh (30)					22.16	<0.0001	
	A	6: 7	-16.8 ± 0	a			
	J	2: 2	-16.8 ± 0	a			
	B	4: 4	-16.7 ± 0.1	b			
	C	5: 4	-16.6 ± 0.02	c			
Whole fish (30)			**33.57**	**<0.0001**	**33.19**	**<0.0001**	
	A	6: 7	-17.9 ± 0	a	10.7 ± 0		a
	J	2: 2	-17.9 ± 0	a	10.7 ± 0		a
	B	4: 4	-18.05 ± 0.1	b	10.7 ± 0.03		b
Continues …	C	5: 4	-18.2 ± 0.1	c	10.5 ± 0.1		c

Sup. Table 1 continued

Prey tissue (days incorporated into tissue)	Age class	n♀: n♂	$\delta^{13}C$ W value (‰)	p homogenous groups	$\delta^{15}N$ W value (‰)		p homogenous groups
Delipidated whole fish (30)					25.35	0.0001	
	A	6: 7	-16.4 ± 0	a			
	J	2: 2	-16.4 ± 0	a			
	B	4: 4	-16.4 ± 0.1	b			
	C	5: 4	-16.5 ± 0.2	c			
Flesh (15)			**32.16**	**<0.0001**	**30.12**	**0.0001**	
	A	6: 7	-17.5 ± 0	a	11.3 ± 0		a
	J	2: 2	-17.5 ± 0	a	11.3 ± 0		a
	B	4: 4	-17.6 ± 0.1	b	11.2 ± 0.1		b
	C	5: 4	-17.8 ± 0.02	c	11.0 ± 0.02		c
Delipidated flesh (15)					30.005	<.0001	
	A	6: 7	-16.4 ± 0	a			
	J	2: 2	-16.4 ± 0	a			
	B	4: 4	-16.5 ± 0.03	b			
	C	5: 4	-16.5 ± 0.01	c			
Whole fish (15)			**32.34**	**<0.0001**	**31.64**	**<0.001**	
	A	6: 7	-17.8 ± 0	a	10.7 ± 0		a
	J	2: 2	-17.8 ± 0	a	10.7 ± 0		a
	B	4: 4	-17.9 ± 0.1	b	10.7 ± 0.04		b
	C	5: 4	18.1 ± 0.01	c	10.6 ± 0.01		c
Delipidated whole fish (15)					30.12	<00001	
	A	6: 7	-16.3 ± 0	a			
	J	2: 2	-16.3 ± 0	a			
	B	4: 4	-16.3 ± 0.	b			
	C	5: 4	-16.4 ± 0.01	c			

Prey for delipidated plasma is the same for plasma so not shown

Sup. Table 2: Stable-carbon and nitrogen isotope values (mean ± SD) of blood components among penguins of different ages: A – adults, J – juveniles, B-blues and C – chicks.

			$\delta^{15}N$					$\delta^{13}C$				
Tissue	Age class	n♀: n♂	value (‰)	homogenous groups	F (W)[1] t (H)[2]	p		value (‰)	homogenous groups	F (W)[1] t (H)[2]	p	
Whole blood					***33.648***	***<0.0001***	*1*			***15.55***	***<0.0001***	*1*
	A	6: 7	13.7 ± 0.1	a	**2.34**	**0.04**	2	-16.2 ± 0.1	a	-0.23	0.82	2
	J	2: 2	13.8 ± 0.1	a	-0.50	0.70	2	-16.2 ± 0.2	a	-1.41	0.39	2
	B	4: 4	13.3 ± 0.2	b	0.78	0.46	2	-16.5 ± 0.1	b	-0.07	0.95	2
	C	5: 4	13.4 ± 0.1	b	**-3.44**	**0.02**	2	-16.4 ± 0.1	b	-2.18	0.07	2
Red blood cells					***(24.31)***	***<0.0001***	*1*			***9.93***	***<0.0001***	*1*
	A	6: 7	13.6 ± 0.1	a	2.13	0.06	2	-16.1 ± 0.1	a	1.06	0.32	2
	J	2: 2	13.8 ± 0.1	a	-2.05	0.19	2	-16.1 ± 0.1	a	-1.43	0.36	2
	B	4: 4	13.2 ± 0.2	b	1.72	0.13	2	-16.3 ± 0.1	b	-0.50	0.63	2
	C	5: 4	13.2 ± 0.18	b	-1.66	0.16	2	-16.3 ± 0.1	b	-1.41	0.21	2
Plasma					*1.57*	*0.22*	*1*			***4.63***	***<0.01***	*1*
	A	6: 7	14.2 ± 0.2	a	-1.67	0.13	2	-16.9 ± 0.2	a	-0.47	0.65	2
	J	2: 2	14.2 ± 0.2	a	-0.42	0.75	2	-16.9 ± 0.2	ab	0.40	0.76	2
	B	4: 4	14.2 ± 0.2	a	1.22	0.26	2	-17.0 ± 0.1	ab	-2.04	0.08	2
	C	5: 4	14.1 ± 0.2	a	-1.36	0.22	2	-17.1 ± 0.2	b	-1.44	0.19	2
Delipidated plasma										***(8.17)***	***0.04***	*1*
	A	6: 7						-15.9 ± 0.2	a	-1.65	0.14	2
	J	2: 2						-15.9 ± 0.3	a	0.55	0.65	2
	B	4: 4						-15.7 ± 0.4	a	-0.79	0.48	2
	C	5: 4						-15.7 ± 0.2	a	-0.26	0.80	2

[1] ANOVA (Kruskal Wallis) between age classes

[2] t-test (Mann-Whitney U test) between sex within each age classes

Sup. Table 3: Comparison of C: N ratio of three blood components across four age classes of penguins

			C:N				
Tissue	Age class	n♀: n♂	df value (‰)	Error SD	t	p Homogenous groups	
Whole blood			3	32	**12.021**	**0.00**	[1]
	A	6: 7	3.32	0.03		a	[2]
	J	2: 2	3.33	0.02		ab	[2]
	B	4: 4	3.38	0.02		bc	[2]
	C	5: 4	3.40	0.05		c	[2]
Red blood cells			3	32	0.891	0.46	[1]
	A	6: 7	3.24	0.02			[2]
	J	2: 2	3.24	0.02			[2]
	B	4: 4	3.23	0.01			[2]
	C	5: 4	3.24	0.02			[2]
Plasma			3	31	8.792	0.000	[1]
	A	6: 7	4.01	0.12		a	[2]
	J	2: 2	4.06	0.15		ab	[2]
	B	4: 4	4.22	0.12		b	[2]
	C	5: 4	4.22	0.09		b	[2]

[1] ANOVA (Kruskal Wallis) between age classes

[2] t-test (Mann-Whitney U test) between sex within each age classes

Sup. Table 4: Discrimination factors for stable-nitrogen and carbon isotope values (mean ± SD) of blood components and fish components

			$\Delta^{15}N$		$\Delta^{13}C$	
DF = Penguin tissue - prey tissue	Age class	n♀: n♂	F (W) value (‰)	p homogenous groups	F (W) value (‰)	p homogenous groups
Whole blood - flesh			**17.28**	**<0.0001**	**5.00**	**<0.01**
	A	6: 7	2.5 ± 0.1	a	1.3 ± 0.1	ab
	J	2: 2	2.6 ± 0.1	a	1.4 ± 0.2	ab
	B	4: 4	2.1 ± 0.2	b	1.2 ± 0.1	b
	C	5: 4	2.4 ± 0.1	a	1.4 ± 0.1	a
Whole blood - delipidated flesh					**12.35**	**<0.0001**
	A	6: 7			0.4 ± 0.1	a
	J	2: 2			0.4 ± 0.2	a
	B	4: 4			0.1 ± 0.1	b
	C	5: 4			0.1 ± 0.1	b
Whole blood - whole fish			**23.65**	**<0.0001**	**5.00**	**<0.01**
	A	6: 7	3.0 ± 0.1	a	1.6 ± 0.1	ab
	J	2: 2	3.08 ± 0.1	a	1.7 ± 0.2	ab
	B	5: 5	2.60 ± 0.2	b	1.5 ± 0.1	b
	C	5: 4	2.8 ± 0.1	b	1.7 ± 0.1	a

Continues …

Sup. Table 4
continued

DF = Penguin tissue - prey tissue	Age class	n♀: n♂	$\Delta^{15}N$ F (W) value (‰)	$\Delta^{15}N$ p homogenous groups	$\Delta^{13}C$ F (W) value (‰)	$\Delta^{13}C$ p homogenous groups
Whole blood - delipidated whole fish					**7.86**	**<0.001**
	A	6: 7			0.1 ± 0.1	a
	J	2: 2			0.1 ± 0.2	a
	B	4: 4			-0.1 ± 0.1	b
	C	5: 4			-0.02 ± 0.1	ab
Red blood cell - flesh			**12.32**	**<0.0001**	(3.20)	0.36
	A	6: 7	2.4 ± 0.1	a	1.6 ± 0.1	a
	J	2: 2	2.6 ± 0.1	a	1.6 ± 0.1	a
	B	4: 4	2.1 ± 0.2	b	1.6 ± 0.2	a
	C	5: 4	2.3 ± 0.2	a	1.7 ± 0.2	a
Red blood cell - delipidated flesh					**14.42**	**<0.0001**
	A	6: 7			0.6 ± 0.1	a
	J	2: 2			0.6 ± 0.1	a
	B	4: 4			0.4 ± 0.2	b
	C	5: 4			0.3 ± 0.1	b
Red blood cell - whole fish			**21.83**	**<0.0001**	1.65	0.20
	A	6: 7	2.9 ± 0.1	a	1.8 ± 0.1	a
	J	2: 2	3.1 ± 0.1	a	1.8 ± 0.1	a
	B	4: 4	2.5 ± 0.2	b	1.8 ± 0.1	a
	C	5: 4	2.6 ± 0.1	b	1.9 ± 0.1	a
Red blood cell - delipidated whole fish					**3.33**	**0.03**
	A	6: 7			0.2 ± 0.1	a
	J	2: 2			0.3 ± 0.1	a
	B	4: 4			0.1 ± 0.1	a
	C	5: 4			0.1 ± 0.1	a
Plasma - flesh			2.31	0.10	0.80	0.50
	A	6: 7	2.9 ± 0.2	a	0.6 ± 0.2	a
	J	2: 2	2.9 ± 0.2	a	0.5 ± 0.2	a
	B	4: 4	3.1 ± 0.1	a	0.6 ± 0.3	a
	C	5: 4	3.1 ± 0.2	a	0.7 ± 0.2	a
Plasma - delipidated flesh					2.42	0.09
	A	6: 7			-0.4 ± 0.2	a
	J	2: 2			-0.5 ± 0.2	a
	B	4: 4			-0.6 ± 0.2	a
	C	5: 4			-0.6 ± 0.2	a
Plasma - whole fish			0.74	0.53	0.46	0.72
	A	6: 7	3.4 ± 0.2	a	1.0 ± 0.2	a
	J	2: 2	3.4 ± 0.2	a	0.9 ± 0.2	a
	B	4: 4	3.5 ± 0.2	a	0.9 ± 0.2	a
Continues …	C	5: 4	3.4 ± 0.2	a	1.0 ± 0.2	a

Sup. Table 4 Sup.
Table 4 *continued*

DF = Penguin tissue - prey tissue	Age class	n♀: n♂	$\Delta^{15}N$ F (W) value (‰)	$\Delta^{15}N$ p homogenous groups	$\Delta^{13}C$ F (W) value (‰)	$\Delta^{13}C$ p homogenous groups
Plasma -delipidated whole fish					1.37	0.27
	A	6: 7			-0.6 ± 0.2	a
	J	2: 2			-0.7 ± 0.2	a
	B	4: 4			-0.7 ± 0.2	a
	C	5: 4			-0.7 ± 0.2	a
Delipidated plasma - flesh					**7.05**	**<0.001**
	A	6: 7			1.5 ± 0.2	a
	J	2: 2			1.6 ± 0.3	a
	B	4: 4			1.9 ± 0.4	ab
	C	5: 4			2.1 ± 0.2	b
Delipidated plasma - delipidated flesh					**10.56**	**0.01**
	A	6: 7			0.5 ± 0.2	a
	J	2: 2			0.6 ± 0.3	ab
	B	4: 4			0.8 ± 0.4	b
	C	5: 4			0.8 ± 0.1	b
Delipidated plasma - whole fish					**5.33**	**<0.01**
	A	6: 7			1.9 ± 0.2	a
	J	2: 2			2.0 ± 0.3	ab
	B	4: 4			2.2 ± 0.4	ab
	C	5: 4			2.4 ± 0.2	b
Delipidated plasma - delipidated whole fish					**13.34**	**<0.01**
	A	6: 7			0.3 ± 0.2	a
	J	2: 2			0.4 ± 0.3	ab
	B	4: 5			0.6 ± 0.4	b
	C	5: 4			0.7 ± 0.1	b

Sup. Table 5: Stable-nitrogen isotope values (mean ± SD) of whole blood of males and females.

Tissue	Age class	n♀: n♂	Male $\delta^{15}N$ F value (‰)	Male $\delta^{15}N$ p homogenous groups	Female $\delta^{15}N$ W value (‰)	Female $\delta^{15}N$ p homogenous groups
Whole blood			**16.81**	**<0.0001**	**12.90**	**<0.01**
	A	6: 7	13.6 ± 0.1	a	13.8 ± 0.1	a
	J	2: 2	13.8 ± 0.1	a	13.8 ± 0.02	a
	B	4: 4	13.2 ± 0.2	b	13.3 ± 0.2	b
	C	5: 4	13.4 ± 0.1	b	13.3 ± 0.04	b

Sup. Table 6: Stable-nitrogen isotope values (mean ± SD) of fish components eaten by males and female penguins

			Male $\delta^{15}N$		Female $\delta^{15}N$	
Tissue	Age class	n♀: n♂	W value (‰)	p homogenous groups	W value (‰)	p homogenous groups
Flesh			16.4	<0.001	16.23	<0.005
	A	6: 7	11.2 ± 0.02	a	11.2 ± 0.02	a
	J	2: 2	11.2 ± 0.02	a	11.2 ± 0.02	a
	B	4: 4	11.1 ± 0.1	b	11.2 ± 0.1	b
	C	5: 4	11.0 ±0.04	c	10.95 ± 0.11	c
Whole fish			16.4	<0.001	16.23	<0.005
	A	6: 7	10.7 ± 0.02	a	10.7 ± 0.02	a
	J	2: 2	10.7 ± 0.02	a	10.7 ± 0.02	a
	B	4: 4	10.7 ± 0.04	b	10.7 ± 0.03	b
	C	5: 4	10.6 ± 0.02	c	10.6 ± 0.1	c

Sup. Table 7: Discrimination factors for stable-nitrogen and carbon isotope values (mean ± SD) of male and female whole blood and fish components

DF = Penguin tissue - prey tissue	Age class	n♀: n♂	Male $\Delta^{15}N$ F value (‰)	p homogenous groups	Female $\Delta^{15}N$ F value (‰)	p homogenous groups
Whole blood - flesh			8.78	<0.01	13.40	<0.001
	A	6: 7	2.4 ± 0.1	a	2.5 ± 0.1	a
	J	2: 2	2.6 ± 0.1	a	2.6 ± 0.02	a
	B	4: 4	2.1 ± 0.2	b	2.2 ± 0.2	b
	C	5: 4	2.5 ± 0.04	a	2.3 ± 0.05	ab
Whole blood - whole fish			11.80	<0.001	17.53	<0.0001
	A	6: 7	2.9 ± 0.1	a	3.05 ± 0.1	a
	J	2: 2	3.1 ± 0.1	a	3.06 ± 0.02	a
	B	4: 4	2.6 ± 0.2	b	2.7 ± 0.2	b
	C	5: 4	2.8 ± 0.1	a	2.7 ± 0.04	b

Sup. Table 8: Results of ANCOVA on $\delta^{13}C$ of blood components at three (four for WB) different egg production phases (EPP): initial yolk deposition (IYD), rapid yolk deposition (RYD), albumen production (AlbProd – only in WB), and after laying.

		Whole blood - WB			Red blood cells - RBC			Plasma - PL			Delipidated PL		
Parameters	Effect	df	F	p [A]	df	F	p	df	F	p [A]	df	F	p
Sex*EPP	Fixed	3	**3.981**	**0.022**	2	1.446	0.262	2	2.799	0.091	2	0.083	0.921
EPP	Fixed	3	1.371	0.280	2	1.609	0.228	2	1.422	0.270	2	1.404	0.274
Sex	Fixed	1	**6.693**	**0.014**	1	2.222	0.147	1	**15.714**	**0.000**	1	1.582	0.220
Bird	Random	14	2.237	0.046	14	1.360	0.266	14	**2.902**	**0.022**	14	0.970	0.518
$\delta^{15}N$	Fixed	1	4.293	0.051	1	0.017	0.898	1	0.236	0.634	1	1.838	0.194
Flesh - FL	Fixed	1	**6.848**	**0.017**	1	**15.174**	**0.001**	1	0.383	0.545	1	1.270	0.276
Whole fish -WF	Fixed	1	2.575	0.124	1	2.286	0.148	1	0.405	0.534	1	3.174	0.094
Weight	Fixed	1	1.412	0.249	1	0.015	0.903	1	**11.914**	**0.003**	1	2.249	0.153

[A] asterisk shows where log~ log transformations have been used as residuals better conform to homoscedasticity

Bonferroni corrected to alpha = 0.025, as the same numbers are used for the two $\delta^{13}C$ analyses

Sup. Table 9: Results of ANCOVA on $\delta^{13}C$ of blood components at three (four for WB) different egg production phases (EPP): initial yolk deposition (IYD), rapid yolk deposition (RYD), albumen production (AlbProd – only in WB), and after laying.

		Whole blood - WB			Red blood cells - RBC			Plasma - PL			Delipidated PL		
Parameters	Effect	df	F	p [A]	df	F	p	df	F	p [A]	df	F	p
Sex*EPP	Fixed	3	**3.973**	**0.023**	2	1.421	0.267	2	2.799	0.081	2	0.083	0.921
EPP	Fixed	3	1.021	0.404	2	1.343	0.286	2	1.422	0.178	2	1.404	0.274
Sex	Fixed	1	**6.937**	**0.013**	1	2.157	0.153	1	**15.714**	**0.000**	1	1.582	0.220
Bird	Random	14	2.261	**0.047**	14	1.344	0.274	14	**2.902**	**0.019**	14	0.970	0.518
$\delta^{15}N$	Fixed	1	4.294	0.051	1	0.033	0.859	1	0.236	0.524	1	1.838	0.194
Delipidated FL	Fixed	1	**6.214**	**0.022**	1	**15.943**	**0.001**	1	0.383	0.439	1	1.270	0.276
Delipidated WF	Fixed	1	0.995	0.330	1	0.345	0.564	1	0.405	0.399	1	3.174	0.094
weight	Fixed	1	1.551	0.227	1	0.013	0.912	1	**11.914**	**0.003**	1	2.249	0.153

[A] asterisk shows where log~ log transformations have been used as residuals better conform to homoscedasticity

Bonferroni corrected to alpha = 0.025, as the same numbers are used for both of the $\delta^{13}C$ analyses

Sup. Table 10: Results of ANCOVA on $\delta^{15}N$ of blood components at three (four for WB) different egg production phases (EPP): initial yolk deposition (IYD), rapid yolk deposition (RYD), albumen production (AlbProd – only in WB), and after laying.

		Whole blood - WB			Red blood cells - RBC			Plasma - PL		
Parameters	Effect	df	F	p	df	F	p	df	F	p [A]
Sex*EPP	Fixed	3	0.994	0.416	2	0.107	0.899	2	0.026	0.971
EPP	Fixed	3	0.086	0.967	2	0.578	0.571	2	0.038	0.963
Sex	Fixed	1	0.066	0.800	1	2.590	0.121	1	2.162	0.157
Bird	Random	14	**9.074**	**0.000**	14	**7.711**	**0.000**	14	1.490	0.220
$\delta^{13}C$	Fixed	1	3.795	0.066	1	0.248	0.625	1	0.319	0.850
Flesh - FL	Fixed	1	0.638	0.434	1	0.138	0.714	1	2.788	0.114
Whole fish - WF	Fixed	1	0.405	0.532	1	0.285	0.600	1	2.863	0.110
Weight	Fixed	1	2.129	0.160	1	4.024	0.060	1	1.709	0.210

[A] asterisk shows where log~ log transformations have been used as residuals better conform to homoscedasticity

Sup. Table 8: Stable-carbon and nitrogen isotope values (mean ± SD) of blood components of four seabird species

Tissue	Species	n	Stable isotopes	
			$\delta^{13}C$	$\delta^{15}N$
Whole blood				
	Cape cormorant	10	-16.9 ± 0.5	14.2 ± 0.2
	Hartlaub's gull	4	-16.6 ± 0.2	14.1 ± 0.2
	Kelp gull	3	-16.5 ± 0.1	13.7 ± 0.2
	Swift tern	2	M = -17.2 F = -18.7	M = 13.5 F = 14.7
Red blood cells				
	Cape cormorant	10	-16.9 ± 0.5	14.2 ± 0.2
	Kelp gull	3	-16.3 ± 0.1	13.5 ± 0.02
Plasma				
	Cape cormorant	10	-16.2 ± 0.1	13.6 ± 0.1
	Kelp gull	3	-17.7 ± 0.1	14.3 ± 0.001

Sup. Table 9: Discrimination factors for carbon – and nitrogen stable isotope values (mean ± SD) between blood components and fish components for four sardine:sardinella diets. Fish components include flesh (FL), whole fish (WF), delipidated FL and delipidated WF. Diet 1 – 50 % sardine:50 %sardinella, diet 2 – 75 % sardine: 25 % sardinella, diet 3 - 100% sardine, and most likely diet which was different for each species (Cape cormorants – 90:10, Hartlaub's gulls – 75:25, kelp gulls – 85:15 and greater crested terns 80:20).

		$\Delta^{13}C$				$\Delta^{15}N$			
Bird species	Fish tissue	Diet 1	Diet 2	Diet 3	Most likely diet	Diet 1	Diet 2	Diet 3	Most likely diet
Cape cormorants	FL	1.3 ± 0.5	1.1 ± 0.5	0.9 ± 0.5	1.0 ± 0.5	2.7 ± 0.2	2.9 ± 0.2	3.1 ± 0.2	3.0 ± 0.2
	WF	1.7 ± 0.5	1.4 ± 0.5	1.1 ± 0.5	1.2 ± 0.5	3.1 ± 0.2	3.3 ± 0.2	3.5 ± 0.2	3.4 ± 0.2
	Delipidated FL	0.3 ± 0.5	-0.00 ± 0.5	-0.3 ± 0.5	-0.6 ± 0.5	2.3 ± 0.2	2.5 ± 0.2	2.8 ± 0.2	2.7 ± 0.2
	Delipidated WF	0.1 ± 0.5	-0.3 ± 0.5	-0.6 ± 0.5	-0.5 ± .05	2.8 ±0.2	3.0 ± 0.2	3.2 ± 0.2	3.1 ± 0.2
Hartlaub's gulls	FL	1.6 ± 0.2	1.5 ± 0.2	1.3 ± 0.2	1.5 ± 0.2	2.6 ± 0.2	2.8 ± 0.2	3.0 ± 0.2	2.8 ± 0.2
	WF	2.1 ± 0.2	1.8 ± 0.2	1.4 ± 0.2	1.8 ± 0.2	3.0 ± 0.2	3.2 ± 0.2	3.4 ± 0.2	3.2 ± 0.2
	Delipidated FL	0.6 ± 0.2	0.4 ± 0.2	0.1 ± 0.2	0.4 ± 0.2	2.2 ± 0.2	2.4 ± 0.2	2.7 ± 0.2	2.4 ± 0.2
	Delipidated WF	0.4 ± 0.2	0.1 ± 0.2	-0.2 ± 0.2	0.1 ± 0.2	2.7 ± 0.2	2.9 ± 0.2	3.1 ± 0.2	2.9 ± 0.2
Kelp gulls	FL	1.7 ± 0.1	1.5 ± 0.1	1.4 ± 0.1	1.5 ± 0.1	2.2 ± 0.2	2.4 ± 0.2	2.6 ± 0.2	2.5 ± 0.2
	WF	2.2 ± 0.1	1.9 ± 0.1	1.5 ± 0.1	1.7 ± 0.1	2.6 ± 0.2	2.8 ± 0.2	3.0 ± 0.2	2.9 ± 0.2
	Delipidated FL	0.7 ± 0.1	0.4 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	1.8 ± 0.2	2.1 ± 0.2	2.3 ± 0.2	2.1 ± 0.2
	Delipidated WF	0.5 ± 0.1	0.2 ± 0.1	-0.2 ± 0.1	0.04 ± 0.1	2.3 ± 0.2	2.5 ± 0.2	2.7 ± 0.2	2.6 ± 0.2
		M = 1.0	M = 0.9	M = 0.7	M = 0.8	M = 2.0	M = 2.2	M = 2.4	M = 2.2
Greater crested terns	FL	F = -0.6	F = -0.7	F = -0.9	F = -0.8	F = 3.2	F = 3.4	F = 3.6	F = 3.5
		M = 1.5	M = 1.2	M = 0.8	M = 1.1	M = 2.4	M = 2.6	M = 2.8	M = 2.6
	WF	F = -0.1	F = -0.4	F = 0.7	F = -0.5	F = 3.6	F = 3.8	F = 4.1	F = 3.9
		M = 0.0	M = -0.3	M = -0.5	M = -0.3	M = 1.6	M = 1.8	M = 2.1	M = 1.9
	Delipidated FL	F = -1.6	F = -1.8	F = -2.1	F = -1.9	F = 2.9	F = 3.1	F = 3.3	F = 3.1
		M = -0.2	M = -0.5	M = -0.8	M = -0.6	M = 2.1	M = 2.3	M = 2.5	M = 2.3
	Delipidated WF	F = -1.8	F = -2.1	F = -2.4	F = -2.2	F = 3.3	F = 3.5	F = 3.7	F = 3.6