Stable Isotopes of Estuarine Fish: Experimental Validations and Ecological Investigations

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Abstract

Stable isotopes of carbon and nitrogen are commonly used in ecological research to determine food webs and trace anthropogenic inputs. These applications rely on understanding isotope signature differences between an animal and its food. When an animal consumes a food item, or changes diet, it does not instantaneously reflect the isotope ratios of that food item. The isotopic signature of animal tissue gradually approaches equilibrium with the isotopic signature of its food, as molecules are turned over and new food items are assimilated into tissues. Stable isotope ratios also change between food consumed and animal tissues that are commonly sampled. The difference in stable isotope ratios between an animal‟s tissue and the food it consumes is called discrimination. The rate of change, or tissue turnover, and discrimination of stable isotopes varies among and within animals, and with environmental factors. I investigated the effects of temperature and diet on these isotope parameters for two fish species and applied results to improve determination of autotrophic sources within estuaries.

I studied two common, omnivorous, estuarine fishes found in South Australia: black bream (*Acanthopagrus butcheri*) and yellow-eye mullet (*Aldrichetta forsteri*). Temperature and diet affected both tissue turnover rates and discrimination of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotope ratios in fish muscle. Fish reared at warmer temperatures generally had faster tissue turnover rates and smaller discrimination factors than fish reared at cooler temperatures. However, temperature interacted with diet quality to affect $\delta^{13}C$ discrimination. Fish fed diets with low C:N ratios had larger $\delta^{13}C$ discrimination at warmer temperatures than at cooler temperatures. This may be caused by fish catabolising more protein for energy and therefore being able to store more lipids at cooler temperatures

than warmer temperatures. Fish fed diets with high C:N ratios were the opposite, with larger $\delta^{13}C$ discrimination at cooler temperatures than at warmer temperatures.

Compound-specific $\delta^{15}N$ analyses were performed on amino acids from experimental black bream muscle tissues to see if the change in $\delta^{15}N$ of amino acids could explain the bulk change in $\delta^{15}N$ of whole muscle tissue. Some amino acid $\delta^{15}N$ results mirrored those of bulk $\delta^{15}N$ analyses suggesting that they may be non-essential amino acids, although there was large variation among individual fish.

Wild fish commonly consume more than one dietary item, necessitating the use of mixing models to determine source contributions to diets. Omnivores consume animal and plant matter that can differ greatly in their elemental composition and this can affect the uptake of isotopic signatures from different food sources. I tested the importance of using elemental concentration in mixing models by combining two diets with different carbon and nitrogen concentrations and feeding them to yellow-eye mullet. I compared measured $\delta^{13}C$ and $\delta^{15}N$ of fish muscle with predicted values calculated with and without using elemental concentration. Using elemental concentration in mixing models improved estimates of predicted isotopic signatures.

The experimentally derived discrimination factors for black bream and yellow-eye mullet were used to investigate the relative importance of autotrophic sources to their diets in four estuaries in South Australia. Isotope signatures of carbon and nitrogen can also be used to investigate ecological niches of animals, as isotope signatures reflect what an animal has eaten from different habitats and environments. I expected the isotopic niches of black bream and yellow-eye

mullet to overlap, due to their shared environmental tolerances and feeding habits, as they are commonly found in the same estuaries. However, I found no overlap in isotopic niches between black bream and yellow-eye mullet. In some estuaries the autotrophic sources that black bream and yellow-eye mullet relied on were similar, however, in these estuaries fish appeared to be either feeding at different trophic levels or were likely not in competition with one another as they were caught in different areas within estuaries. The separate isotopic niches of black bream and yellow-eye mullet may be caused by habitat partitioning or interspecific competition within the estuaries studied.

I used $\delta^{15}N$ of black bream muscle to trace anthropogenic inputs of nutrients across a range of estuaries and related nutrient concentrations of estuarine waters to black bream abundance and recruitment. Black bream abundance and recruitment showed subsidy-stress responses to nutrient concentrations of ammonia, oxidised nitrogen and orthophosphorus, with peaks in abundance and recruitment occurring at low concentrations. A positive linear relationship was found between ammonia concentration of estuarine waters and $\delta^{15}N$ of black bream. This suggests that anthropogenic ammonia was being taken up into the food web, or directly by black bream, and affecting black bream abundance and recruitment.

In summary, I found environmental factors affected stable isotope signatures of fish muscle tissue. These results further show how important it is to quantify isotope parameters for individual species. Future research should focus on how to quantify influences on isotope signatures that cannot be determined in the field, such as ration intake, and how to account for these factors in field studies.

Declaration

I, Alexandra Louise Bloomfield certify that this work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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Archie „helping“ me study.

Chapter One: General Introduction

Harriet River mouth, Kangaroo Island, October 2008.

General Introduction

Stable isotopes were once almost exclusively used in the earth sciences. However, with the advent of on-line isotopic analyses some 20 years ago and improved rates of analysis, biologists began to take interest in their applications. In recent years the study of stable isotopes and their applications in biology and ecology have multiplied, coinciding with the gradual reduction in cost of analyses. Applications of stable isotopes have even been the subject of entire books (e.g. Lajtha and Michener, 1994; Dawson and Siegwolf, 2007) and include studying plant and animal physiology (Farquhar et al., 1989; Carleton et al., 2008), food webs (Rush et al., 2010), animal movements (Herzka, 2005), human impacts on ecosystems (Schlacher et al., 2005), and environmental change (Dawson and Siegwolf, 2007). Although stable isotopes can be used to trace elements and molecules through ecosystems, and detect change over time, experimental validation of isotope parameters is needed to improve our interpretations of field studies.

Isotope chemistry and terminology

Isotopes are naturally existing atoms of the same element with different mass. Stable isotopes are known to be stable and not decay radioactively as unstable isotopes do. They behave in a similar chemical way to atoms of the same element, forming the same types of compounds. Stable isotopes exist in natural abundances in varying proportions to their elemental counterparts. The proportion of stable isotopes relative to elemental atoms can be measured using a mass spectrometer, in relation to world-wide standards[1], and are expressed as the following:

$$\delta^{X}Y\ (‰) = ((R_{sample}/R_{standard}) - 1) \times 10^{3}$$

[1] The international standard for carbon is the Peedee Belemnite and the standard for nitrogen is air (N_2).

where X is the heavy stable isotope of element Y, measured in parts per thousand (‰). R is the ratio of the heavy isotope (X) to the light isotope of the element Y for the sample and the international standard. These proportions, or ratios, of isotopes can be altered by chemical reactions and biological processes. The change in isotope ratios caused by chemical reactions is called fractionation and results in different isotopic ratios or signatures in different compounds.

Fractionation occurs through chemical and kinetic effects on chemical reactions whereby the heavier isotope is concentrated into a particular compound. In chemical equilibrium reactions the heavier isotope is generally concentrated into molecules with the greatest bond strength (Dawson and Brooks, 2001). Kinetic effects occur in biological reactions and physical processes, such as diffusion, where the heavier isotopes/molecules move slower than their lighter counterparts, making them less available for chemical reactions. These two processes combine together to create varying concentrations of stable isotopes in different compounds, cells and organisms.

There has been some confusion over the use of the term fractionation in ecological studies, with a large number of alternatives being used: fractionation, fractionation factor, apparent fractionation, enrichment, trophic enrichment, trophic fractionation, discrimination, trophic discrimination, discrimination factor, and tissue-diet discrimination factor (Martínez del Rio et al., 2009). Martinez del Rio and Wolf (2005) suggested that the term fractionation be used strictly with regard to chemical reactions, and the equilibrium and kinetic effects that cause differences in stable isotope ratios between reactants and products. Martínez del Rio et al. (2009) argued that trophic fractionation encompasses the difference in stable isotope ratios between an entire animal and its diet, by viewing an animal

as a collection of elements and considering assimilation and excretion as chemical reactions in which an animal participates. They further proposed that the term discrimination factor be used when referring to the difference in stable isotope ratios between an animal"s tissue and its diet. The stable isotope ratio of an animal"s specific tissue (e.g. muscle) is the sum total of many chemical reactions and physical processes within the animal, which can be influenced by what the animal is experiencing physiologically (e.g. temperature, dehydration, stress). As individual tissues are influenced by what happens in the rest of the animal"s body, using the term „fractionation" would be misleading as it refers to physical and chemical influences and not necessarily physiological. The term discrimination is more encompassing for tissue-diet differences and I will use it here, as all following chapters use isotopic measurements of animal muscle tissue. Discrimination factors are commonly denoted by Δ, and are calculated by:

$$\Delta^{X}Y_{tissue\text{-}diet} = \delta^{X}Y_{tissue} - \delta^{X}Y_{diet}$$

where X is the heavy stable isotope of element Y (e.g. $\delta^{13}C$), and this will be used throughout this thesis.

Isotopes and their applications

The elements that are commonly used in stable isotope ecological research are hydrogen, carbon, nitrogen, oxygen, and sulphur. Hydrogen and oxygen isotopes are used in studies involving water and have been used, among other things, to trace migrations of birds and to determine past climatic histories (Hobson, 2007 and references therein). Sulphur isotopes ($\delta^{34}S$) are useful in both pollution and food web investigations. However analyses of hydrogen and sulphur isotopes are still relatively expensive and require larger sample amounts to gain good data. Carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotopes, on the other hand, are relatively

inexpensive to analyse and are abundant in organisms as they provide the building blocks of life, allowing us to trace nutrients and energy through systems. This thesis will focus on carbon and nitrogen isotopes only from this point on.

Carbon

Carbon isotopes vary among autotrophs due to different photosynthetic pathways, ratios of demand to supply of carbon, and the source of inorganic carbon (Farquhar et al., 1989; Marshall et al., 2007). Plants with C_3 photosynthesis (e.g. mangroves) preferentially select ^{12}C over ^{13}C for photosynthesis by RUBISCO, resulting in $\delta^{13}C$ values of approximately -27 ‰ (Farquhar et al., 1989). Plants using C_4 photosynthesis incorporate carbon dioxide into bundle sheath cells first, storing it as bicarbonate. The heavier isotope concentrates into bicarbonate compared to carbon dioxide, which is then used for photosynthesis by RUBISCO. As ^{13}C is concentrated in the bicarbonate, more is incorporated into plant tissue, leading to a higher $\delta^{13}C$ of approximately -13 ‰ (Farquhar et al., 1989). Crassulacean Acid Metabolism (CAM) plants fix carbon during the night and store it as malate before releasing the CO_2 to RUBISCO during the day, leading to large variation in $\delta^{13}C$ in these plants (Farquhar et al., 1989; Griffiths, 1992). Environmental conditions, such as water, light and nutrient availability, can also affect $\delta^{13}C$ of plant matter (Dawson et al., 2002) through diffusion affects from stomatal opening and plant health.

Photosynthesis of aquatic plants is predominantly through the C_3 pathway, however, $\delta^{13}C$ values of aquatic plants can be different to terrestrial C_3 plants. This is due to fractionation of CO_2 dissolving in water to form bicarbonate (predominately in the sea), which preferentially contains more ^{13}C. Freshwater dissolved inorganic carbon varies in $\delta^{13}C$ depending on the source of dissolved

CO_2: carbonate rock weathering, mineral springs, atmospheric CO_2, or organic matter respiration (Fry, 2006). There are also boundary affects on the uptake of carbon by aquatic plants and algae whereby plants remove dissolved inorganic carbon from the water surrounding them, which is then replaced by diffusion. However diffusion rates in water are slower than in air, resulting in supply of carbon being much lower than demand, giving RUBISCO less molecules to preferentially select ^{12}C from (Fry, 1996).

Carbon isotopes also vary among algae. Phytoplankton typically has values of -19 to -24 ‰ due to kinetic effects on fractionation (Fry, 2006). Macroalgae $\delta^{13}C$ values vary due to differential uptake of CO_2 and bicarbonate from surrounding waters (Finlay and Kendall, 2007). Dissolved inorganic carbon $\delta^{13}C$ also varies spatially depending on its source, as mentioned above, which affects $\delta^{13}C$ values of algae.

Nitrogen

Isotopic composition of N_2 in the atmosphere is 0 ‰ by definition. The rate of supply of nitrogen often limits reaction rates, such as plant growth and bacterial mineralization, resulting in smaller differences in $\delta^{15}N$ of nitrogen pools than $\delta^{13}C$ among carbon pools. The limiting rate of supply reduces the possibility of fractionation, as all nitrogen is consumed and one isotope cannot be preferentially incorporated over another. This results in $\delta^{15}N$ ranging from -10 to 10 ‰ in many biogeological pools (Fry, 2006). However, there are some distinct patterns of $\delta^{15}N$ in biological pools. Nitrification and denitrification can both lead to substantial $\delta^{15}N$ differences of up to 10 to 40 ‰ in the open ocean (Fry, 2006). A faster loss of ^{14}N over ^{15}N during particulate N decomposition leads to $\delta^{15}N$ increasing with increasing depth of soil and water (Fry, 2006). However, the most important and

widely used change in $\delta^{15}N$ is the retention of ^{15}N over ^{14}N by animals through excretion (Martínez del Rio et al., 2009 and references therein).

Isotopic discrimination

Discrimination of $\delta^{15}N$ by animals is on average 3.4 ‰ per trophic level (Post, 2002) and this has been used to determine trophic position or level within a food web (e.g. Kelleway et al., 2010). However, this is a grand average across a range of species and tissues, and $\Delta^{15}N$ is known to vary among animals as well as among tissues (DeNiro and Epstein, 1981). Although $\Delta^{13}C$ has previously been accepted to be negligible between an animal and its diet, increasingly it is being shown to be highly variable (Pinnegar and Polunin, 1999; Gaston et al., 2004; Caut et al., 2009). Therefore there have been calls for more experiments to determine discrimination factors for individual species and tissues, for $\delta^{15}N$ and $\delta^{13}C$, that can be applied to field studies (Gannes et al., 1997; Martínez del Rio et al., 2009). Environmental factors have also been shown to affect discrimination (e.g. temperature: Bosley et al., 2002; Barnes et al., 2007). Therefore experiments are needed to determine discrimination factors for species of interest under relevant environmental conditions.

Tissue turnover

Animals do not instantaneously reflect the stable isotope signatures of their diet. When an animal consumes a food item it is digested, or broken down into macromolecules, and absorbed into the blood stream. The macromolecules are then used in the body for growth and metabolism. Molecules that are assimilated into cell matter through growth and metabolism will reflect the isotopic signature of the animal‟s diet. However, the isotopic signature of animal tissue is only

diluted by the amount of food consumed when their diet changes. The mass of an animal is usually much greater than the amount of food eaten, therefore it can take some time for isotopic signatures to change when diets change. Feed rates usually depend upon the rates of growth and metabolism and these in turn determine tissue turnover rates. Therefore it is important to measure tissue turnover rates of animal tissue as well as discrimination of isotopes in experiments. This is typically done in diet switching experiments where animals are raised on one diet and then switched to another diet, of different isotopic signature (e.g. Hobson and Clark, 1992; Mirón et al., 2006; Guelinckx et al., 2007). The change in isotopes over time is recorded and when the rate of change levels off, the animal is said to be in equilibrium with its diet as isotopic signatures are no longer changing.

Ecological applications of stable isotopes

The variation in $\delta^{13}C$ of primary producers, $\delta^{15}N$ increase with increasing trophic level, and tissue turnover rates have been used to determine modern and historical diets (Bocherens et al., 2004; Koch, 2007), to study physiology (Carleton et al., 2008), track animal migrations (Hobson, 2007), and document land-use change over time (Martinelli et al., 2007). Nitrogen isotopes have also been used to trace anthropogenic inputs in aquatic ecosystems, particularly sewage inputs (Schlacher et al., 2005; Hadwen and Arthington, 2007). Sewage and animal wastes contain high concentrations of urea, which is hydrolysed to ammonia. The ammonia is then either lost as gas or is dissolved as ammonium in solution. The gas that is lost as ammonia is strongly depleted in ^{15}N through kinetic affects, leaving behind ^{15}N-enriched ammonium (Heaton, 1986). Therefore ecosystems that are affected by sewage inputs have high $\delta^{15}N$ values.

It has been suggested that stable isotopes of carbon and nitrogen can be used to investigate ecological niches (Newsome et al., 2007). Ecological niches are comprised of two sets of dimensions: scenopoetic, those that define the area an animal lives in, and bionomic, those that define the resources an animal requires to sustain its existence (Hutchinson, 1957, 1978). Carbon and nitrogen isotopes reflect resources used by an animal and where they have gained these resources. Therefore stable isotopes of carbon and nitrogen can be used to investigate ecological niches, although few studies have capitalised on these attributes to date (e.g. Genner et al., 1999; Olsson et al., 2009; Quevedo et al., 2009).

Compound-specific isotope analysis

A recent advance in the field of stable isotope ecology is that of compound-specific analyses of isotopes. It is possible to analyse individual compounds in a solution by coupling a chromatograph to a mass spectrometer so that isotopic ratios are measured as each compound is eluted out of the chromatograph. Fatty acids and amino acids are compounds focused on to date as they are able to be separated using chromatography, and analysed for $\delta^{13}C$ and $\delta^{15}N$ (McClelland and Montoya, 2002; Howland et al., 2003), although not simultaneously yet. The analysis of compound-specific isotopic signatures of amino acids enables researchers to determine trophic position of animals without needing to sample autotrophic sources, a great advantage for open ocean studies where sampling is more challenging (e.g. McClelland and Montoya, 2002; Hannides et al., 2009; Lorrain et al., 2009). There is great potential for compound-specific isotope analyses in the field of physiology as well as ecology, with few experimental studies published to date.

Mixing models

Food web studies commonly involve measuring multiple sources, or dietary items, and a target species for which the diet is to be determined. Linear mixing models can be used to determine the proportional contributions of two sources to a target using isotopes from a single element, or for three sources using two elements (Phillips and Gregg, 2001). However, there are often multiple sources and not enough elements to analyse to allow linear mixing models to find a unique solution; where the number of sources can only exceed the number of elements analysed by one. Linear models also assume that the proportional contribution of a source is equal for all elements analysed. However, elemental concentration may vary among the elements analysed within the sample (e.g. typical elemental concentration of carbon in plant matter is approximately 37 % and nitrogen concentration is approximately 2 %, Chapter 4). Therefore it seems unlikely that both elements equally contribute to mixing. Phillips and Koch (2002) described a concentration dependent linear mixing model that assumed a source"s contribution is proportional to its elemental concentration. Few experimental tests have been done on the importance of elemental concentration in mixing models (Caut et al., 2008), yet it could play an important role in determining isotopic signatures of target organisms (Pearson et al., 2003; Mirón et al., 2006).

To improve on linear mixing models when multiple sources are present, Phillips and Gregg (2003) devised a method where all possible combinations of sources (0-100 %) are calculated in increments (e.g. 1 %). The combinations that sum to the target isotopic signatures, within a tolerance to allow for variation (e.g. 0.1 %), are considered feasible solutions. The frequency and range of feasible solutions can then be determined. Phillips and Gregg (2003) wrote a computer

program, IsoSource, to perform the calculations for ecologists. IsoSource has been extremely popular and much used (e.g. Connolly et al., 2005b; Hadwen et al., 2007), however it does not take into account the inherent variability in isotope data, nor does it account for elemental concentration of sources.

Bayesian inference methods can incorporate source and discrimination variability and calculate proportional contributions as true probability distributions, unlike IsoSource (Moore and Semmens, 2008; Parnell et al., 2010). Parnell et al. (2010) recently published an open source R package called SIAR: Stable Isotope Analysis in R. SIAR uses Bayesian inference methods to analyse stable isotope data and incorporates all sources of uncertainty within data sets. It also accounts for elemental concentration of sources, encompassing the known variability and discrepancies in isotope mixing models.

Sample preparation

There are various protocols that have been used for preparation of ecological samples for stable isotope analysis. It is widely accepted that samples cannot be preserved with formalin or ethanol without affecting $\delta^{13}C$ or $\delta^{15}N$ values (Bosley and Wainright, 1999) therefore freezing of samples is required for short-term preservation. However, it is either not clear or there is some debate over other treatment protocols and when it is appropriate to use them. The two key areas relate to whether samples should be acidified and whether lipids should be extracted prior to isotope determination.

Acidification

When using stable isotope analyses to investigate diets we only want to analyse the carbon and nitrogen sources that are relevant to the diet of the study species.

This usually does not include dissolved inorganic carbon, since only organic carbon is of interest for dietary studies (Carabel et al., 2006). However, when collecting samples containing calcium carbonate deposits, such as molluscs, some algae and sediment, there is debate over whether acidification of samples should be performed to remove the inorganic carbonates, as it can affect $\delta^{15}N$ values (Carabel et al., 2006; Mazumder et al., 2010).

It is generally accepted that samples of animals that do not incorporate an exoskeleton, such as fish, will not need acidification as there will not be any dissolved inorganic carbon present. Indeed no significant effect of acidification has been found on fish tissues (Bosley and Wainright, 1999; Pinnegar and Polunin, 1999; Carabel et al., 2006). However, animals which are too small to have their carbonate structures removed before analysis, such as molluscs and crustaceans, or algae may need to be acidified to remove the inorganic carbon. Carabel et al. (2006) found a significant affect of acidification on $\delta^{13}C$ values of some plankton samples and sedimentary organic matter but no affect on a macroalga (*Laminaria hyperborea*). They also found no affect of acidification on cephalopod muscle tissue $\delta^{13}C$, but there was a significant effect on crab muscle tissue and on whole crab samples, although variable results for other invertebrate samples have been obtained by others (Jaschinski et al., 2008b; Mazumder et al., 2010). A further decrease in $\delta^{13}C$ values was observed when crab muscle and whole crab samples were washed with deionised water after acidification (Carabel et al., 2006), leading to recommendations against this practice. Acidification is not required for tissues that do not incorporate a carbonate structure, however it is advisable for plants and animals that do, such as molluscs, crustaceans, plankton, articulated coralline algae and sediment samples.

Acidification can affect $\delta^{15}N$ values as well as $\delta^{13}C$ values and this is not desirable. Pinnegar and Polunin (1999) found a significant difference in $\delta^{15}N$ values between fish samples that were acidified and those that were not, however the differences were very small (0.6-0.8‰) and are unlikely to affect trophic studies greatly. Other studies have failed to detect a difference (Bosley and Wainright, 1999; Jaschinski et al., 2008b), or found a difference in only a few species or bulk samples (Carabel et al., 2006; Mazumder et al., 2010). Therefore acidification of samples needs to be based on specific results of published studies for similar samples, or separately investigated.

Lipid extraction

Lipids are relatively depleted in ^{13}C compared to proteins and carbohydrates in an organism (DeNiro and Epstein, 1977; Post et al., 2007) and differential storage of lipids among tissue types may cause different discrimination values (Focken and Becker, 1998). Animals that have a high feed rate may also have increased lipid storage compared to other individuals of the same species (Gaye-Siessegger et al., 2004b). This may cause greater variation among samples and reduce accuracy of data interpretation.

There has been some discussion in the literature about when to account for lipids. Post et al. (2007) recommends to account for lipids when lipid content is high (when C:N > 3.5 for aquatic animals or > 4 for terrestrial animals) or variable among consumers and when differences in $\delta^{13}C$ values between end members is less than 10-12 ‰. This is the case for most food web studies. They also recommend accounting for lipid content in plants, however, in food web studies this does not necessarily make sense. If an animal consumes a plant they will assimilate much of what they need, be it lipid or protein or carbohydrate.

Therefore it makes no sense to extract lipids from plant samples in food webs studies as animals may assimilate or metabolise the lipids they consume from plants and may not discriminate against them. Dietary investigations into the affect of diet composition on discrimination should help to account for varying lipid intake and eliminate the need for lipid extraction of plant samples in food web studies (Gaye-Siessegger et al., 2005).

Similarly to acidification of samples, chemical lipid extraction can affect the $\delta^{15}N$ values of samples (Pinnegar and Polunin, 1999; Trueman et al., 2005; Post et al., 2007). This has led scientists to explore mathematical relationships of $\delta^{13}C$ and lipid content (McConnaughey and McRoy, 1979). Mathematical normalization of lipids may be more desirable than chemical extraction to preserve $\delta^{15}N$ values and to reduce sample preparation (Post et al., 2007).

Estuaries

An estuary is defined as "a partially closed coastal body of water that is either permanently, periodically, intermittently or occasionally open to the sea within which there is a measurable variation in salinity due to the mixture of seawater with water derived from on or under the land" by the Natural Resources Management Act SA (2004). Estuaries are highly complex and diverse ecosystems that vary in their size, hydrology, salinity, tidal characteristics, geomorphology, sedimentation and ecosystem energetics (Kennish, 2002; Gillanders, 2007). They are often made up of a range of habitats with varying autotrophic sources including saltmarshes, mangroves, seagrass, reefs, paperbark swamps, non-vegetated habitats and open water (Gillanders, 2007), providing a range of valuable ecosystem services (Costanza et al., 1997). Estuaries are thought to provide greater food abundance than the surrounding freshwater and marine

environments and therefore can support a high biomass of fish and invertebrates (Kennish, 2002).

Fish are known to move in and out of estuaries (Elsdon and Gillanders, 2006) and among habitats (Russell and McDougall, 2005) throughout their lives. Estuaries are thought to act as nurseries for many fish species, providing enhanced food and shelter (e.g. Shaw and Jenkins, 1992; Levin et al., 1997). However, our ability to make direct observations of fish in estuaries can be hampered by turbidity. Estuaries are important habitats for fish, however, determining the ultimate source of nutrition for fish and their migratory habits can be challenging. Stable isotope analysis provides a novel way to determine the sources of nutrition for fish, trophic relationships, migration or settlement of larvae, and pollution sources in estuaries (Herzka et al., 2002; Melville and Connolly, 2003; Schlacher et al., 2005; Hadwen et al., 2007).

Estuaries are one of the most heavily impacted ecosystems by human activities (Kennish, 2002), having been a focus for human settlement and activity throughout the world. They provide fresh water, fertile floodplains, shipping ports and abundant seafood (Saenger, 1995), attracting human settlement. Kennish (2002) argues that nutrient load increases and sewage pollution, as a result of human settlements, are two of the greatest impacts on estuaries. He further predicts that estuaries with low fresh water flows, or minimal flushing and therefore high tendency to retain nutrients, are most likely to be greatly affected by nutrient increases and recent studies have supported this view (Hadwen and Arthington, 2007). Unfortunately this also describes estuaries in South Australia (SA). SA is the driest state in Australia with average annual rainfall of just 236 mm (National Water Commission, 2007), and estuaries are rarely flushed and

highly likely to be impacted by nutrient increases and other human activities. There is a dearth of information on estuaries in South Australia and stable isotope analyses of fish, to elicit ecological information, can help us fill this gap.

Thesis outline

This thesis summarises my doctoral research on stable isotopes of carbon and nitrogen in fish and their applications in estuarine research. I used experiments to determine discrimination factors and tissue turnover rates for my study species, black bream (*Acanthopagrus butcheri* (Munro, 1949)) and yellow-eye mullet (*Aldrichetta forsteri* (Valenciennes, 1836)), and investigated the affects of temperature and diet on discrimination and tissue turnover rates. I then used these discrimination factors to determine which autotrophic sources the fishes relied on in four estuaries in South Australia, using mixing models. I investigated the isotopic niches of black bream and yellow-eye mullet in these four estuaries to see if their niches overlapped. I also used $\delta^{15}N$ of black bream muscle across a broader selection of estuaries to determine if black bream abundance and recruitment were affected by anthropogenic sources of nitrogen in estuaries. Specific aims were to:

- Determine discrimination factors and tissue turnover rates of $\delta^{15}N$ and $\delta^{13}C$ for black bream and yellow-eye mullet.
- Investigate the effects of temperature and diet on $\delta^{15}N$ and $\delta^{13}C$ discrimination and tissue turnover rates.
- Determine if compound-specific analyses of amino acid $\delta^{15}N$ can indicate the causes of $\delta^{15}N$ discrimination.
- Test the importance of using elemental concentration in mixing models.

- Determine the relative importance of autotrophic sources to black bream and yellow-eye mullet in four estuaries using stable isotopes.
- Use stable isotopes to determine niche overlap of two omnivorous fishes.
- Determine if anthropogenic sources of nitrogen in estuaries are affecting abundance and recruitment of black bream, by using $\delta^{15}N$ to trace human-influenced molecules.

The following four chapters (2-5) are original data, written as articles for publication in scientific journals. The first chapter has been published, with the second chapter currently under review. The last two chapters will be submitted to journals for peer review and publication shortly. While there may be some inconsistency among chapters with regards to notation or language, such as using scientific or common names for fish species, this is due to chapters being submitted to different journals and the need to adhere to individual journal style. Tables and figures are imbedded within the text and cited references are listed at the end of the thesis in the *Bibliography*. The following is a brief overview of each chapter:

Chapter 2

Discrimination factors and tissue turnover rates can be affected by temperature and dietary composition and are known to vary among species. This chapter describes an experiment done on black bream to determine discrimination factors and tissue turnover rates, with treatments of temperature and diet composition. After initial bulk isotopic analyses of $\delta^{13}C$ and $\delta^{15}N$, compound-specific $\delta^{15}N$ of amino acids were analysed to see if they could explain the results of bulk $\delta^{15}N$ changes.

Chapter 3

Elemental concentration of diet can affect stable isotope incorporation rates and discrimination factors. This chapter describes an experiment on yellow-eye mullet to determine discrimination factors and tissue turnover rates for fish fed diets varying in elemental concentration and reared at different temperatures. The use of elemental concentration in mixing models was also tested by mixing the diets that varied in elemental concentration and feeding the mixed diets to fish. Measured results were compared with predicted values from linear mixing models, with and without accounting for elemental concentration of diets.

Chapter 4

One of the most common applications of stable isotopes is determining diets or food webs. However, few studies quantify discrimination factors for study species, with even fewer accounting for environmental influences on discrimination factors, such as temperature. This chapter summarises the results of isotopic analyses of black bream and yellow-eye mullet in four estuaries in South Australia, using the experimentally derived discrimination factors from Chapters 2 and 3. Autotrophic sources, black bream, and yellow-eye mullet were sampled within estuaries and analysed for $\delta^{13}C$ and $\delta^{15}N$. The mixing model SIAR was used to determine proportional contributions of autotrophic sources to black bream and yellow-eye mullet diets. The overlap of isotopic niches of black bream and yellow-eye mullet in each estuary were also analysed, as these two fishes are both common and omnivorous and are frequently found in the same estuaries in high abundance.

Chapter 5

Nitrogen compounds that are influenced by human activities, particularly sewage, commonly show high $\delta^{15}N$ signatures. This premise was used to detect human nutrient inputs in estuaries in South Australia where black bream reside and reproduce. Black bream recruitment to a metapopulation from estuaries was determined using otolith chemistry. Black bream abundance and nutrient concentrations of water were measured for each estuary. Responses of black bream abundance and recruitment to nutrient concentrations were documented. Relationships of black bream muscle $\delta^{15}N$ with ammonia and oxidised nitrogen concentrations of estuaries were sought to determine if anthropogenic sources of nutrients were being taken up into the food web and affecting black bream recruitment and abundance.

Chapter 6

Chapter 6 is a general discussion of the results of the proceeding chapters, with future directions given for isotope research.

Chapter Two: Temperature and diet affect carbon and nitrogen isotopes of fish muscle: can amino acid nitrogen isotopes explain effects?

Experimental fish samples: *Acanthopagrus butcheri.*

Chapter 2 Preamble

This chapter is a co-authored paper published in the *Journal of Experimental Marine Biology and Ecology*, and as such, is written in the plural throughout. It is included with permission from Elsevier (see Appendix A), and can be cited as: Bloomfield, A.L., Elsdon, T.S., Walther, B.D., Gier, E.J., Gillanders, B.M., 2011. Temperature and diet affect carbon and nitrogen isotopes of fish muscle: can amino acid nitrogen isotopes explain effects? Journal of Experimental Marine Biology and Ecology 399(1), 48-59.

In this chapter Travis Elsdon, Benjamin Walther, Bronwyn Gillanders and I developed the experimental design and supplied the funding. Travis Elsdon, Benjamin Walther and I did the experiment, caring for the fish. I prepared the samples and assisted with the analyses of bulk isotopic signatures. I further prepared most of the samples for compound-specific $\delta^{15}N$ analyses. Elizabeth Gier prepared some samples and did the compound-specific $\delta^{15}N$ analyses. I did all of the statistical analyses and wrote the accepted manuscript with input from all co-authors.

I certify that the statement of contribution is accurate

Alexandra Bloomfield (Candidate)

Signed

I herby certify that the statement of contribution is accurate and I give permission for the inclusion of the paper in the thesis

Professor Bronwyn Gillanders

Dr Travis Elsdon

Dr Benjamin Walther

Dr Elizabeth Gier

Temperature and diet affect carbon and nitrogen isotopes of fish muscle: can amino acid nitrogen isotopes explain effects?

Abstract

Stable isotope ratios of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) are widely used in food web studies to determine trophic positioning and diet sources. However in order to accurately interpret stable isotope data the effects of environmental variability and dietary composition on isotopic discrimination factors and tissue turnover rates must be validated. We tested the effects of temperature and diet on tissue turnover rates and discrimination of carbon and nitrogen isotopes in an omnivorous fish, black bream (*Acanthopagrus butcheri*). Fish were raised at 16°C or 23°C and fed either a fish-meal or vegetable feed to determine turnover rates in fish muscle tissue up to 42 days after exposure to experimental treatments. Temperature and diet affected bulk tissue $\delta^{15}N$ turnover and discrimination factors, with increased turnover and smaller discrimination factors at warmer temperatures. Fish reared on the vegetable feed showed greater bulk tissue $\delta^{15}N$ changes and larger discrimination factors than those reared on a fish-meal feed. Temperature and diet affected bulk tissue $\delta^{13}C$ values, however the direction of effects among treatments changed. Analyses of $\delta^{15}N$ values of individual amino acids found few significant changes over time or treatment effects, as there was large variation at the individual fish level. However glutamic acid, aspartic acid and leucine changed most over the experiment and results mirrored those of

treatment effects in bulk $\delta^{15}N$ tissue values. The results demonstrate that trophic discrimination for $\delta^{15}N$ and $\delta^{13}C$ can be significantly different than those typically used in food web analyses, and effects of diet composition and temperature can be significant. Precision of compound-specific isotope analyses (0.9 ‰) was larger than our effect size for bulk $\delta^{15}N$ diet effects (0.7 ‰), therefore future experimental work in this area will need to establish a large effect size in order to detect significant differences. Our results also suggest that compound-specific amino acid $\delta^{15}N$ may be useful for determining essential and non-essential amino acids for different animals.

Introduction

Understanding where animals derive their energy and nutrition from is important for management of ecosystems and reconstructing food web dynamics. Traditional descriptions of aquatic animal diets have come from feeding observations or gut-content analysis (e.g. Webb, 1973; Gillanders, 1995; Sarre et al., 2000; Platell et al., 2006). However, results from these methods may not reflect the actual source of energy and nutrients assimilated in aquatic food webs, but rather reflect ingested dietary items at one point in time. Stable isotope ratio analysis, on the other hand, allows assimilated energy and nutrients to be tracked back to dietary sources (e.g. Melville and Connolly, 2003; Gaston and Suthers, 2004; Connolly et al., 2005b), providing a more complete and time integrated description of trophic structures.

Stable isotope ratios have been broadly employed to investigate ecological processes, such as food web dynamics (Michener and Schell, 1994) and larval settlement (Herzka, 2005). Isotope ratios of ^{13}C to ^{12}C (expressed as $\delta^{13}C$) and ^{15}N to ^{14}N (expressed as $\delta^{15}N$) are particularly informative, with $\delta^{13}C$ being used to

trace primary producers and $\delta^{15}N$ being used to determine trophic positioning of consumers (see Smit, 2001; e.g. Connolly et al., 2005b). The isotopic discrimination factor, or the difference in isotopic composition between a consumer"s tissue and its food (Martínez del Rio et al., 2009), varies among tissue types (DeNiro and Epstein, 1978, 1981). Bulk isotopic discrimination of $\delta^{15}N$ is generally considered to be 2-4 ‰ for most soft tissues, such as muscle and liver (DeNiro and Epstein, 1981; Minagawa and Wada, 1984; Post, 2002; McCutchan et al., 2003) and this has been applied to estimate relative trophic positions of species and individuals (e.g. Melville and Connolly, 2003; Hadwen and Arthington, 2007). Bulk isotopic discrimination of ^{13}C has been found to be small compared to the range in $\delta^{13}C$ values in nature (DeNiro and Epstein, 1978; Peterson and Fry, 1987; Post, 2002; McCutchan et al., 2003) and researchers have often omitted applying a discrimination factor when identifying baseline compositions of carbon sources in food webs (e.g. Melville and Connolly, 2003; Connolly et al., 2005b; Hadwen and Arthington, 2007; Hadwen et al., 2007). However, bulk isotopic discrimination values of $\delta^{13}C$ and $\delta^{15}N$ have been found to vary significantly in both laboratory experiments (e.g. Bosley et al., 2002; Gaston and Suthers, 2004; Trueman et al., 2005; Barnes et al., 2007; Elsdon et al., 2010) and field studies (e.g. Connolly et al., 2005a; Mill et al., 2007). Applying inappropriate and untested bulk isotopic discrimination values could lead to erroneous estimates of both trophic position and baseline sources of food webs (McCutchan et al., 2003). This has led to calls for more experiments to refine the magnitude of bulk isotopic discrimination (Gannes et al., 1997; Robbins et al., 2005; Martínez del Rio et al., 2009).

Tissue isotope turnover rate is the speed at which isotopic signatures of animal tissues change following a dietary shift to a new food (Herzka, 2005). Tissue turnover rates also vary among species and among tissue types (e.g. bone collagen turnover takes longer than muscle DeNiro and Epstein, 1978, 1981; Tieszen et al., 1983; Hesslein et al., 1993; MacNeil et al., 2006; Guelinckx et al., 2007), which is thought to relate to the relative activity of metabolism and growth. More metabolically active tissue has faster turnover rates than tissue that is less metabolically active (Guelinckx et al., 2007). Likewise, actively growing tissue has faster turnover rates compared to tissue that is not actively growing, although this is largely due to dilution effects (Herzka, 2005). Water temperature can affect the tissue turnover rate of fish as their metabolism and growth slows in colder water, and temperature also affects isotopic fractionation and subsequently discrimination factors[2] (Bosley et al., 2002; Witting et al., 2004; Perga and Gerdeaux, 2005; Barnes et al., 2007). The composition of the diet does also affect the allocation of nutrients and therefore the tissue turnover rate (Focken and Becker, 1998) and discrimination factor (Gaye-Siessegger et al., 2004a; Gaye-Siessegger et al., 2006). It is vital that the causes of variation in tissue turnover rates and discrimination factors are understood in order to accurately interpret field-collected data on stable isotopes in food webs.

Discrimination of carbon and nitrogen isotopes, and assimilation of nutrients and energy, may also be dependent on physiological factors including how elements are sourced: such as carbon from sugars or lipids; nitrogen directly from proteins in the diet, recycled within the animal or synthesised *de novo*

[2] Note that here we use the term „fractionation" to refer to the chemical process where reactant and product isotopic signatures differ; and we use the term „discrimination factor" to refer to the difference in isotopic signatures between a consumer"s tissue and its diet, as Martínez del Rio et al. (2009) recommend.

(Hobson et al., 1993; Focken and Becker, 1998; Post et al., 2007), and these are related to diet quality and intake factors. Bulk tissue nitrogen discrimination is thought to be largely related to protein intake, with the more protein an animal eats, the more enriched in ^{15}N it becomes (Vander Zanden and Rasmussen, 2001; Martínez del Rio et al., 2009; Kelly and Martínez del Rio, 2010) because ^{14}N is preferentially excreted. The excreted nitrogen comes from catabolism of amino acids. If an animal is eating a protein rich diet it will catabolise more amino acids for energy creating more depleted excreta and more enriched tissue (Gannes et al., 1998 and references therein). However, if an animal is eating a protein poor diet, or it is fasting, it is forced to manufacture its own amino acids by transamination using proteins already in the tissue and therefore tissue ^{15}N is still enriched (Hobson et al., 1993; Gannes et al., 1998 and references therein). Theoretically though, if an animal is consuming a diet that matches its requirements then $\delta^{15}N$ enrichment would be at a minimum (Robbins et al., 2005) as amino acids would be used directly, with little catabolism or transamination.

Animals are limited in their ability to manufacture amino acids, and essential amino acids must be obtained from food. Therefore, $\delta^{15}N$ values of essential amino acids should theoretically be preserved in a food web and provide a conservative tool for identifying food web dynamics. In practise $\delta^{15}N$ of all essential amino acids may not be conserved up the food chain (McClelland and Montoya, 2002). However different groupings of amino acids, that contain essential and non-essential amino acids, may yield the same information and enable us to determine nitrogen sources and trophic position (Schmidt et al., 2004; Popp et al., 2007; Hannides et al., 2009; Lorrain et al., 2009; Olson et al., 2010). McClelland and Montoya (2002) found that the $\delta^{15}N$ discrimination of some

amino acids (i.e. phenylalanine, glycine, serine, threonine, lysine and tyrosine) by zooplankton consumers was approximately the same or less than the bulk discrimination between food and consumer (1.7 ‰ in that study). They also found that several amino acids were enriched in $\delta^{15}N$ by more than the bulk discrimination; they were enriched by ~3-7 ‰ (i.e. alanine, aspartic acid, glutamic acid, isoleucine, leucine, proline and valine). It is thought that those amino acids that remain similar to food sources in $\delta^{15}N$ undergo dominant metabolic processes that neither cleave nor form carbon-nitrogen bonds (Chikaraishi et al., 2007); and these have been called „source amino acids" (Popp et al., 2007). Alternatively those amino acids that are enriched in ^{15}N undergo metabolic processes that cleave carbon-nitrogen bonds (Chikaraishi et al., 2007); and these have been called „trophic amino acids" (Popp et al., 2007). This has led to the theory that some amino acids, which may or may not be essential, can be used to trace the source of nutrients whilst others can indicate trophic position and therefore consumer samples alone can be used to define trophic position (Popp et al., 2007).

Seasonal variation in $\delta^{15}N$ of amino acids in oceanic zooplankton have been reported (Hannides et al., 2009) with variation in basal $\delta^{15}N$ of inorganic nitrogen being identified as the reason for the variability. However the extent to which temperature influences the incorporation and subsequent enrichment of $\delta^{15}N$ in amino acids in animals has not been tested. Although some experimental work analysing the $\delta^{15}N$ values of amino acids has been done, it has focused on invertebrates (McClelland and Montoya, 2002; Chikaraishi et al., 2009) with little work on fish (Chikaraishi et al., 2009) and it has been acknowledged that more laboratory experiments are needed to test the broader applications of compound-specific isotope analyses of amino acids (Hannides et al., 2009; Martínez del Rio

et al., 2009; Naito et al., 2010; Olson et al., 2010). To our knowledge no manipulative experiments have been done to test if environmental or dietary factors affect $\delta^{15}N$ of amino acids, as they are assumed to be unaffected by these factors.

To increase our understanding of variation in isotopic discrimination factors and muscle tissue isotope turnover rates an experiment was done on an omnivorous fish, *Acanthopagrus butcheri*. We tested the hypotheses that fish reared at warmer temperatures would have a faster bulk tissue isotope turnover rate and a smaller bulk isotopic discrimination compared with fish kept at colder temperatures. We also tested the hypothesis that fish fed a diet based on fish-meal will have a faster tissue turnover rate and smaller discrimination than those fed a diet based on vegetable protein. The $\delta^{15}N$ of individual amino acids were further analysed to elucidate the causes of variation in discrimination factors among treatments. We assumed that $\delta^{15}N$ values of certain amino acids do not change or fractionate, and therefore record the $\delta^{15}N$ value of the sources of amino acids, while other amino acids are highly fractionated and provide a direct estimate of trophic level as shown by others (i.e. McClelland and Montoya, 2002; Popp et al., 2007).

Methods

Fish rearing

Juvenile black bream, *A. butcheri*, were obtained from a hatchery and acclimated to either 16°C or 23°C to reflect local winter and summer temperatures (Elsdon et al., 2009). During acclimation fish were maintained on hatchery feed and were fed this feed for approximately 100 days before the start of the experiment.

Treatments consisted of orthogonal combinations of two temperatures, two diets, and five rearing periods, making a total of 20 combinations with two replicate tanks per combination.

Fish were randomly allocated to 40L tanks at densities of 6-10 fish per tank. At this time twenty fish were sacrificed (day 0) to measure initial mean sizes (68 ± 2 (SE) mm; standard length and 11.52 ± 0.89 (SE) g; mass), with five of these being analysed for $\delta^{13}C$ and $\delta^{15}N$ of fish muscle tissue. A subset of 10 fish was maintained on the hatchery feed for a further 29 days at 16°C, with five fish sacrificed after seven days and the remaining five after 29 days, to test if fish muscle was in an isotopic steady state with the hatchery feed. Of the two feeds that were used during the experimental phase, one was based on fish-meal with a high protein content, and the other was vegetable based and had lower protein content (see Table 2.1). Stable isotope signatures of diets were not artificially enriched so that diets reflected natural situations and results are applicable to field studies. We acknowledge that protein quality and quantity have varied concurrently in this experiment, however we believe that this is a realistic approach as protein quality and quantity are likely to vary concurrently in nature. Fish were switched to experimental feeds and reared for 2, 7, 14, 28, and 42 days with entire tanks being sacrificed on these days, as there were replicate tanks for each time and treatment combination (total of 40 tanks, with 8 tanks sacrificed each time). Fish were fed two to three times a day to satiation; no dominance effects were observed.

Table 2.1 Attributes of the hatchery feed and the two feeds used in the experiment. Proximal composition information is taken from the manufacturers‟ packaging. Note: NA = information not available.

Feed Attribute	Hatchery feed	Fish-meal feed	Vegetable feed
$\delta^{13}C$ (mean ‰ ± SE)	-20.2 ± 0.1	-21.1 ± 0.0	-22.2 ± 0.1
$\delta^{15}N$ (mean ‰ ± SE)	9.6 ± 0.1	8.8 ± 0.2	1.4 ± 0.3
Protein (%)	45	43	28
Fat (%)	22	9	4
Carbohydrates (%)	NA	15	NA
Fibre (%)	NA	2	7
Ash (%)	NA	13	14
Moisture (%)	NA	12	12
Unaccounted for (%)	33	6	35

Bulk tissue stable isotope ratios: sample preparation and analysis

Fish were sacrificed, weighed (mass (g)) and measured (standard length and total length (mm)) before having dorsal muscle samples taken. Muscle samples were frozen to -80°C, then freeze-dried and ground into a powder using a mortar and pestle, with bones and scales being removed prior to grinding. Feed samples were oven dried at 85°C for 96 hrs and ground into a powder using a mortar and pestle. Lipids were not extracted from any samples; C:N for fish muscle averaged a low 3.52 ± 0.04 (SE) and, as we are only comparing samples within a species and not across a food web, we decided that lipid extraction would introduce more variation in $\delta^{15}N$ and was unnecessary (Post et al., 2007). Lipids were not extracted for feed samples as fish consume and metabolise the entire feed, including the lipids. Samples of fish muscle (n = 5/tank) and feeds (n = 5/diet)

were weighed into tin capsules for $\delta^{13}C$ and $\delta^{15}N$ analyses, which were done on a SerCon ANCA SL/20-20 continuous flow isotope ratio mass spectrometer. International and internal laboratory reference materials (EDTA, ammonium sulphate, glycine, and sucrose) were run every 10 samples for calibration of the instrument readings. Average precision of the machine was 0.18 ‰ for $\delta^{15}N$ values and 0.29 ‰ for $\delta^{13}C$ values (1 SD). Average accuracy was 0.14 ‰ for $\delta^{15}N$ values and 0.36 ‰ for $\delta^{13}C$ values.

Amino acid analysis

Fish muscle samples (n = 3/treatment group) were randomly picked from within a subset of treatment groups and prepared for analysis of $\delta^{15}N$ values of amino acids. Fish from the start of the experiment (time = 0) and fish from each of the treatment groups (n = 4 groups) at the end of the experiment (time = 42 days) were analysed. Fish from days 7, 14 and 28 reared on the vegetable feed at 23°C were also analysed because the bulk $\delta^{15}N$ values showed the greatest nitrogen discrimination factor and change over time (see results). Within each treatment group at least one fish from each replicate tank was chosen for analysis. The three feeds (hatchery, vegetable, and fish-meal) were also prepared and analysed for $\delta^{15}N$ of amino acids.

Acid hydrolysis and derivatization

Samples were prepared similar to Hannides et al. (2009) for amino acid hydrolysis and derivitization with minor deviations. Sub-samples of approximately 5-10 mg of freeze-dried fish muscle and 20 mg of oven dried fish food were weighed into 8 mL screw-cap glass vials for acid hydrolysis. Each vial had 0.5 mL of 6N HCl acid (sequanal grade, constant boiling) added. Vials were flushed with N_2, sealed

with Teflon-lined caps and heated to 150°C for 70 min to hydrolyse proteins into amino acids. The vials were cooled and then evaporated until dry at 55°C under a stream of N_2. The dried hydrolysate was then re-dissolved in 1 mL of 0.01N HCl acid and filtered through low protein-binding, 0.22 μm, hydrophilic filters. Vials were rinsed with a further 1 mL of 0.01N HCl and filtered. The hydrolysate was purified further through 5 cm of a cation-exchange column made of Dowex 50WX8-400 ion exchange resin (Metges and Petzke, 1996) suspension using 0.01N HCl acid, in a Pasteur pipette, blocked with glass wool. Amino acids were eluted through the column by adding repeated rinses of 2N NH_4OH totalling 4 mL in volume. The elutate was evaporated until dry at 80°C under a stream of N_2. The samples were re-acidified with 0.5 mL of 0.2N HCl acid, vials were flushed with N_2 and heated to 110°C for 5 min.

Amino acids were derivatized by esterification of the carboxyl terminus and trifluoroacetylation of the amine group (Macko et al., 1997; Popp et al., 2007). Samples were dried under a stream of N_2 at 55°C. To each sample, 2 mL of 4:1 isopropanol:acetyl chloride was added, then vials were flushed with N_2 and heated to 110°C for 60 min. After the vials cooled, excess solvent was evaporated to dryness under a stream of N_2 at 60°C. To each sample, 800 μL of a 3:1 methylene chloride:trifluoracetic anhydride (TFAA) was added and heated to 100°C for 15 min, with the vial caps on, to acylate amino acids. Vials were cooled and the liquid evaporated to dryness under N_2.

Samples were further purified by solvent extraction through re-dissolving them in 2 mL of phosphorus-buffer (KH_2PO_4 + Na_2HPO_4 in ultra pure water, pH 7) and 1 mL of chloroform, mixed vigorously for 60 s and centrifuged at 600 g for approximately 2 min (Ueda et al., 1989). The chloroform layer containing the

acylated amino acid esters was extracted into a clean vial and 1 mL of chloroform was further added to the remaining phosphorus-buffer mixture. The phosphorus-buffer and chloroform mixture was mixed vigorously and centrifuged again. The chloroform layer was extracted and added to the first extraction. The chloroform solution was evaporated until dry under a stream of N_2 at room temperature. Another 800 µL of 3:1 methylene chloride:trifluoracetic anhydride (TFAA) was added to each sample to ensure complete derivatization and heated to 100°C for 15 min, with vial caps on. Vials were stored in this solution at < 4°C until they were analysed.

Compound-specific stable nitrogen isotope analysis

The $\delta^{15}N$ values of amino acid derivatives were analysed using a Delta XP mass spectrometer interfaced to a Trace gas chromatograph through a GC-C III combustion furnace at 980°C, reduction furnace at 680°C, with a liquid nitrogen cold trap. All samples were evaporated until dry at room temperature under a stream of N_2 and then re-dissolved in 100 to 1,000 µL ethyl acetate before analysis. Samples were co-injected with 0.5 µL of standards (norleucine and aminoadipic acid) (split/splitless 10:1 split ratio) at 180°C injector temperature under constant helium flow rate for 2 mL min^{-1}. The column oven was initially held at 50°C for 2 min, ramped to 190°C at 8° min^{-1}, and then to 280°C at 10° min^{-1}, and finally held at 280°C for 10 minutes. Samples were analysed multiple times and calibrated against standards. This method gave $\delta^{15}N$ measurements for alanine, glycine, threonine, valine, serine, leucine, isoleucine, proline, aspartic acid, glutamic acid, phenylalanine, and lysine. Note that asparagine and glutamaine are converted to aspartic acid and glutamic acid respectively during acid hydrolysis and that they are included in $\delta^{15}N$

measurements of these amino acids. Tyrosine was present and $\delta^{15}N$ values were measured in some samples, but not all, therefore it was excluded from analyses. Average precision was 0.94 ‰ (1 SD) and average accuracy was 0.30 ‰.

Statistical analysis

One-way ANOVAs were performed on $\delta^{13}C$ and $\delta^{15}N$ values of fish fed the hatchery feed for varying periods of time. Four factor ANOVAs, with main factors of temperature, diet, and time (excluding time 0) (all fixed factors) and tank as a nested random factor within treatments, were used to investigate differences in $\delta^{15}N$ and $\delta^{13}C$ values, lengths, and weights of fish among treatments. Isotopic discrimination factors of fish reared for 42 days were calculated by deducting the respective feed $\delta^{15}N$ and $\delta^{13}C$ values from individual fish values. Discrimination factors of fish reared for 42 days were analysed using a three factor ANOVA of diet and temperature treatments (fixed factors), with tank as a random nested factor within treatments. Post-hoc comparisons were done using Student-Newman-Keuls (SNK) tests. Exponential decay relationships of $\delta^{15}N$ and $\delta^{13}C$ values over time were investigated based on the equation of Guelinckx et al. (2007):

$$\delta_{(t)} = \delta_{final} + (\delta_{inital} - \delta_{final})\exp(vt)$$

Where $\delta_{(t)}$ is the value at the time in question; δ_{final} is the final value of tissue after reaching a steady state; δ_{inital} is the value at the beginning of the experiment; t is the time fish had been reared on experimental feeds (days); v is a measure of the turnover rate and has units of t^{-1}.

Permutation multivariate analysis of variance (PERMANOVA) (Anderson, 2001) was performed to investigate differences in the nitrogen isotopic composition of amino acids in feeds. Data were not transformed,

resembled using Euclidean similarity distance matrices, and permutations were unrestricted. Individual ANOVAs on isotopic results of amino acids in fish were done, as some amino acids were expected to change and others were expected to remain unchanged from treatment effects. Two-way, nested ANOVAs on the $\delta^{15}N$ values of amino acids in fish were done on the samples generated at the end of the experiment (42-day samples) to see if there was an effect of diet or temperature on the $\delta^{15}N$ of amino acids. One-way, nested ANOVAs were done on amino acid $\delta^{15}N$ from the initial fish and fish fed the vegetable feed at 23°C over time to see if $\delta^{15}N$ values of amino acids changed during the experiment. Replicate injections of individual fish were nested within fish, which were nested within tank and treatments (diet × temperature for two-way ANOVA; time for one-way ANOVA) to account for analytical variation. The nitrogen discrimination factors, i.e. the difference in $\delta^{15}N$ values between tissue and diet for each amino acid, was calculated from mean values for each feed being deducted from mean values for each fish. The discrimination factors were then analysed by a three-factor PERMANOVA, as above, with diet and temperature as fixed factors and tank as a random factor nested within them.

Results

There was large size variation among fish sampled initially (53 – 82 mm SL; 4.77 – 18.5 g). However, data of average sizes of fish reared for 2 and 42 days, grouped by treatment, show that fish grew over the experiment (Table 2.2) and fish size (length and mass) was affected by rearing time (Table 2.3). Fish mass was not affected by treatments of diet, temperature, or tank (Table 2.3). The standard lengths of fish were affected by temperature (Tables 2.2 & 2.3), with fish reared at 23°C being longer on average than fish reared at 16°C.

Table 2.2 Average size of analysed fish (n = 5/treatment; mass and standard length (SL)) for fish reared for 2 or 42 days and fed either fish-meal or vegetable feed and reared at 16°C or 23°C.

Diet	Temperature	Day	Mass (mean ± SE, g)	SL (mean ±SE, mm)
Fish-meal	16°C	2	10.27 ± 1.26	65 ± 3
		42	10.68 ± 1.53	66 ± 4
	23°C	2	11.83 ± 1.97	68 ± 3
		42	13.94 ± 1.19	74 ± 2
Vegetable	16°C	2	8.89 ± 0.81	64 ± 3
		42	11.74 ± 0.95	69 ± 2
	23°C	2	10.84 ± 0.59	68 ± 1
		42	11.06 ± 1.35	71 ± 2

Table 2.3 Four factor analysis of variance (ANOVA) of treatment effects on fish mass and standard length. Bolded numbers indicate significant effect of treatment ($p < 0.05$). Data were not transformed.

Source of Variation		Mass		Standard Length	
	df	MS	p	MS	p
Day	4	94.480	**0.004**	401.950	**0.001**
Diet	1	48.637	0.103	60.082	0.283
Temperature	1	59.999	0.067	1017.700	**0.001**
Day × Diet	4	25.463	0.200	63.364	0.397
Day × Temperature	4	28.625	0.195	90.441	0.232
Diet × Temperature	1	29.517	0.178	41.628	0.416
Day × Diet × Temperature	4	40.441	0.074	115.420	0.133
Tank (Diet × Temp. × Day)	20	15.915	0.705	56.484	0.836
Residual	212	19.681		81.413	
Total	251				

Tissue isotope turnover

Temperature affected tissue isotope turnover rate, with a significant interaction between temperature and day for $\delta^{15}N$ values (see Table 2.4, Fig. 2.1a). Fish reared at 16°C appeared to have reached a steady state with their new feeds after 14 days, as there were no significant differences in $\delta^{15}N$ values among fish reared for 14, 28, and 42 days (in post-hoc tests when pooled across diets) (Fig. 2.1a). Fish reared at 23°C, however, appeared to have taken much longer to equilibrate with the isotopic values of their feeds. In fact fish may not have reached a steady state or isotopic equilibrium during the experiment, with significant differences

detected among most rearing times when pooled across diets. It was not possible to fit exponential equations to the 23°C treatments with reasonable r^2 values, therefore no regression lines are shown (Fig 2.1a). Diet had a significant effect on $\delta^{15}N$ values of fish tissue (Table 2.4, Fig. 2.1a), with fish fed the fish-meal feed having higher $\delta^{15}N$ values than those fed the vegetable feed. Fish maintained on the hatchery feed did not change significantly in $\delta^{15}N$ or $\delta^{13}C$ over time ($F_{2,12} = 0.54$, $p = 0.60$; $F_{2,12} = 0.14$, $p = 0.87$ respectively).

Table 2.4 Four factor ANOVA of treatment effects on $\delta^{15}N$ and $\delta^{13}C$. Bolded numbers indicate significant effect of treatment ($p < 0.05$). Data were not transformed.

Source of Variation		$\delta^{15}N$		$\delta^{13}C$	
	df	MS	p	MS	p
Day	4	9.487	**0.001**	1.182	**0.001**
Diet	1	8.989	**0.001**	0.001	0.896
Temperature	1	4.031	**0.002**	0.181	0.092
Day × Diet	4	0.252	0.455	0.217	**0.027**
Day × Temperature	4	2.752	**0.001**	0.116	0.155
Diet × Temperature	1	0.973	0.080	2.943	**0.001**
Day × Diet × Temperature	4	0.257	0.447	0.190	**0.034**
Tank (Diet × Temp. × Day)	20	0.267	0.373	0.060	0.533
Residual	157	0.248		0.063	
Total	196				

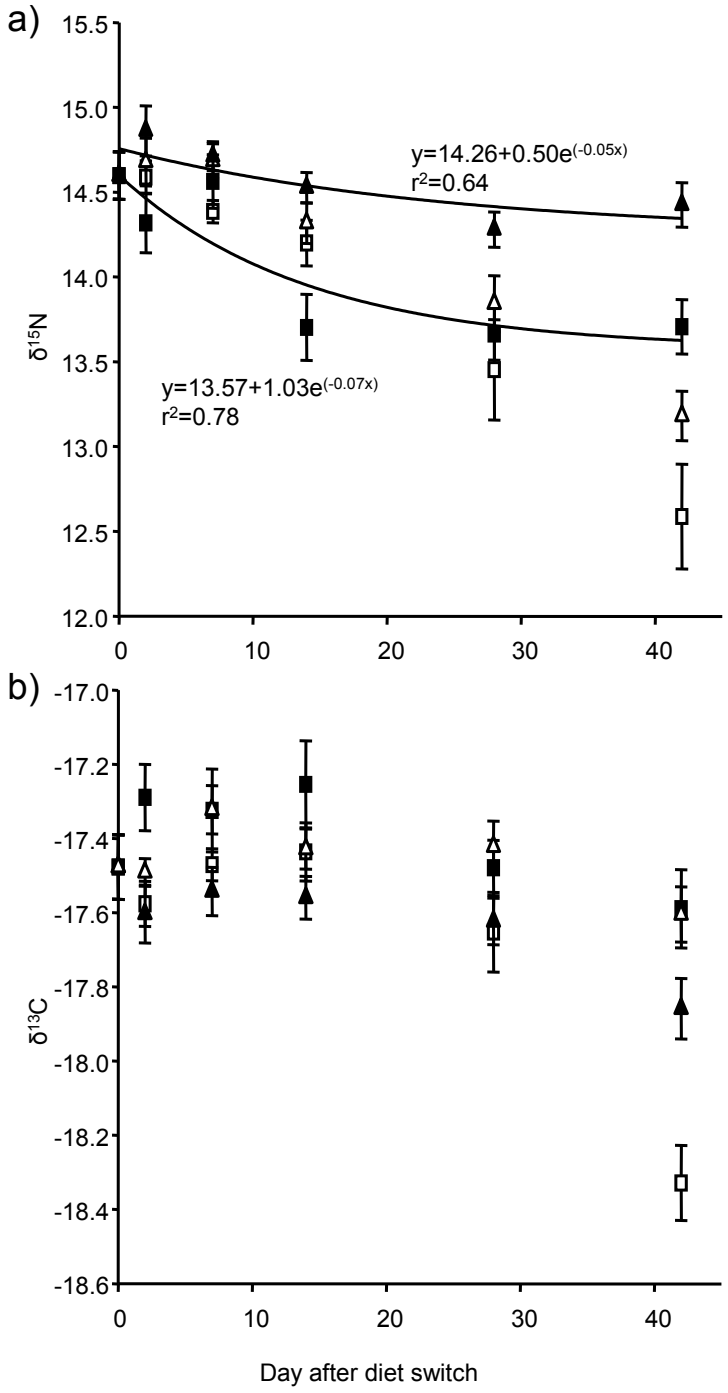

Figure 2.1 Average a) $\delta^{15}N$ and b) $\delta^{13}C$ (‰ ± SE) of fish muscle tissue from fish reared at 16°C and fed vegetable (■) or fish-meal feed (▲) and fish reared at 23°C and fed vegetable (□) or fish-meal feed (Δ) over 42 days. Note: as there was no effect of tank (Table 2.4) tanks have been pooled; exponential decay curves are fitted to 16°C treatments for $\delta^{15}N$ only.

Fish tissue $\delta^{13}C$ values changed during the experiment and were affected by both diet and temperature treatments, with significant interactions between day, diet and temperature (Table 2.4, Fig. 2.1b). Tissue $\delta^{13}C$ values initially increased or stayed the same for the first 14 days overall, with fish fed the vegetable feed at 16°C increasing the most, although the increase was still very small (<0.3 ‰) and within the bounds of our precision. Tissue $\delta^{13}C$ values then generally decreased over the last 28 days of the experiment, with fish fed the vegetable feed at 23°C decreasing the most.

Discrimination factor

Diet affected $\delta^{15}N$ and $\delta^{13}C$ discrimination (Table 2.5, Figs 2.2a and b), and fish fed the vegetable feed had a greater discrimination factor than fish fed the fish-meal feed. Temperature affected $\delta^{15}N$ discrimination with fish reared at 16°C having a greater discrimination of $\delta^{15}N$ than those reared at 23°C. Although a tank effect was detected in the ANOVA of $\delta^{15}N$ discrimination values (Table 2.5), significant differences were only detected between one pair of tanks and this did not alter main effects. This pair of tanks did not vary in fish size (mass: $p = 0.19$ (2-tailed T-test); SL: $p = 0.27$ (2-tailed T-test)).

An interaction between diet and temperature treatments was detected for $\delta^{13}C$ discrimination (Table 2.5). The directions of temperature effects on $\delta^{13}C$ discrimination switched depending on which feed fish were fed. Fish fed the vegetable feed had a greater $\delta^{13}C$ discrimination factor at 16°C than at 23°C (Fig. 2.2b), however, fish fed the fish-meal feed had a significantly greater $\delta^{13}C$ discrimination factor at 23°C than at 16°C (Fig. 2.2b).

Table 2.5 Three factor ANOVA of treatment effects on $\delta^{15}N$ and $\delta^{13}C$ discrimination (Δ) (tissue-diet). Bolded numbers indicate significant effect of treatment ($p < 0.05$). Data were not transformed.

Source of Variation		$\Delta^{15}N$		$\Delta^{13}C$	
	df	MS	p	MS	p
Diet	1	418.500	**0.001**	6.769	**0.003**
Temperature	1	13.324	**0.020**	0.534	0.162
Diet × Temperature	1	0.062	0.809	2.294	**0.014**
Tank (Diet × Temp.)	4	1.020	**0.024**	0.154	0.125
Residual	30	0.319		0.074	
Total	37				

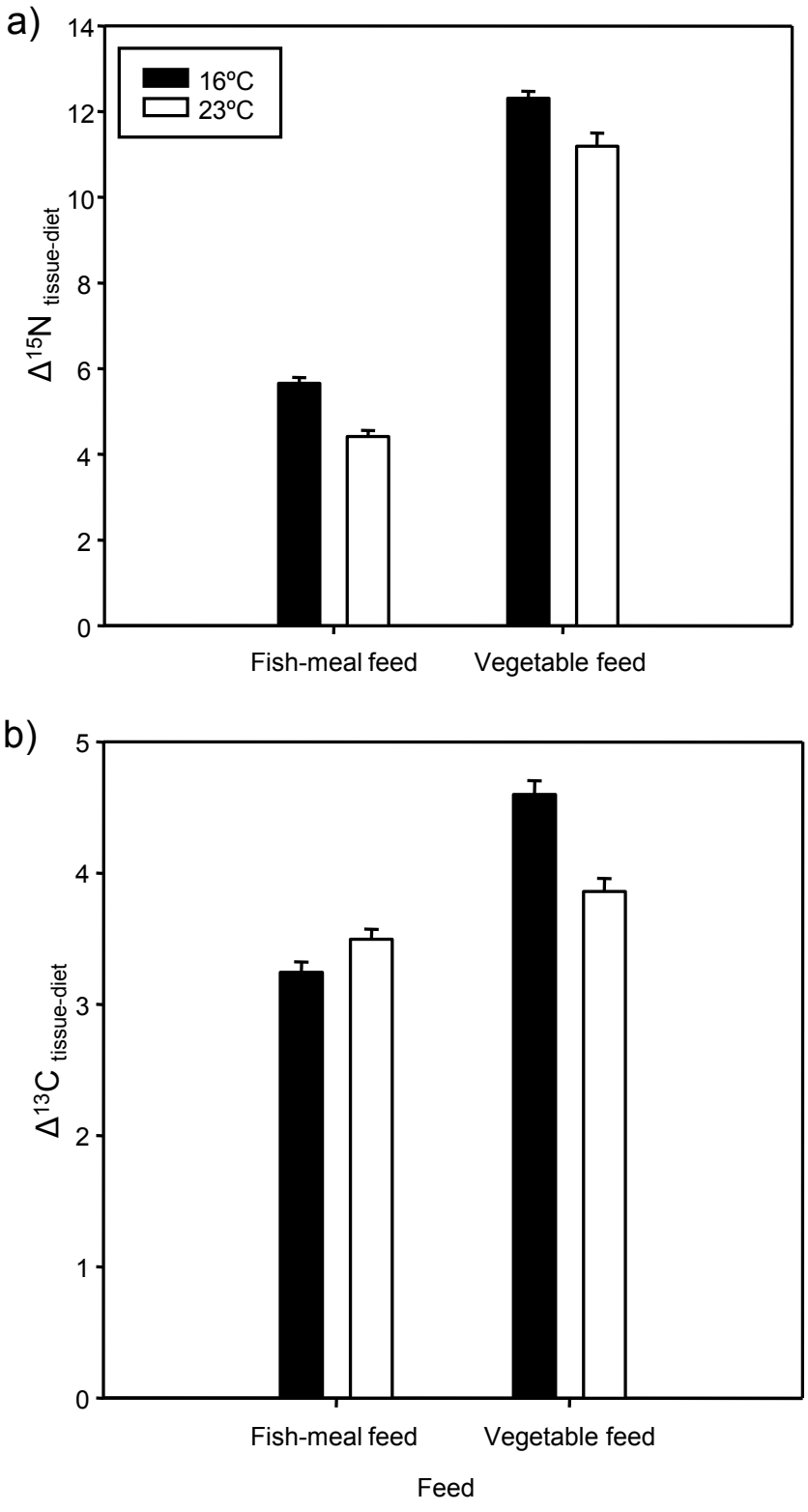

Figure 2.2 Average discrimination factor (Δ) of a) $\delta^{15}N$ and b) $\delta^{13}C$ (tissue-diet) (‰ ± SE) after 42 days of rearing on either vegetable or fish-meal feed at either 16°C or 23°C.

Amino acids

The $\delta^{15}N$ composition of amino acids in the three feeds were significantly different from each other (PERMANOVA $F_{2,6} = 411.02$, $p < 0.05$) (Fig. 2.3). The vegetable feed generally had lower $\delta^{15}N$ values for most amino acids compared to both the hatchery and fish-meal feeds. The exception to this pattern was threonine, for which the $\delta^{15}N$ values of the hatchery and vegetable feeds were more similar and enriched compared to the fish-meal feed (Fig. 2.3).

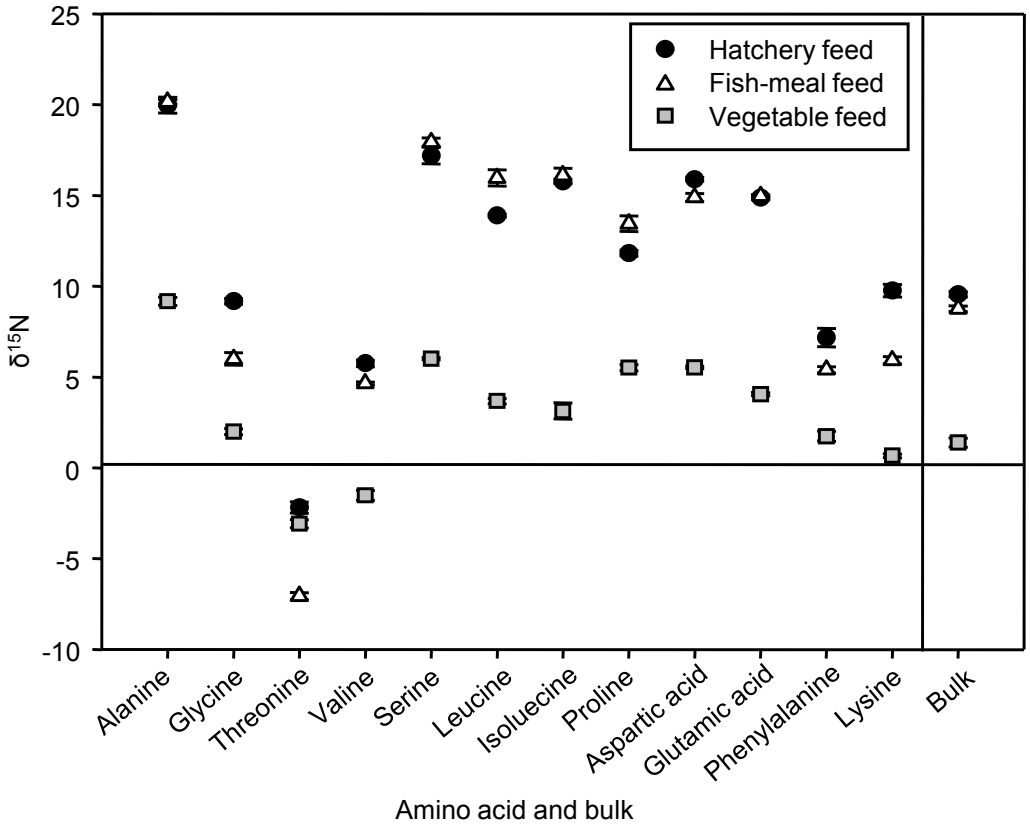

Figure 2.3 Average amino acid and bulk $\delta^{15}N$ values (‰ ± SE) of the three feeds used during the experiment.

The differences in $\delta^{15}N$ values of amino acids between experimental feeds (vegetable and fish-meal) were not reflected in fish after 42 days (Fig. 2.4). There was no significant effect ($p > 0.05$) of diet or temperature on the $\delta^{15}N$ values of amino acids in fish reared for 42 days, except for glutamic acid which was affected by diet treatments ($p = 0.015$). Tissue $\delta^{15}N$ values of leucine, aspartic acid, and glutamic acid had similar patterns of treatment effects after 42 days to bulk tissue $\delta^{15}N$ (Fig. 2.4). The $\delta^{15}N$ for fish fed the fish-meal feed were higher than fish fed the vegetable feed. Tissue $\delta^{15}N$ of leucine, aspartic acid, and glutamic acid were also lower for fish kept at 23°C than those kept at 16°C. There was a significant effect at the individual fish level for every amino acid analysed ($p < 0.05$). This led us to investigate the variance components as per Kingsford and Battershill (1998). Diet and temperature treatments were grouped into one treatment of four factors because there were no significant effects of treatments, except for glutamic acid. Variance associated with individual fish explained at least 30 % of the total variation (Table 2.6). Two amino acids, glycine and lysine, had the greatest variation among replicate injections for individual fish, although variation at the fish level was still greater than 30 %.

Fish fed the vegetable feed and reared at 23°C showed no significant change through time in $\delta^{15}N$ of amino acids ($p > 0.05$), except for glycine ($p < 0.05$, Fig. 2.5). There was an overall trend of decreasing $\delta^{15}N$ values with time for most amino acids: alanine, glycine, valine, leucine, isoleucine, proline, aspartic acid, and glutamic acid (see Fig. 2.5 and Appendix B). However, there was large variation within day groups for most amino acids except glycine. All ANOVAs found a significant effect of individual fish on $\delta^{15}N$ of amino acids ($p < 0.01$), similar to the above analyses for fish after 42 days. Variance

components indicated that individual fish explained at least 30 % of total variation for all amino acids and it was the greatest source of variation, except for glycine, which had the greatest variance associated with replicate injections (Table 2.7).

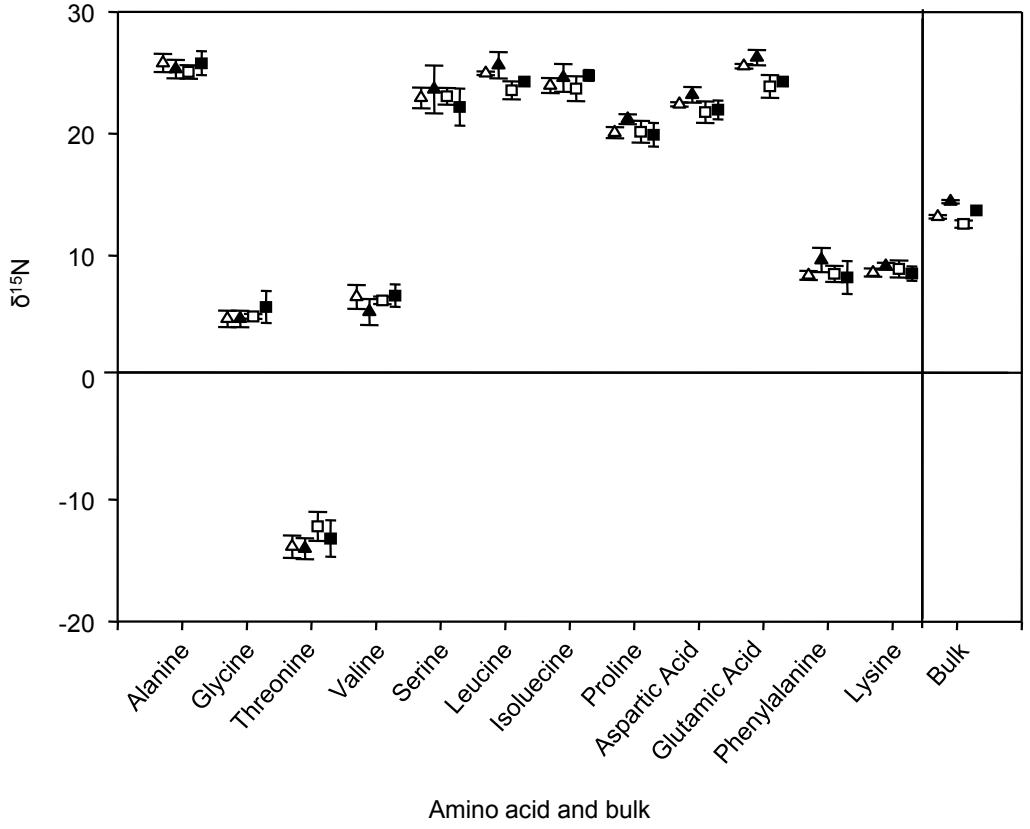

Figure 2.4 Average $\delta^{15}N$ (‰ ± SE) of amino acids and bulk in fish muscle from fish reared at 16°C and fed vegetable (■) or fish-meal feed (▲) and fish reared at 23°C and fed vegetable (□) or fish-meal feed (Δ) after 42 days.

Table 2.6 Variance components (%) of experimental treatments (diet × temperature), tank, fish and replicate injections for $\delta^{15}N$ of amino acids in fish muscle after 42 days being fed two different feeds (fish-meal or vegetable) at two temperatures (16°C or 23°C). Ala = alanine, Gly = glycine, Threon = threonine, Val = valine, Ser = serine, Leu = leucine, Isoleu = isoleucine, Pro = proline, Aspa = aspartic acid, Glu = glutamic acid, Phenyl = phenylalanine, Lys = lysine. Bolded numbers highlight the source of the greatest variation per amino acid.

Amino Acid	Ala	Gly	Threon	Val	Ser	Leu	Isoleu	Pro	Aspa	Glu	Phenyl	Lys
Experimental treatments (Diet × Temp.)	0	0	0	0	0	5.60	0	0	0	28.51	0	0
Tank (D × T)	**36.73**	33.25	39.14	28.57	21.42	14.35	31.37	8.44	31.50	0	0	15.16
Fish(Tank(D × T))	31.18	32.60	**43.07**	**42.58**	**64.70**	**47.29**	**55.80**	**61.85**	**46.30**	**52.28**	**85.07**	39.12
Residual (replicate injections)	32.08	**34.16**	17.80	28.85	13.88	32.76	12.83	29.71	22.20	19.21	14.93	**45.72**

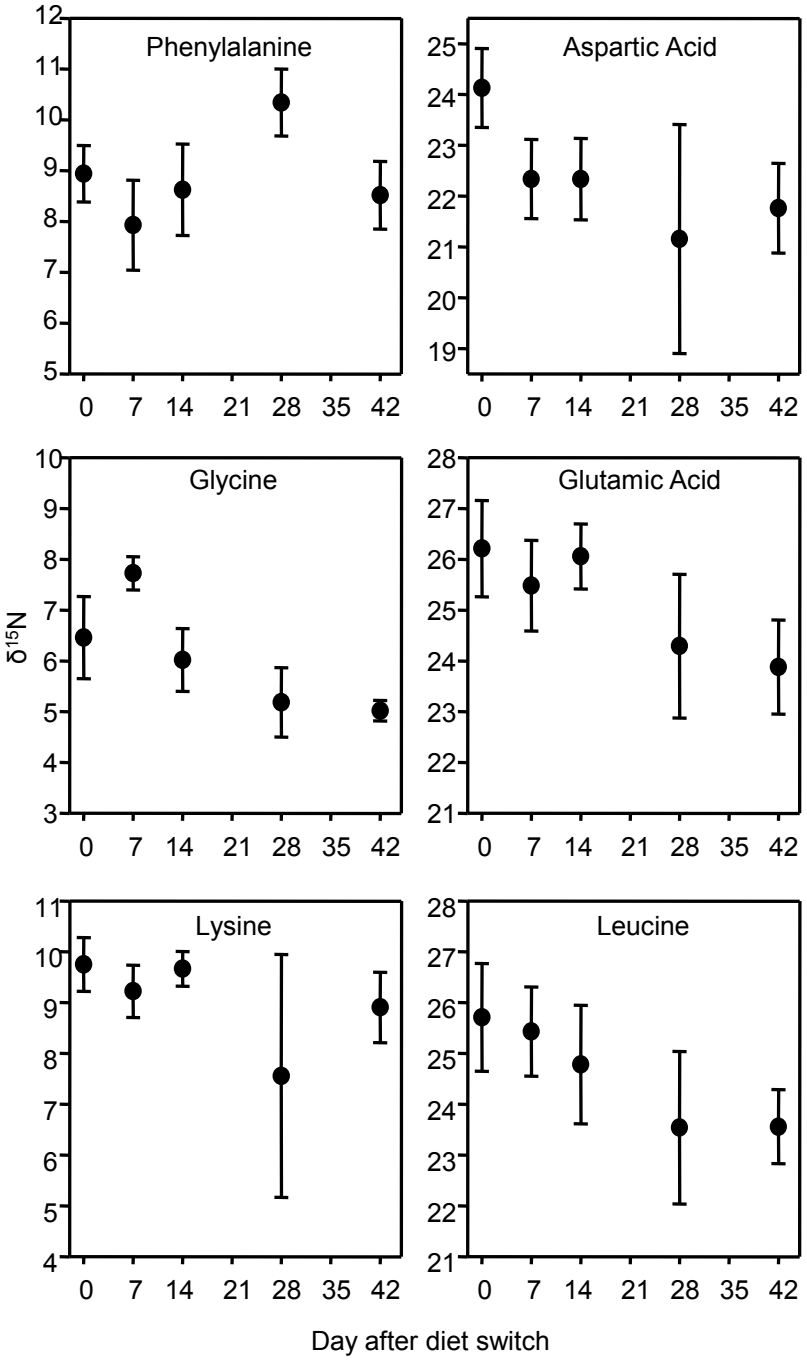

Figure 2.5 Average $\delta^{15}N$ of a selection of amino acids (‰ ± SE) in fish muscle from fish fed the vegetable feed at 23°C over time, including initial fish (day = 0). Amino acids on the left are „source" amino acids, those on the right are „trophic" amino acids as per Popp et al. (2007). Note all graphs have y-axes that span 7‰, although they are not the same values.

Table 2.7 Variance components (%) of time (day), tank, fish and replicate injections for $\delta^{15}N$ of amino acids in fish muscle from fish fed a vegetable feed, reared at 23°C over time. Abbreviations are the same as for Table 2.6. Bolded numbers highlight the source of the greatest variation per amino acid.

Amino Acid	Ala	Gly	Threon	Val	Ser	Leu	Isoleu	Pro	Aspa	Glu	Phenyl	Lys
Day	8.07	23.31	0	10.10	0	1.68	0	0	0	4.45	6.85	0
Tank(Day)	0.48	0	0	0	0	0	30.26	13.71	8.11	0	0	0
Fish(Tank(Day))	**70.16**	34.72	**82.73**	**59.51**	**79.10**	**82.58**	**44.41**	**58.34**	**85.46**	**81.55**	**70.66**	**87.56**
Residual (replicate injections)	21.28	**41.97**	17.27	30.39	20.90	15.74	25.33	27.94	6.43	14.00	22.50	12.44

There was a significant affect of diet on the discrimination of $\delta^{15}N$ for amino acids (PERMANOVA; $F_{2,12} = 62.663$, $p = 0.001$) (Fig. 2.6). The vegetable feed had the largest discrimination factors for nearly all amino acids, with the exception of threonine for which the fish-meal feed had the largest discrimination factor.

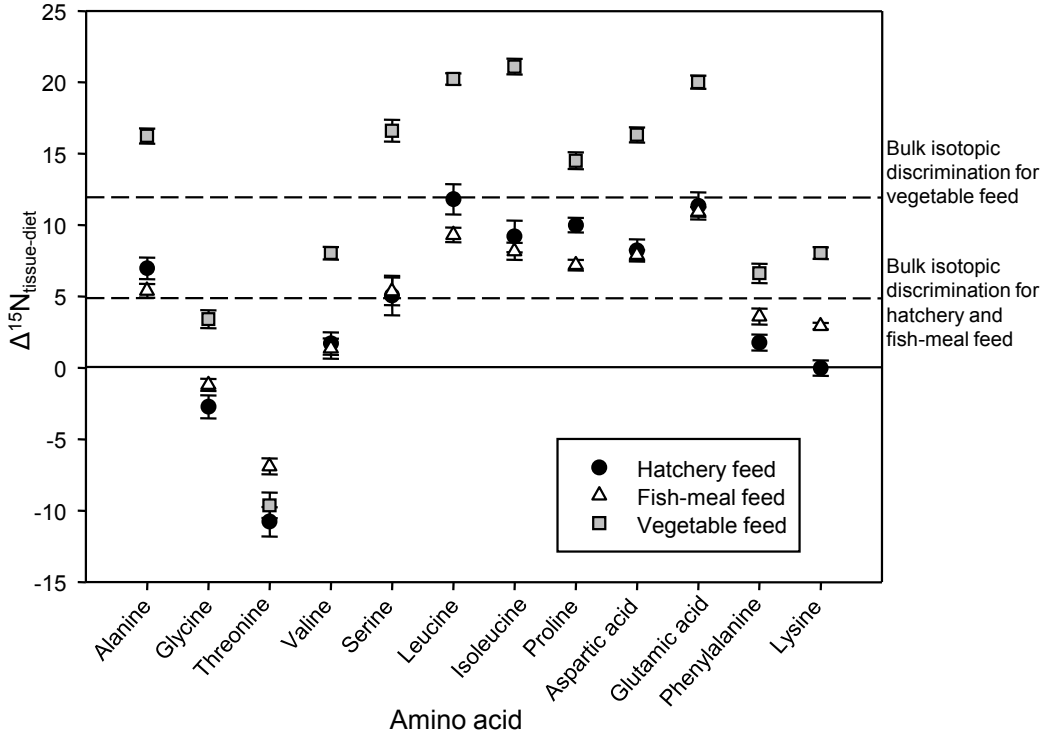

Figure 2.6 Average amino acid $\delta^{15}N$ discrimination factors (tissue-diet) (Δ ‰ ± SE) for fish sacrificed at the beginning of the experiment (hatchery feed), and those sacrificed after 42 days of rearing on vegetable or fish-meal feeds. Note: results are pooled over temperature as there was no effect of temperature.

Discussion

Bulk tissue $\delta^{15}N$ and $\delta^{13}C$

Both temperature and diet affected $\delta^{13}C$ and $\delta^{15}N$ values of fish muscle tissue. Fish reared at warmer temperatures had faster turnover of $\delta^{15}N$ than fish reared at cooler temperatures, which was expected. However, unexpected variation was found for when fish reached a steady state or isotopic equilibrium with their diet for the two temperature treatments. Fish reared at warmer temperatures do not appear to have reached a steady state after 42 days, whereas those reared at cooler temperatures appear to have reached a steady state after 14 days. This contrasts with findings of others (Bosley et al., 2002; Witting et al., 2004), who generally found faster tissue turnover rates and shorter times to reach a steady state for fish at warmer temperatures than fish at cooler temperatures. Isotopic turnover is due to the combined processes of growth dilution and metabolic reworking (Fry and Arnold, 1982; Hesslein et al., 1993; Herzka et al., 2002) and it is generally considered that growth is the main contributor to isotope turnover of muscle for growing poikilotherms (Fry and Arnold, 1982; Bosley et al., 2002; Witting et al., 2004; Trueman et al., 2005; Carleton and Martínez del Rio, 2010). However, metabolic activity also contributes to isotope turnover to varying degrees. Tarboush et al. (2006) found that metabolism contributed over 65 % to isotope turnover in young adult fish indicating that the contribution of metabolism to isotope turnover may vary with age or growth rates of fish. In our experiment, fish reared at 16°C on average were smaller than fish reared at 23°C and did not grow as fast, if at all. Therefore the rates of change detected at 16°C may be more similar to turnover rates due to metabolism than rates due to growth and metabolism combined (Carleton and Martínez del Rio, 2010). If this is the case,

fish reared at 16°C appear to have retained an historical dietary signature from the previously consumed diet. In contrast, fish reared at 23°C grew faster leading to more rapid dilution of their historical dietary signature through the addition of bulk tissue. However in order to reach a steady state, or isotopic equilibrium, at 23°C the experimental period for *A. butcheri* would need to be longer. These results support the theory that isotopic signatures only reflect food consumed while animals are growing (Perga and Gerdeaux, 2005; Carleton and Martínez del Rio, 2010). Fish are known to grow faster in warmer waters, and therefore isotopic signatures of wild fish tissue may be weighted towards diets consumed during growth periods in summer seasons (Perga and Gerdeaux, 2005; Carleton and Martínez del Rio, 2010).

Some previous researchers have identified durations required for juvenile and mature fish to reach an isotopic steady state with their diet that exceed the six weeks of our experimental period. Trueman et al. (2005) found that a 300 % increase in mass of one year old Atlantic salmon (starting at an average weight of 48.5g) was required to achieve complete muscle tissue turnover, which took eight months. Zuanon et al. (2007) reared Nile tilapia fingerlings (starting at 3.5g) for nearly two months to reach an isotopic steady state, after which fish had more than doubled their mass. Partridge and Jenkins (2002) found *A. butcheri* to double in mass over a period of approximately one month, for fish of similar size to those used in our experiment. Therefore it was expected that fish in our experiment would have at least doubled in mass over six weeks and reached an isotopic steady state. However Partridge and Jenkins (2002) fed fish supplemental fresh food (prawns, muscles, and whitebait) in addition to commercial aquaculture feed and we were not able to do this as it would interfere with our isotope results.

Unfortunately, *A. butcheri* in this experiment (starting at an average mass of 11.52 g) increased in size by less than 50 % and although it was evident that fish grew there was size variation among fish from the outset. It would have been desirable to track individual fish through time to determine growth more accurately; however, this was not possible as excessive handling causes stress and mortality. Quantitative relationships between mass and $\delta^{15}N$ and $\delta^{13}C$ were sought, however no meaningful relationships were found. It is apparent that fish did not grow sufficiently over six weeks to more than double their size and dilute the isotopic signature of their previous diet with their new diet.

Diet significantly altered $\delta^{15}N$ values of fish tissue over time largely because the two experimental feeds differed in $\delta^{15}N$ values. Fish fed the vegetable feed had lower $\delta^{15}N$ values than fish fed the fish-meal feed and this was due to the vegetable feed having a much lower $\delta^{15}N$ value (1.4 ‰) than the fish-meal feed (8.8 ‰) and the hatchery feed (9.6 ‰), which were similar. The rates of change of $\delta^{15}N$ for fish fed the two experimental feeds were different and this was again due to the vegetable feed having a much lower $\delta^{15}N$. All fish were reared on the same diet before the experiment started and would have had similar $\delta^{15}N$ values to begin with. However fish fed the vegetable feed had a greater change in $\delta^{15}N$ of their diet, causing the $\delta^{15}N$ in fish muscle to change more dramatically as fish muscle isotopes approached a steady state at a lower $\delta^{15}N$. This is contrary to our initial predictions that suggested isotope turnover would be faster in fish fed the fish-meal feed, as it should more closely match the dietary requirements of *A. butcheri*. However, the isotope concentrations in the two feeds were different and appear to have had more of an effect on the rates of change than the dietary composition or protein content over the short time period monitored.

A significant interaction among day, diet and temperature treatments was detected for $\delta^{13}C$ values. Tissue $\delta^{13}C$ values initially increased or stayed the same for the first 14 days, they then generally decreased over the last 28 days of the experiment. The initial increase in $\delta^{13}C$ may have been due to metabolism of internally stored lipids. Lipids are known to be depleted in ^{13}C and therefore have more negative $\delta^{13}C$ values compared to proteins and carbohydrates (DeNiro and Epstein, 1977; Post et al., 2007). The two experimental feeds both had much lower fat content compared to the hatchery feed, and the initial change to lower-fat feeds may have stimulated fish to metabolise stored lipids, thus increasing in $\delta^{13}C$ as ^{13}C-depleted lipids are metabolised. After 14 days the decrease in $\delta^{13}C$ may be explained by fish beginning to store lipids derived from their new diets. These observations and hypotheses are supported by C:N ratio data, with C:N values initially decreasing and then increasing after 14 days (unpublished data). Fish reared at 23°C generally grew more than fish reared at 16°C, therefore it would be expected that their $\delta^{13}C$ values at 23°C would be more negative if they put on more fat than fish reared at 16°C. However, this trend was only observed for fish fed the vegetable feed. The fish fed the fish-meal feed had lower $\delta^{13}C$ values at 16°C than at 23°C after 42 days. The vegetable feed had slightly lower $\delta^{13}C$ values (-22.2 ‰) than the fish-meal feed (-21.1 ‰) and fish $\delta^{13}C$ values at 23°C reflected this. However, the trend was reversed for the 16°C treatment. The carbohydrate composition of the hatchery and vegetable feeds is not known and this may affect the incorporation, or relative assimilation efficiency of carbon compounds into fish muscle and therefore the $\delta^{13}C$ (Kelly and Martínez del Rio, 2010).

Discrimination factors of $\delta^{15}N$ were greater for fish reared at 16°C than 23°C and it appeared that fish reared at 16°C were in an isotopic steady state. However if fish were not growing at 16°C, or were growing slowly, their tissue would have retained more isotopic signature from the previous diet than fish reared at 23°C. Therefore we cannot presume to quantify a discrimination factor for $\delta^{15}N$ for either feed at 16°C because of the historic isotopic signature. Fish reared at 23°C had a smaller $\delta^{15}N$ discrimination factor than those reared at 16°C after 42 days, as would be expected due to increased metabolism and growth and decreased fractionation through kinetic effects on chemical reactions. This agrees with previous research, which has shown temperature effects on $\delta^{15}N$ discrimination for European sea bass muscle (Barnes et al., 2007), with a greater discrimination at 11°C (4.41 ‰) than at 16°C (3.78 ‰). However some caution should be taken in making numerical conclusions regarding the discrimination factor for $\delta^{15}N$ at 23°C in this experiment because $\delta^{15}N$ values have not reached a steady state.

Diet composition affected $\delta^{15}N$ discrimination by fish muscle tissue, as fish fed the fish-meal feed at 23°C changed in $\delta^{15}N$ (1.4 ‰ from day 0 to day 42) more than the difference between the two feeds (0.8 ‰) and this is beyond precision and error rates. The fish-meal feed and the hatchery feed were quite similar in their protein proximal composition and amino acid $\delta^{15}N$, and fish maintained on the hatchery feed did not change in $\delta^{15}N$ over 29 days. If there was no effect of diet composition on discrimination factors then the change expected between fish at day 0 and fish fed the fish-meal feed at day 42 should only be 0.8 ‰. This study shows that there are affects of diet on $\delta^{15}N$ discrimination and similar results have been found previously (Robbins et al., 2005; Tsahar et al.,

2008; Robbins et al., 2010). There is evidence that as dietary protein quality increases, with regards to how well the protein matches an animal‟s requirements, the discrimination of $\delta^{15}N$ decreases (Robbins et al., 2005). Therefore it may be that the fish-meal feed matched the dietary requirements of *A. butcheri* better than the hatchery feed, as the discrimination at 42 days was less than the discrimination for the hatchery diet. This would also indicate that the vegetable feed was a poor match for *A. butcheri*‟s dietary requirements, as the discrimination factors were large. However part of these large discrimination factors may be due to historical isotopic signatures. The $\delta^{15}N$ discrimination values we found for fish fed the hatchery (5.0 ‰) and fish-meal feeds (4.4-5.7 ‰) are greater than the average 3.4 ‰ used by field researchers (Post, 2002). However, our values for $\delta^{15}N$ discrimination for these diets are within the range of results for various organisms (Adams and Sterner, 2000; Gaston and Suthers, 2004; Connolly et al., 2005a; Mill et al., 2007). Therefore a grand mean of our estimates for the hatchery and fish-meal feed $\delta^{15}N$ discrimination for *A. butcheri* muscle (5.1 ± 0.7 (1SD) ‰) may be more appropriate to use than 3.4 ‰ to encompass the potential variation in diet quality and temperature that cannot be quantified *a-priori* for food web or dietary studies. However, we acknowledge that this may be high due to historic feed effects and limited growth, particularly at 16°C.

Discrimination factors of $\delta^{13}C$ were greater than the assumed 1 ‰ for all diets. Fish muscle discrimination factors of $\delta^{13}C$ for the two experimental diets were greater than 3 ‰ at 42 days and discrimination for the hatchery diet was 2.7 ‰. Ratios of C:N were low (3.52), therefore lipid extraction or mathematically de-fatting $\delta^{13}C$ values should not greatly affect results. Regardless of whether fish in this experiment were in an isotopic steady state or not, these

values are similar to discrimination factors that have been found by others. Gaston and Suthers (2004) experimentally derived a discrimination value of 3.7 ‰ for muscle $\delta^{13}C$ of the marine fish Australian mado and Barnes et al. (2007) derived a value of 3.13 ‰ for European sea bass muscle. Carbon sources in field situations may be separated by 1 to 5 ‰; therefore source identification in food web studies may be effected if no discrimination factor is applied. We recommend that a discrimination factor for $\delta^{13}C$ of 3.5 ± 0.7 ‰ (grand mean of all results ± SD) for *A. butcheri* muscle be used in food web studies to encompass variation in diet and temperature conditions.

Compound-specific amino acid $\delta^{15}N$

There was relatively large variation in $\delta^{15}N$ of amino acids among fish within treatment groups. Some amino acids (leucine, aspartic acid, and glutamic acid) appear to have responded to diet and temperature treatments in a similar way to the bulk $\delta^{15}N$. However there was much larger variation within treatment groups for amino acid $\delta^{15}N$ than for the bulk $\delta^{15}N$, obscuring our ability to detect significant treatment effects. The accuracy and precision for bulk $\delta^{15}N$ is much better than compound-specific $\delta^{15}N$ analyses, enabling us to detect significant differences that are very small (0.7 ‰ was the average difference in bulk $\delta^{15}N$ between diet treatments across temperatures at 42 days). However, the precision of our compound-specific $\delta^{15}N$ analyses (0.9 ‰) is larger than the differences we detected among treatment groups (minimum of 0.7 ‰) using bulk $\delta^{15}N$. Therefore we cannot expect to detect such small differences using compound-specific $\delta^{15}N$ analysis. This indicates that if we are to use statistical tests on compound-specific $\delta^{15}N$ of amino acids the effect size needs to be very large to detect a difference, much larger than precision.

Although change over time in $\delta^{15}N$ of amino acids was only statistically significant for glycine, there were several amino acids which showed decreasing tends in $\delta^{15}N$ over time: alanine, glycine, valine, leucine, isoleucine, proline, aspartic acid, and glutamic acid. Most of the amino acids that decreased in $\delta^{15}N$ over time were „trophic amino acids" (Popp et al., 2007), with glycine the only one classified in the „source amino acid" group. Although on average there were temporal changes in $\delta^{15}N$ of some amino acids as large a change as the temporal change in bulk tissue $\delta^{15}N$ (i.e. leucine, aspartic acid, and glutamic acid), there was too much variation to detect significant differences. We expected that $\delta^{15}N$ of essential amino acids would take longer to respond to a diet switch as the amino acids already incorporated into cells would have the $\delta^{15}N$ of previous diets and could only be renewed by replacing them with amino acids from the diet through tissue renewal. Turnover of non-essential amino acids was expected to be faster as animals can continuously manufacture non-essential amino acids as well as incorporate them directly from their diet. Leucine is considered to be an essential amino acid for fish (National Research Council (U.S.) and Committee on Animal Nutrition, 1993) while glutamic acid and aspartic acid are considered non-essential, yet all three changed by more than the average bulk tissue $\delta^{15}N$ change suggesting they are non-essential amino acids. It could be that we are in fact identifying essential and non-essential amino acids through compound-specific isotope analyses (Martínez del Rio et al., 2009 and references therein) and that leucine is actually a non-essential amino acid for *A. butcheri*.

An effect of diet on $\delta^{15}N$ of amino acids was only detected for glutamic acid, although similar effects occurred for leucine and aspartic acid. Aspartic acid, glutamic acid, and leucine changed the most in $\delta^{15}N$ of all the amino acids over

time and would therefore be more likely to show treatment effects at 42 days. Other amino acid $\delta^{15}N$ values may have been still changing, particularly as the bulk $\delta^{15}N$ of muscle tissue was still changing for fish reared at 23°C. If the experiment had continued we may have found more treatment effects across other amino acids that were not changing as fast as leucine, aspartic acid, and glutamic acid. Although we did not detect a significant effect of temperature on $\delta^{15}N$ of amino acids, it appears that temperature may have an effect as some amino acid $\delta^{15}N$ mirrored that of bulk tissue $\delta^{15}N$, for which there was an affect of temperature. Therefore future research that includes seasonality within the sampling regime and $\delta^{15}N$ of amino acids may need to consider temperature, and possibly growth effects.

The amino acids that were isotopically discriminated by fish, or those that became more enriched than the bulk discrimination factor for each diet were alanine, serine, leucine, isoleucine, proline, aspartic acid, and glutamic acid. The amino acids that were isotopically discriminated less than the bulk discrimination factor by fish were glycine, threonine, valine, phenylalanine, and lysine. These patterns largely agree with those found by previous studies (McClelland and Montoya, 2002; Popp et al., 2007). One exception to other researchers‟ findings is that serine has previously been labelled a source amino acid, which is isotopically discriminated less than the bulk discrimination factor for $\delta^{15}N$. Valine is another exception, as it was labelled a trophic amino acid but here it groups out with the source amino acids. It could be that serine is in fact a non-essential amino acid for *A. butcheri* and that valine is essential for *A. butcheri*. The traditional classification of essential and non-essential amino acids were derived from experiments on mice and other researchers have found that these groupings do not

necessarily apply to other taxa (Zubay et al., 1995). However, whether compound-specific isotope analyses can identify essential amino acids for animals requires further testing.

Previous work on compound-specific isotope analysis of $\delta^{15}N$ in amino acids has focused on oceanic and near shore settings, to determine trophic position of consumers in their food web from consumer samples alone (Popp et al., 2007; Hannides et al., 2009; Olson et al., 2010). They have largely relied on McClelland and Montoya"s (2002) observation that $\delta^{15}N$ of glutamic acid, or trophic amino acids are enriched by 7 ‰ on average for each trophic level and have applied this to estimate trophic position (see Hannides et al., 2009 for trophic position equations). Our results showed that glutamic acid was enriched by 11.3 ± 1.0 (mean ± SE) ‰ for the hatchery feed, after approximately 100 days rearing, which gives our best estimate of enrichment. This would put the experimental fish in a 0.6 higher trophic position than they really are and this was found across all amino acid trophic position equations used to date, such that trophic position of *A. butcheri* was consistently over estimated by approx. 0.6.

The lack of significant effects of treatments on $\delta^{15}N$ of some amino acids may be due to large within group variation or it could be because there really are no differences for particular amino acids. Many animals, particularly herbivores and omnivores, have symbiotic relationships with gut microbes that break down ingested food into soluble molecules, or manufacture essential amino acids that are taken up by the host into their tissues (e.g. Torrallardona et al., 1996; Metges, 2000). It is possible that *A. butcheri* have gut microbes that digest their food, particularly proteins, or manufacture particular amino acids such that they receive an isotopically constant supply of particular amino acids regardless of what the

fish are actually ingesting. Our glutamic acid enrichment and over estimation of trophic position support *A. butcheri* being supplied with amino acids by microbes. There is also evidence that fish may have nitrogen conserving systems that involve gut microbial activity (Singer, 2003), however Singer (2003) speculated that this could only occur in ureotelic[3] fish. Moeri et al. (2003) showed that the $\delta^{15}N$ of fish muscle and liver were not determined solely by diet, but that ambient ammonia was taken in at the protein level in ammonotelic[4] and ureotelic fish. Therefore it is possible that the ammonia excreted by fish within tanks was being re-absorbed into muscle at the protein level and potentially obscuring significant differences in $\delta^{15}N$ of amino acids. Fish were reared in tanks without flow-through water, retaining excreted ammonia for up to two days at a time until a portion of the water was changed. We speculate that results for $\delta^{15}N$ of amino acids may be different if this experiment had been carried out in flow-through tanks. This would provide further evidence for ambient ammonia uptake into proteins. Research into the gut microbes of *A. butcheri* and their digestive and assimilative capacity would also further our understanding of why the $\delta^{15}N$ of some amino acids did not vary significantly.

This experiment and analyses of compound-specific amino acid $\delta^{15}N$ has shown that precision is a limiting factor in being able to detect significant differences among groups. Therefore effect sizes will need to be large if statistical analyses are to be applied successfully to such data. The results for individual amino acids largely concur with previous groupings of source and trophic amino acids. However, several differences occurred that have further raised the question of using compound-specific $\delta^{15}N$ of amino acids to determine essential and non-

[3] Ureotelic organisms excrete excess nitrogen as urea.
[4] Ammonotelic organisms produce soluble ammonia as a result of deamination. Many fish are ammoniotelic.

essential amino acids for different species. As few significant differences were found in this experiment we echo the call for further experiments or analysis of past experimental samples to clarify effects on amino acid $\delta^{15}N$ and to validate how applicable trophic position equations are across a range of species.

Conclusions

Our results highlight the need to experimentally derive isotopic discrimination values for individual species, particularly at appropriate temperatures and on a variety of diets as treatment effects were found. Temperature effects indicated that fish muscle isotopic signatures reflect diets consumed during warmer growth periods. Therefore isotope food web studies involving fish muscle should consider sampling during summer, particularly towards the end of summer after fish have grown and incorporated dietary isotopes. Compound-specific $\delta^{15}N$ of amino acids partially explained the treatment effects found on bulk tissue $\delta^{15}N$, with several amino acids mirroring bulk tissue $\delta^{15}N$ results. This indicates that $\delta^{15}N$ of some amino acids is also affected by temperature and diet, but whether this affects the outcomes of trophic position calculations needs further investigation. Further investigation into whether $\delta^{15}N$ of amino acids can be used to determine essential and non-essential amino acids is also required. Without sound experimental validations of factors influencing isotope ratios, field applications may provide misrepresentations of trophic relationships.

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Chapter Three: The influence of temperature and elemental concentration of diet on carbon and nitrogen stable isotopes in fish muscle, with a test of the concentration dependent mixing model

Experimental fish samples: *Aldrichetta forsteri.*

Chapter 3 Preamble

This chapter is a co-authored paper currently under peer-review with the *Journal of Fish Biology*, with Bronwyn Gillanders and Travis Elsdon as co-authors. As such it is written in plural.

In this chapter Travis Elsdon, Bronwyn Gillanders and I developed the experimental design and supplied the funding. I did the experiment, caring for the fish, with some help from other students (see acknowledgements). I prepared the samples for analyses. I did all of the statistical analyses and wrote the manuscript with input from co-authors.

I certify that the statement of contribution is accurate

Alexandra Bloomfield (Candidate)

I herby certify that the statement of contribution is accurate and I give permission for the inclusion of the paper in the thesis

Professor Bronwyn Gillanders Dr Travis Elsdon

The influence of temperature and elemental concentration of diet on carbon and nitrogen stable isotopes in fish muscle, with a test of the concentration dependent mixing model

Abstract

The effects of temperature and elemental concentration of diet on $\delta^{13}C$ and $\delta^{15}N$ incorporation rates and discrimination factors were investigated for yellow-eye mullet *Aldrichetta forsteri*. Fish were reared at 16°C and 24°C and fed diets of varying elemental concentration over 85 days. Fish reared at 24°C generally had faster isotope incorporation, with the best estimate of half-life of $\delta^{15}N$ being 27.2 days at 24°C. Fish reared at 24°C also had smaller $\delta^{15}N$ discrimination, reflecting increased growth and lower fractionation during chemical reactions. Values of fish muscle $\delta^{13}C$ reflected nutritional status of fish to a certain degree, with those in good condition and high C:N ratios (and therefore higher lipid content) having lower $\delta^{13}C$. No linear relationship was found between elemental concentration of diet and isotope discrimination factors. Diet treatments of varying elemental concentration showed potentially complex interactions of protein sparing, nitrogen recycling and changes in metabolism of carbohydrates or proteins for energy. Evidence of little change in $\delta^{15}N$ during poor nourishment was also found. When elemental concentration was used in mixing models, predictions of stable isotope values were improved and were closer to measured isotopic values than when elemental concentration was omitted. This work highlights the importance

of using elemental concentration in mixing models and supports the concentration dependent mixing model.

Introduction

Stable isotopes of carbon and nitrogen are frequently used in ecological research and are powerful tools for deciphering diets and food web dynamics, documenting aquatic larval settlement, and detecting human impacts (e.g. Herzka et al., 2001; Gaston et al., 2004; Connolly et al., 2005b). Stable isotope ratios of carbon (^{13}C to ^{12}C; $\delta^{13}C$) are known to vary among plants and algae with biological and chemical processes (e.g. Peterson and Fry, 1987; Boon and Bunn, 1994; Smit, 2001). The differences in $\delta^{13}C$ among plants and algae enable researchers to determine diets and track movements of fish (e.g. Herzka et al., 2002; Melville and Connolly, 2003; Hadwen and Arthington, 2007). Stable isotope ratios of nitrogen (^{15}N to ^{14}N; $\delta^{15}N$) increase with trophic level (Minagawa and Wada, 1984) and show traces of human impacts through higher $\delta^{15}N$ across food webs (Heaton, 1986; Gaston and Suthers, 2004; Hadwen and Arthington, 2007).

Ecological studies often rely on predictable differences in isotopic ratios between a consumer and its diet, i.e. discrimination factor. Discrimination of stable isotopes occurs as a result of chemical and biological processes that alter isotope ratios between sources (diet) and sinks (animal tissue), and are the total of many fractionating processes (see Martínez del Rio et al., 2009 for more discussion). Errors in discrimination factors used in ecological studies can translate to erroneous contributions of dietary items, or misplacing organisms in food webs (Post, 2002; Caut et al., 2009). Researchers use an average discrimination factor of 3.4 ‰ for $\delta^{15}N$ and 0-1 ‰ for $\delta^{13}C$ per trophic level, as recommended by Post (2002), when more specific values are unavailable.

However, the discrimination factors presented by Post (2002) are averages across species yet discrimination factors vary among species and within species (DeNiro and Epstein, 1978, 1981) leading to calls for researchers to experimentally quantify discrimination factors for study species whenever possible (Martínez del Rio et al., 2009). Simply quantifying discrimination factors will lead to specific values for experimental conditions and these may not be applicable to wild animals that experience a variety of environmental conditions. Therefore, when deriving discrimination factors researchers should aim to encompass environmental variability that will affect discrimination factors and to quantify the variation.

Isotopic signatures of animal tissue do not instantaneously reflect diet. Isotopes are gradually incorporated into animal tissue through growth and metabolic activity (Fry and Arnold, 1982; Hesslein et al., 1993; Carleton and Martínez del Rio, 2010), so the isotopic signature of animal tissue actually reflects the animal‟s time-integrated diet. Muscle tissue is one of the most common tissue types sampled for isotopic studies (Caut et al., 2009). Isotope incorporation rates of muscle are thought to be dominated by growth, with little effect of metabolism on isotopic change, particularly for fish (Chapter 2, Perga and Gerdeaux, 2005; Zuanon et al., 2006; Bloomfield et al., 2011). However, growth of fish is also affected by environmental factors including temperature, which in turn affects isotope incorporation rates (Bosley et al., 2002; Witting et al., 2004; Barnes et al., 2007). Discrimination factors are affected by temperature too, through kinetic effects on chemical reactions, with warmer temperatures leading to smaller discrimination factors and cooler temperatures resulting in larger discrimination factors (Chapter 2, Bosley et al., 2002; Barnes et al., 2007; Bloomfield et al.,

2011). Temperatures of water bodies that fish inhabit vary seasonally and spatially (e.g. Jones et al., 1996; Elsdon et al., 2009). Therefore, researchers need to quantify the variability in discrimination factors and isotope incorporation rates caused by temperature to improve the accuracy of ecological field studies using stable isotopes.

Elemental concentration, or the percentage of atoms, in sources greatly affects the results of isotope mixing models (Phillips and Koch, 2002). Isotope mixing models are widely used to determine proportional source (diet) contributions to a target organism (consumer) for dietary and ecological research (e.g. Melville and Connolly, 2003; McClellan et al., 2010; Rush et al., 2010). In the mixing model by Phillips and Koch (2002) the contribution of each source was assumed to be proportional to the contributed mass multiplied by the elemental concentration of the source. Phillips and Koch (2002) tested their model by using isotope data from other published studies and extrapolated carbon and nitrogen elemental concentrations of diet items in those studies from other sources. Validation of the concentration dependent mixing model has received little attention, with few experimental tests (Caut et al., 2008). Although more complicated mixing models now exist, that provide the option of including elemental concentration (e.g. Parnell et al., 2010), not all studies use elemental concentration in analyses (e.g. Rush et al., 2010). Using elemental concentration in mixing models has been supported by other published studies (Pearson et al., 2003; Mirón et al., 2006). Elemental concentration can affect isotope discrimination and incorporation rates (Pearson et al., 2003; Mirón et al., 2006). There is, however, contradictory evidence as to whether increasing elemental concentration causes an increase (Pearson et al., 2003) or decrease (Mirón et al.,

2006) in isotope discrimination, with some evidence suggesting that isotope incorporation is also faster (Mirón et al., 2006). These studies have been done on birds (Pearson et al., 2003) and bats (Mirón et al., 2006), with no published research focused on elemental concentration in fish diets. Therefore further research is warranted into the effects of elemental concentration on the discrimination factors and isotope incorporation rates of fish.

Omnivores are excellent candidates for isotopic investigations as they tend to eat a range of dietary components that are readily available to them (e.g. Webb, 1973; Sarre et al., 2000; Chuwen et al., 2007; Hadwen et al., 2007). Dietary components of omnivores can vary greatly in their elemental concentration (Pearson et al., 2003), making research into effects of elemental concentration on isotopes in omnivores pertinent. Yellow-eye mullet *Aldrichetta forsteri* (Valenciennes, 1836) are a common, omnivorous, euryhaline fish that can be found in bays, estuaries, and open coastlines around New Zealand, the Chatham Islands, and Australia: from Newcastle, NSW, along the south coast to Shark Bay, WA, including around Tasmania (Kailola et al., 1993; Armitage et al., 1994). The abundance and large geographical distribution of *A. forsteri* make them ideal fish for ecological investigations. Previous dietary studies, or stomach content analyses, of *A. forsteri* have been hampered by the high frequency of empty stomachs and high proportions of unidentifiable matter and detritus in the gut (Webb, 1973; Platell et al., 2006). Isotope studies have the potential to generate dietary data more efficiently over large areas than traditional stomach content analyses.

The effects of temperature and dietary elemental concentration on $\delta^{13}C$ and $\delta^{15}N$ incorporation rates and discrimination factors of *A. forsteri* muscle were

experimentally tested. Fish were reared at two temperatures and fed two diets that differed in their elemental concentrations. It was predicted that fish reared at warmer temperatures would have smaller discrimination factors and faster incorporation rates than fish reared at cooler temperatures (Barnes et al., 2007). It was also expected that fish fed a diet with high nitrogen and carbon concentration would have faster isotope incorporation rates and larger isotope discrimination factors than fish fed a diet with lower nitrogen and carbon concentration (Mirón et al., 2006). Experimental feeds were mixed together in varying proportions and fed to fish to test if there was a linear relationship between dietary elemental concentration and isotopic signatures of fish (Adams and Sterner, 2000). To test the importance of elemental concentration in mixing models predictions of mixing models were compared, with and without elemental concentration, for $\delta^{13}C$ and $\delta^{15}N$ of fish fed mixed diets with measured isotopic values (Caut et al., 2008).

Methods

Treatments

There were two components of this study: one focusing on isotope incorporation and discrimination factors; and the other focusing on elemental concentration of diet in mixing models. For the isotope incorporation and discrimination factors component fish were reared under orthogonal treatments of two temperatures (16°C or 24°C), two diets (100 % chicken or 100 % *Artemia*; encompassing different C and N concentrations), and seven rearing times (2, 7, 14, 28, 42, 60, and 85 days after diet switch) with two replicate tanks per treatment (56 tanks in total) and ten fish per tank. Entire tanks were sacrificed on days mentioned above so that fish density was not varied. Temperatures were chosen to reflect local

summer and winter temperatures of environments where *A. forsteri* have been caught in the past (Jones et al., 1996). We acknowledge that daily water temperatures can vary by as much as 10°C in shallow estuaries, where *A. forsteri* are found, and that oscillating temperatures may influence incorporation rates and discrimination factors in intricate ways. However, the effect of temperature on these parameters needs to be established first before investigating how oscillating temperature affects isotope incorporation and discrimination.

To further investigate effects of elemental concentration in diets on isotopes in fish and discrimination factors, fish were fed mixtures of chicken and *Artemia* (see diet details below) for 85 days at 24°C. There were also two replicate tanks for each mixed diet treatment, with ten fish per tank. For the component focusing on using elemental concentration in mixing models, data from the above experiment were used to test how accurate mixing models were at predicting isotope ratios with and without elemental concentration in their calculations.

Diet details

Chickens, used as fish feed, had been feeding on a diet of approximately 20-30 % corn and were sourced from a commercial chicken farm. For initial diet preparations two whole chickens were boned and fat removed, with muscle being pureed in a food blender. The puree was then frozen into small blocks. Frozen *Artemia* blocks were sourced from a commercial *Artemia* farm. To prepare the mixed diets a sample of pureed chicken and frozen *Artemia* were mixed together in ratios of 25:75, 50:50, and 75:25 measured by wet weight, in a food blender. Hereafter mixes are referred to with the percentage of chicken first such that 25:75 refers to a mixture of 25 % chicken and 75 % *Artemia*. The mixtures were

frozen into small blocks weighing approximately 3-4 g, which were similar in mass to chicken and *Artemia* blocks.

Diets ranged in carbon concentration from 33 % for *Artemia* to 52 % for chicken and nitrogen concentration ranged from 7 % for *Artemia* to 14 % for chicken (see Table 3.1). The elemental concentration of chicken is typical of animal matter (muscle) with carbon usually being 45-50 % and nitrogen usually being 14 % (Bloomfield, unpublished data). The nitrogen concentration of *Artemia* was much lower than that of chicken, however, it is not as low as that usually found in plant matter, which can be as low as 1-3 % (Bloomfield unpublished data, Mill et al., 2007). The carbon concentration in plant matter can be highly variable (40 % ± 7 (1SD); Bloomfield unpublished data) but is often lower than that of animal muscle. Therefore *Artemia* represents dietary items with a lower carbon and nitrogen concentration (%) that *A. forsteri* may encounter in the wild, such as plant-derived matter. However it is acknowledged that the nitrogen concentration of *Artemia* is much higher than typical plant matter. Fish were fed *Artemia* over a pure algal based diet as the amount of algal feed required to sustain fish would be large, leading to substantially different feed rates, and such volumes would be difficult to produce. Even though fish were fed chicken and *Artemia* at different rates, this difference is much less than it would have been if fish had been fed a pure algal based feed.

Table 3.1 Carbon (C) and nitrogen (N) elemental concentration (% mean ± SE) of feeds (*Artemia*, mixed feeds: Chicken:*Artemia* mixed in mass proportions, 25:75, 50:50, and 75:25, Chicken and Worms) used in the experiment.

Feed	C (%)	N (%)
Artemia	32.9 ± 1.8	6.6 ± 0.4
25:75	45.0 ± 0.5	11.7 ± 0.1
50:50	47.0 ± 0.7	12.6 ± 0.1
75:25	48.3 ± 0.4	13.6 ± 0.1
Chicken	51.8 ± 0.9	13.8 ± 0.4
Worms	53.5 ± 0.3	10.1 ± 0.1

Fish rearing

Juvenile (average standard length ± SE of 41 ± 1.5 mm, range = 32 – 51 mm; average mass ± SE of 1.02 ± 0.13 g, range = 0.14 – 1.97 g) *A. forsteri* were collected from Gawler River, South Australia, in October 2008 using a seine net. Fish were placed in 50 L containers with aeration and transported to the University of Adelaide aquarium room for acclimation to experimental conditions. Fifteen fish were sacrificed on the day of collection to represent wild caught *A. forsteri* for size (length and weight) measurements and stable isotope analyses. All fish were sacrificed using an ice water slurry. Fish were housed in two 800 L tanks at an ambient temperature of 17°C and a 12 hr day/night cycle. During acclimation fish were fed live black worms (*Lumbriculus* sp.), or occasionally frozen worms of the same species, two to three times daily for two months. Fish were observed to eat black worms vigorously and competition for food within tanks was high. A group of 20 fish was fed black worms for 116 days total to

allow them to equilibrate with worms as their diet for stable isotope analyses. Water temperature was increased to 20°C over two months, due to increased ambient summer temperatures. Water quality was maintained by daily water changes of approximately 30-50 % during this acclimation period.

After two months fish were randomly allocated to 40 L experimental tanks at a density of ten fish per tank. In the 40 L tanks fish were acclimated to either 16°C or 24°C over several days. Fish were fed black worms during the temperature acclimation period and fish were observed to eat them. On the first day of the experiment fish were fed frozen blocks of chicken or *Artemia*, or a mixture of the two, cut into smaller pieces. Initially fish were fed a quarter of a frozen food block (approximately 1 g) per 40 L tank of 10 fish (equating to approximately 5 % body mass per feed) for each feed (2-3 feeds per day). Not all fish were observed to eat the frozen chicken initially, however, fish fed on chicken after a couple of days. Fish ate most of the frozen *Artemia* and mixed diets. Feeding rates were increased during the experiment to coincide with increased growth. If any food was left in tanks after 10 mins it was removed to maintain water quality. Water quality in 40 L tanks was further maintained by quarter water changes every two days.

Sample preparation and analyses

Sacrificed fish were frozen whole to -20°C and later defrosted for dissection of dorsal muscle tissue. When fish were defrosted they were individually weighed (mass (g)) and measured (standard and total lengths (mm)), with the five largest fish in each tank having dorsal muscle samples taken. The five largest fish were chosen as it was thought that they would be more likely to have consumed more feed and therefore have incorporated more isotopes from their diet into their tissue

than the smaller fish. Random samples of pureed chicken, *Artemia* and mixed diets were prepared for elemental concentration and stable isotope analyses. Black worms were also randomly sampled over the duration of the acclimation period. Samples of fish dorsal muscle and diets were freeze-dried and ground into a powder using an agate mortar and pestle. Fish muscle samples did not have lipids extracted as most samples had C:N below 3.5, the cut off for desired lipid extraction in fish (Post et al., 2007). Also, samples were compared within a species and lipid extraction is known to introduce more variation into $\delta^{15}N$ of samples (Elsdon et al., 2010). Lipids were not extracted from feeds, as fish consume the whole feed and may metabolise lipids. Samples of fish muscle and diets were weighed into tin capsules for elemental concentration and stable isotope analyses of carbon and nitrogen. Elemental concentration and stable isotope analyses were done on a GV Isoprime (Manchester UK) continuous flow isotope ratio mass spectrometer coupled to a Eurovector (Milan Italy) elemental analyser 3000 at Griffith University, Queensland, Australia. International and internal standards (N: Ambient Air, IAEA-305a, C: ANU Sucrose, Acetanilide, Working standards: 'Prawn') were run in parallel with fish and diet samples to calibrate machine results. Average precision of the elemental analyser was 0.61 % for carbon and 0.29 % for nitrogen (1 SD), with average accuracy of 0.18 % for carbon and 0.05 % for nitrogen (average deviation from theoretical value). Average precision of the mass spectrometer was 0.06 ‰ for $\delta^{13}C$ and 0.29 ‰ for $\delta^{15}N$ (1 SD), with average accuracy of 0.02 ‰ for $\delta^{13}C$ and 0.04 ‰ for $\delta^{15}N$ (average deviation of results from known value).

Statistical analyses

Isotopes of experimental feeds were analysed using a one-way PERMANOVA (Euclidean distance used for resemblance matrix, unrestricted permutations of raw data, 999 unique permutations, Anderson, 2001) to see if there was a difference in $\delta^{13}C$ and $\delta^{15}N$ compositions of feeds (excluding worms which were fed to fish prior to treatments). Fish mass, standard length, and Fulton‟s K of all fish reared under experimental conditions were analysed by a four-factor ANOVA to see if experimental treatments affected fish growth and condition. Four-factor ANOVAs consisted of treatments of day (excluding day 0), diet (chicken and *Artemia*-fed fish), and temperature (all factors were treated as fixed factors) with tank as a random nested factor within day × diet × temperature. Fish muscle $\delta^{13}C$, $\delta^{15}N$, and C:N were analysed in a similar manner in separate ANOVAs. C:N ratios can be an indicator of how „fat‟ or lipid-rich an animal is, with increasing C:N ratios indicating more lipids in tissues (Post et al., 2007) and potentially better animal condition (Kaufman and Johnston, 2007).

Effects of mixing diets on $\delta^{13}C$ and $\delta^{15}N$ of fish muscle were tested in separate, two-factor ANOVAs. Factors in the ANOVAs were diet (fixed) and tank as a nested random factor. The ANOVAs were performed on fish tissue $\delta^{13}C$ and $\delta^{15}N$ from fish reared for 85 days at 24°C and fed either chicken or *Artemia* or mixtures of the two (0:100, 25:75; 50:50, 75:25 and 100:0). Diet effects on length, mass, Fulton‟s K, and C:N ratios of fish muscle were also tested in similar two-factor ANOVAs. Relationships between isotopes of fish muscle and elemental concentration in feed were investigated using regression analyses (dynamic fitting) in Sigma Plot 11.0.

Discrimination factors for carbon and nitrogen were calculated for fish reared for the longest period of time on their respective diets (85 days for all diets except worm-fed fish which were reared for 116 days). The average of diet isotopes was subtracted from individual fish tissue $\delta^{13}C$ and $\delta^{15}N$ values. Treatment effects on $\delta^{13}C$ and $\delta^{15}N$ discrimination were tested using separate ANOVAs. Two ANOVA designs were done on discrimination factors, as temperature was not replicated completely across all experimental diets. One ANOVA design had tank as a nested random factor within diet treatments (fixed) of chicken or *Artemia* or mixtures of the two for fish reared at 24°C. Interactive effects of diet and temperature were tested in three-factor ANOVAs, with tank as a nested random factor within diet and temperature (both fixed factors) for fish fed only chicken or *Artemia* (100 %) and reared at 16°C or 24°C.

Mixing models

Effects of carbon and nitrogen elemental concentration on outcomes of isotope mixing models were investigated by calculating the expected isotopic signatures of fish muscle tissue (with and without elemental concentration) and comparing them with measured isotopic signatures. To calculate the expected isotopic signatures it was assumed that all food that was consumed was assimilated in relative proportions. The mass balance model (Phillips and Gregg, 2001) was used to calculate expected isotopic signatures for carbon and nitrogen in fish muscle tissue without elemental concentration:

$$\delta^{13}C_M = f_{Ch}(\delta^{13}C_{Ch} + \Delta^{13}C_{tissue\text{-}Ch}) + f_A(\delta^{13}C_A + \Delta^{13}C_{tissue\text{-}A}) \quad (1)$$

$$\delta^{15}N_M = f_{Ch}(\delta^{15}N_{Ch} + \Delta^{15}N_{tissue\text{-}Ch}) + f_A(\delta^{15}N_A + \Delta^{15}N_{tissue\text{-}A}) \quad (2)$$

$$1 = f_{Ch} + f_A \quad (3)$$

where the subscript M represents the fish tissue from fish fed the mixed diet under question; subscript Ch represents the pureed chicken muscle; subscript A represents the *Artemia* blocks; f represents the fractional contribution of chicken or *Artemia* to the mixed diet under question, by mass; and $\Delta^{13}C_{tissue\text{-}diet}$ is the isotopic discrimination of chicken or *Artemia* experimentally derived from fish reared for 85 days at 24°C. Note that although fish may not have all reached isotopic equilibrium after 85 days (see Results), as fish had been feeding on mixed diets for the same period of time isotope integration and discrimination would be proportionally similar. Therefore the un-equilibrated discrimination factors used are valid for the mixing models as fish had been reared on diets for the same period of time.

The concentration dependent equations of Phillips and Koch (2002) were also used, but they were reduced to two sources. Let $f_{Ch,B}$ and $f_{A,B}$ represent the fractions of consumed biomass (B) of chicken (Ch) and *Artemia* (A) respectively (i.e. the proportion of chicken or *Artemia* in the mixed diet); and $f_{Ch,C}$, $f_{A,C}$, $f_{Ch,N}$ and $f_{A,N}$ represent the fractions of consumed carbon (C subscript) or nitrogen (N subscript) from chicken or *Artemia*. The mass balance equations can then be written as:

$$\delta^{13}C_M = f_{Ch,C}(\delta^{13}C_{Ch} + \Delta^{13}C_{tissue\text{-}Ch}) + f_{A,C}(\delta^{13}C_A + \Delta^{13}C_{tissue\text{-}A}) \quad (4)$$

$$\delta^{15}N_M = f_{Ch,N}(\delta^{15}N_{Ch} + \Delta^{15}N_{tissue\text{-}Ch}) + f_{A,N}(\delta^{15}N_A + \Delta^{15}N_{tissue\text{-}A}) \quad (5)$$

The diet fractional contributions for C, N, and biomass are constrained to sum to 1, as per Phillips and Koch (2002):

$$1 = f_{Ch,C} + f_{A,C} \quad (6)$$

$$1 = f_{Ch,N} + f_{A,N} \quad (7)$$

$$1 = f_{Ch,B} + f_{A,B} \quad (8)$$

Phillips and Koch‟s (2002) concentration dependent mixing model assumes that the contribution of each food or source (chicken or *Artemia*) to the consumer‟s tissue (fish muscle) isotopic signature is proportional to the elemental concentration in the food multiplied by the assimilated biomass. However, here it was assumed that it was proportional to the consumed biomass to enable back-calculations so that they can be compared with measured isotopic signatures. Let $(C)_{Ch}$, $(C)_A$, $(N)_{Ch}$ and $(N)_A$ represent the carbon and nitrogen concentrations in chicken and *Artemia*. Therefore, using Phillips and Koch‟s concentration dependent model the following were derived and used:

$$f_{Ch,C} = \frac{f_{Ch,B}(C)_{Ch}}{f_{Ch,B}(C)_{Ch} + f_{A,B}(C)_A} \quad (9)$$

$$f_{A,C} = \frac{f_{A,B}(C)_A}{f_{Ch,B}(C)_{Ch} + f_{A,B}(C)_A} \quad (10)$$

$$f_{Ch,N} = \frac{f_{Ch,B}(N)_{Ch}}{f_{Ch,B}(N)_{Ch} + f_{A,B}(N)_A} \quad (11)$$

$$f_{A,N} = \frac{f_{A,B}(N)_A}{f_{Ch,B}(N)_{Ch} + f_{A,B}(N)_A} \quad (12)$$

in equations 4 and 5 to derive expected isotopic signatures, accounting for elemental concentration, of fish muscle for fish fed mixed diets. These results were then compared with those derived from strict mass balance calculations and measured isotopic signatures to see if accounting for elemental concentration improves predictions of $\delta^{13}C$ and $\delta^{15}N$ in fish muscle tissue.

Results

Diets

The elemental concentrations of mixed feeds did not vary linearly with proportional mass contribution as expected (see Table 3.1). The elemental concentrations, and consequently the stable isotope signatures, were skewed

toward the chicken puree (see Table 3.1, Fig. 3.1), however, elemental concentration did vary co-linearly between % carbon and % nitrogen.

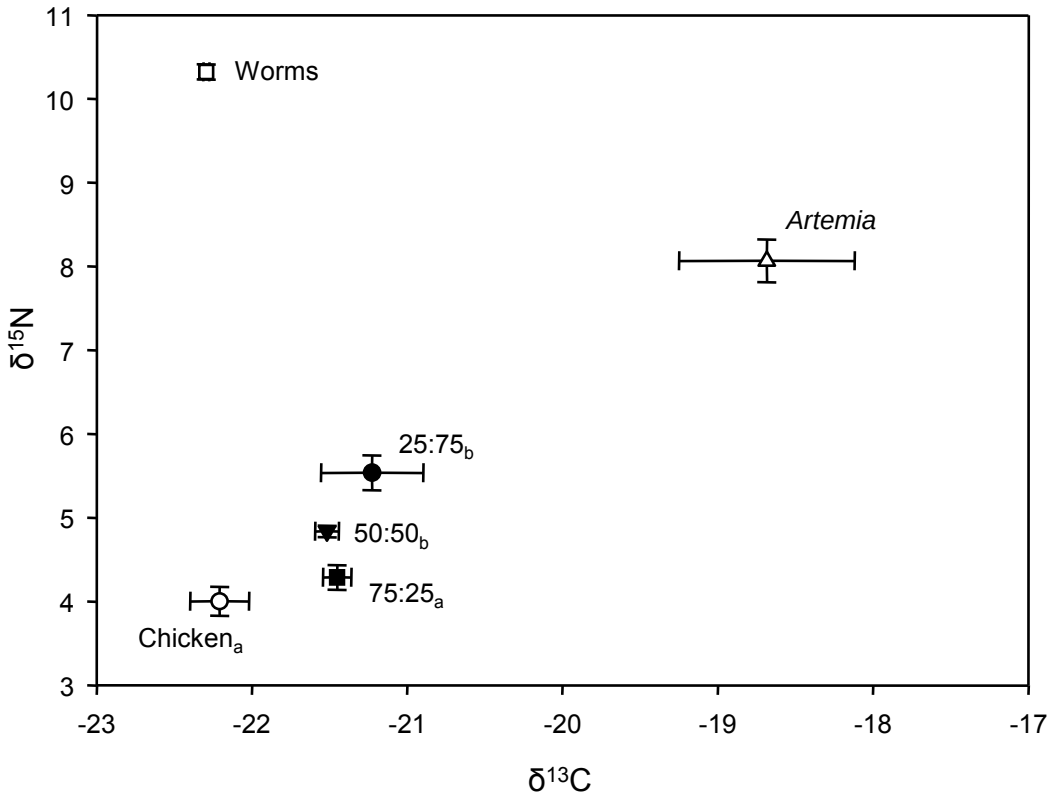

Figure 3.1 Feed $\delta^{15}N$ and $\delta^{13}C$ values (mean ± SE ‰) used in the experiment. Note subscript letters denote diets that were not significantly different in isotopic composition.

Isotope ratios of live black worms did not vary greatly over the duration of the experiment ($\delta^{15}N = 10.3 \pm 0.1$, $\delta^{13}C = -22.29 \pm 0.02$, mean ± SE ‰) nor did isotopes of chicken ($\delta^{15}N = 4.0 \pm 0.2$, $\delta^{13}C = -22.21 \pm 0.19$, mean ± SE ‰). However, isotopes of *Artemia* were more variable ($\delta^{15}N = 8.1 \pm 0.3$, $\delta^{13}C = -18.68 \pm 0.57$, mean ± SE ‰), which subsequently affected the mixed feeds, particularly the 25:75 mix ($\delta^{15}N = 5.5 \pm 0.2$, $\delta^{13}C = -21.23 \pm 0.33$, mean ± SE ‰). There was a significant difference in isotope composition ($\delta^{13}C$ and $\delta^{15}N$) among all feeds

(PERMANOVA $F_{4,34} = 13.035$, $p = 0.001$) except for 25:75 and 50:50, and 75:25 and chicken (see Fig. 3.1), which were similar in pair-wise analyses. The similarities in isotope signatures of particular diets are likely due to more C and N coming from chicken than *Artemia*.

Chicken and Artemia *fed fish (100 %)*

Fish growth

There was an interaction among day, diet, and temperature for fish mass and standard length (Table 3.2). On average fish grew over the duration of the experiment, but, most of this growth occurred after 28 days. Fish fed chicken were generally larger (heavier and longer) than fish fed *Artemia* (Figs 3.2a and b). Fish size (mass and length) was largely unaffected by temperature for the first 28 days, after which fish reared at 24°C were more often larger than fish reared at 16°C, particularly for fish fed chicken (Figs 3.2a and b).

Table 3.2 Four factor analysis of variance (ANOVA) of treatment effects on fish mass, standard length (SL) and condition (Fulton‟s K) for all fish fed 100 % of either chicken or *Artemia* and reared at 16°C or 24°C (excludes day 0). Bolded numbers indicate significant effects ($p < 0.05$). Data were not transformed.

		Mass		SL		Fulton's K	
Source of variation	df	MS	p	MS	p	MS	p
Day	6	28.260	**0.001**	1154.400	**0.001**	0.899	**0.001**
Diet	1	70.730	**0.001**	1155.000	**0.001**	5.417	**0.001**
Temperature	1	5.201	**0.047**	293.120	**0.044**	0.093	0.180
Day x Diet	6	4.600	**0.007**	105.690	0.140	0.264	**0.001**
Day x Temperature	6	2.290	0.116	77.494	0.323	0.049	0.465
Diet x Temperature	1	9.104	**0.010**	43.350	0.407	0.932	**0.001**
Day x Diet x Temp.	6	4.494	**0.008**	190.940	**0.019**	0.175	**0.006**
Tank (Day x Diet x Temp.)	28	1.187	0.359	60.306	0.503	0.051	**0.001**
Residual	476	1.087		63.652		0.012	
Total	531						

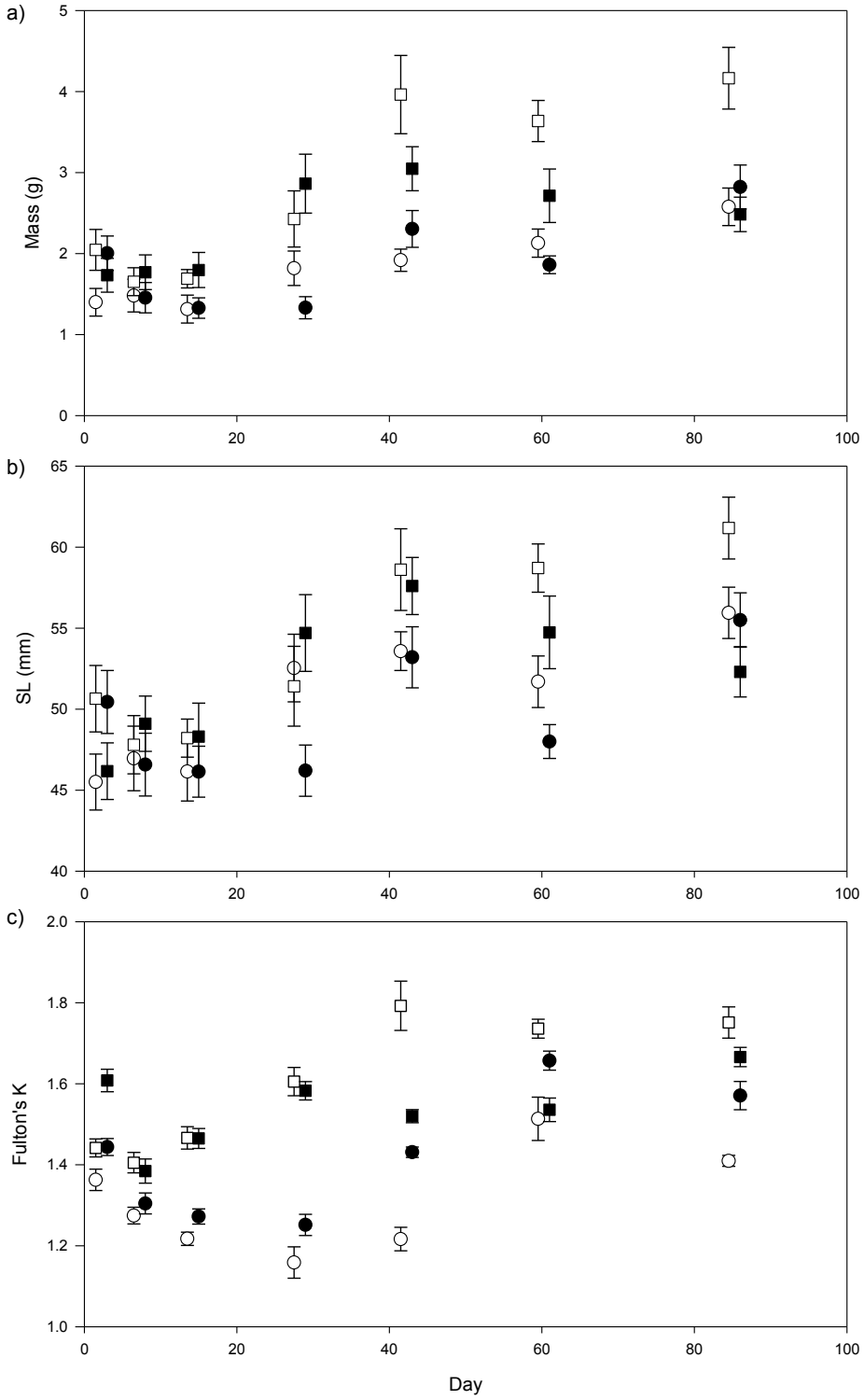

Figure 3.2 Average (± SE) (a) mass (g) (b) standard length (SL, mm) and (c) Fulton‟s K for fish fed chicken (squares) or *Artemia* (circles) and reared at either 16°C (solid symbols) or 24°C (open symbols) pooled over tanks. Note: points are offset slightly around sampling days.

Fish condition

There was an interaction among day, diet, and temperature in four-factor ANOVA of Fulton‟s K (Table 3.2). Generally fish fed chicken and reared at 24°C were in the best condition (highest Fulton‟s K), followed by chicken-fed fish reared at 16°C, then *Artemia*-fed fish reared at 16°C, with *Artemia*-fed fish reared at 24°C in the worst condition (lowest Fulton‟s K) for the duration of the experiment (see Fig. 3.2c). Over the first 28 days, condition of fish fed *Artemia* generally decreased and condition of chicken-fed fish increased (Fig. 3.2c). After 28 days, condition of fish fed *Artemia* increased and levelled off, and this was likely due to increased feeding rates. Another increase in condition for *Artemia*-fed fish was not detected after 65 days, when feed rates were again increased. Despite a significant effect of tank of Fulton‟s K (Table 3.2), data are presented pooled across tanks as there were significant treatment effects of diet and temperature which interacted with time (Fig. 3.2c). Carbon to nitrogen (C:N) ratios of fish muscle showed similar patterns to Fulton‟s K and were affected by diet, but this depended on temperature and time (Table 3.3, Fig. 3.3a).

Table 3.3 Four factor ANOVA of treatment effects on $\delta^{13}C$, $\delta^{15}N$, and C:N ratios of fish muscle (excluding day 0) for fish fed chicken or *Artemia* and reared at either 16°C or 24°C over 85 days. Bolded numbers indicate significant effects ($p < 0.05$). Data were not transformed.

		$\delta^{13}C$		$\delta^{15}N$		C:N	
Source of Variation	df	MS	p	MS	p	MS	p
Day	6	0.985	**0.013**	31.279	**0.001**	0.217	**0.001**
Diet	1	46.738	**0.001**	354.82	**0.001**	5.342	**0.001**
Temperature	1	2.039	**0.014**	20.526	**0.025**	0.675	**0.001**
Day x Diet	6	5.249	**0.001**	5.645	0.154	0.137	**0.001**
Day x Temperature	6	0.368	0.289	4.482	0.292	0.028	0.102
Diet x Temperature	1	1.336	**0.041**	6.891	0.164	0.274	**0.001**
Day x Diet x Temp.	6	0.155	0.756	5.141	0.216	0.077	**0.001**
Tank (Day x Diet x Temp.)	28	0.295	**0.016**	3.279	**0.012**	0.014	0.554
Residual	224	0.172		1.718		0.015	
Total	279						

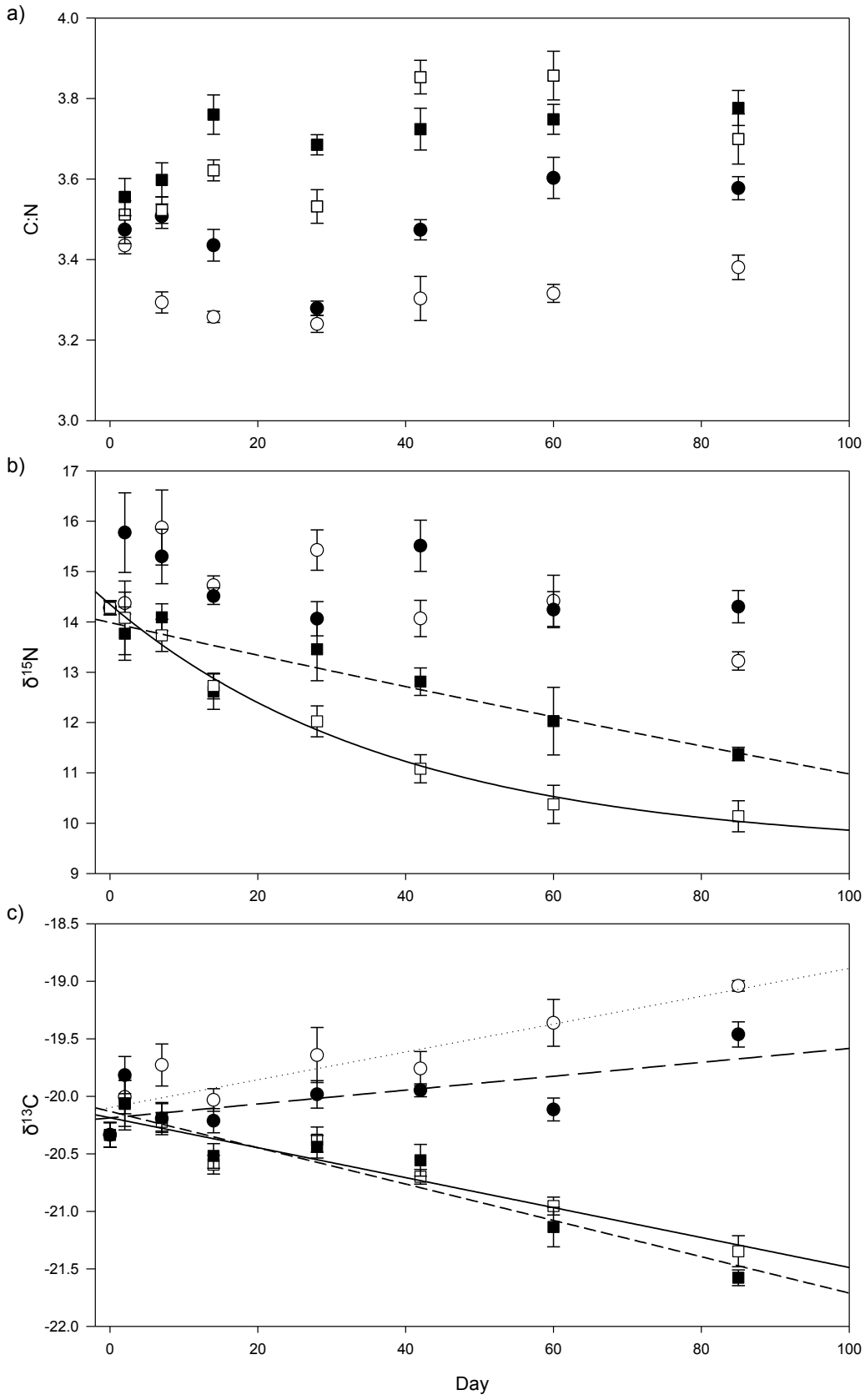
a)
C:N
4.0
3.8
3.6
3.4
3.2
3.0
0
20
40
60
80
100
b)
δ15N
17
16
15
14
13
12
11
10
9
c)
δ13C
-18.5
-19.0
-19.5
-20.0
-20.5
-21.0
-21.5
-22.0
Day

Figure 3.3 Average (± SE) (a) C:N ratios, (b) $\delta^{15}N$ (‰) and (c) $\delta^{13}C$ (‰) of fish muscle tissue over the duration of the experiment for fish fed chicken (squares) or *Artemia* (circles) and reared at either 16°C (solid symbols) or 24°C (open symbols) pooled over tanks. Isotope data for day 0 fish are taken from fish kept on worms for 116 days total. Lines of best fit are shown (solid line – chicken-fed fish reared at 24°C; dashed line – chicken-fed fish reared at 16°C; dotted line – *Artemia*-fed fish reared at 24°C; long dashed line – *Artemia*-fed fish reared at 16°C). No lines are fitted to $\delta^{15}N$ of *Artemia* treatments as the change over time was inconsistent, in differing directions and there was large variation among individuals. See Table 3.4 for regression details.

Table 3.4 Regression analyses details of $\delta^{15}N$ and $\delta^{13}C$ (y, ‰) over time (x, days) in Figs 3.3b) and c) for fish fed chicken or *Artemia* at 16°C or 24°C over 85 days.

Isotope	Diet	Temperature (°C)	Equation	r^2	p
$\delta^{15}N$	Chicken	24	$y = 9.49 + 4.86e^{(-0.026x)}$	0.99	<0.0001
		16	$y = -3.43 + 17.41e^{(-0.0019x)}$	0.83	0.0114
$\delta^{13}C$	Chicken	24	$y = -20.19 - 0.013x$	0.89	0.0004
		16	$y = -20.13 - 0.016x$	0.91	0.0003
	Artemia	24	$y = -20.10 + 0.012x$	0.83	0.0017
		16	$y = -20.19 + 0.006x$	0.44	0.0700

Isotope incorporation

There was a significant effect of day, diet and temperature on nitrogen isotopes of fish muscle (Table 3.3). Generally fish reared at 24°C had lower $\delta^{15}N$ than fish reared at 16°C and fish fed chicken had lower $\delta^{15}N$ than fish fed *Artemia* (Fig. 3.3b). Fish fed chicken at 24°C displayed the classic exponential decay relationship found in many other experiments. These fish had the fastest isotope turnover as $\delta^{15}N$ changed the most over the 85 days of the experiment and the exponential co-efficient is larger than that for fish reared at 16°C and fed chicken (Table 3.4). Fish fed chicken and reared at 24°C appear to be near equilibrium, with regression analysis finding the asymptotic $\delta^{15}N$ value to be 9.49 ± 0.33 (SE) ‰. The half life, or the length of time for half the change in $\delta^{15}N$ to occur, for $\delta^{15}N$ of fish fed chicken at 24°C was 27.2 days. Fish fed chicken and reared at 16°C may be in equilibrium with their diet, as there was no significant difference in $\delta^{15}N$ of fish tissue sampled on days 60 and 85. However, there was a significant difference between tanks on day 60 for this treatment. Therefore it is likely that the similarity in $\delta^{15}N$ between days 60 and 85, for fish fed chicken and reared at 16°C, is due to large variation pooled over tanks on day 60 and that fish $\delta^{15}N$ was still changing. The line of best fit for $\delta^{15}N$ of fish fed chicken at 16°C was more similar to a linear decrease than to an exponential decay curve, which suggests fish would need to be reared longer to determine where an asymptote occurs. The asymptote could be expected to be between 10 and 11 ‰, slightly higher than that for fish reared at 24°C, due to larger discrimination at cooler temperatures (Bosley et al., 2002; Barnes et al., 2007). Exponential decay curves were not fitted to $\delta^{15}N$ of *Artemia*-fed treatments as the change over time was not unidirectional for either temperature (Fig. 3.3b).

There were significant interactions between diet and temperature, and between day and diet on $\delta^{13}C$ of fish muscle (Table 3.3). The diet and temperature interaction reflected a difference between temperature treatments of fish muscle $\delta^{13}C$ from fish fed *Artemia*, but there was no difference between temperature treatments for chicken-fed fish. Fish fed *Artemia* and reared at 24°C generally had higher $\delta^{13}C$ than fish reared at 16°C (Fig. 3.3c). The rate of change of $\delta^{13}C$, measured by the slope of the line, was twice as fast for fish fed *Artemia* and reared at 24°C than those reared at 16°C (see Table 3.4, Fig. 3.3c). The diet and time interaction was due to fish fed *Artemia* increasing in $\delta^{13}C$ over time, whereas fish fed chicken decreased in $\delta^{13}C$. Fish muscle $\delta^{13}C$ for all treatments did not appear to approach an asymptote so no attempt was made to fit exponential decay curves. These data suggest that fish muscle $\delta^{13}C$ is continuing to change and that fish may not have equilibrated with their diets. Lines of best fit were linear, with r^2 being greater than 0.8 for most regressions (Table 3.4), except for *Artemia*-fed fish reared at 16°C ($r^2 = 0.44$).

There was a significant effect of tank on $\delta^{15}N$ and $\delta^{13}C$ of fish muscle (Table 3.3). For $\delta^{15}N$ three pairs of tanks showed significant differences, whereas for $\delta^{13}C$ four pairs of tanks showed significant difference between them. Most of the pairs of tanks that showed significant differences were not the same in $\delta^{13}C$ and $\delta^{15}N$ analyses, except for one pair. Differences between pairs of tanks were generally not biologically meaningful, as they were due to small variation within data sets. Given main effects are the primary concern of this paper, individuals from all tanks were used for analyses and all graphs show averages pooled for tanks.

Mixed diets

There was a significant effect of diet on fish size (mass: $F_{4,84} = 6.10$, $p < 0.05$, and length: $F_{4,84} = 7.97$, $p < 0.05$), Fulton‟s K ($F_{4,84} = 12.95$, $p < 0.01$) and C:N ratios ($F_{4,49} = 13.01$, $p = 0.01$) of muscle from fish reared for 85 days at 24°C on mixed diets (Table 3.5). The effects of diet on fish size were similar for mass and standard length (see Table 3.5) with significant differences in fish size (length and mass) between fish fed high percentages of *Artemia* and those fed low percentages. Fish fed the 25:75 mix were the smallest in mass and length on average, followed by *Artemia*-fed fish, fish fed 50:50 mixture, then chicken-fed fish, and fish fed the 75:25 mix were the largest fish on average. The diet effect on fish condition, or Fulton‟s K, was due to significant differences between *Artemia*-fed fish and those fed all other diets except for 25:75; which were similar in Fulton‟s K to *Artemia*-fed fish but also different from all other diets (see Table 3.5). *Artemia*-fed fish had the lowest condition, based on Fulton‟s K, of all treatment groups. The significant effect of diet on C:N ratios was due to differences between fish fed 25:75 diets and all other diet groups, bar *Artemia*-fed fish (100 %), which were also low in C:N and similar to fish fed the 25:75 diet (see Table 3.5). Chicken-fed fish also had a significantly greater C:N ratio than fish fed 50:50 diets.

Table 3.5 Average (± SE) of mass, standard length (SL), condition (Fulton‟s K), and C:N ratios of muscle from fish fed chicken (C) or *Artemia* (A) or mixtures of the two (C:A; 0:100, 25:75, 50:50, 75:25, 100:0) for fish reared at 24°C after 85 days. Note subscript letters denote diets with values that are not significantly different.

Diet	Mass (g)	SL (mm)	Fulton‟s K	C:N
Artemia (100 %)	$2.58 \pm 0.23_{a,c}$	$56 \pm 2_{a,c}$	$1.41 \pm 0.01_{a}$	$3.4 \pm 0.0_{a,b,c}$
25:75	$2.25 \pm 0.20_{a}$	$53 \pm 2_{a}$	$1.41 \pm 0.02_{a}$	$3.3 \pm 0.0_{a}$
50:50	$3.59 \pm 0.39_{b,c}$	$61 \pm 2_{b,c}$	$1.52 \pm 0.02_{b}$	$3.5 \pm 0.0_{c}$
75:25	$4.57 \pm 0.46_{b}$	$65 \pm 2_{b}$	$1.60 \pm 0.03_{b}$	$3.7 \pm 0.1_{b,c}$
Chicken (100 %)	$4.16 \pm 0.38_{a,b}$	$61 \pm 2_{a,b}$	$1.75 \pm 0.04_{b}$	$3.7 \pm 0.1_{b}$

There was a significant effect of diet on $\delta^{13}C$ ($F_{4,49} = 45.33$, $p = 0.001$) and $\delta^{15}N$ ($F_{4,49} = 19.91$, $p= 0.01$) of fish muscle for fish fed mixed diets (Fig. 3.4). There were significant differences in $\delta^{13}C$ and $\delta^{15}N$ of fish tissue for those fed chicken or *Artemia* (100 %), however, there were not always differences among fish fed mixed diets. Fish fed mixtures with percentage contributions that were next to each other on a proportional scale had similar isotope ratios and more distant mixtures had significantly different isotope ratios (Fig. 3.4).

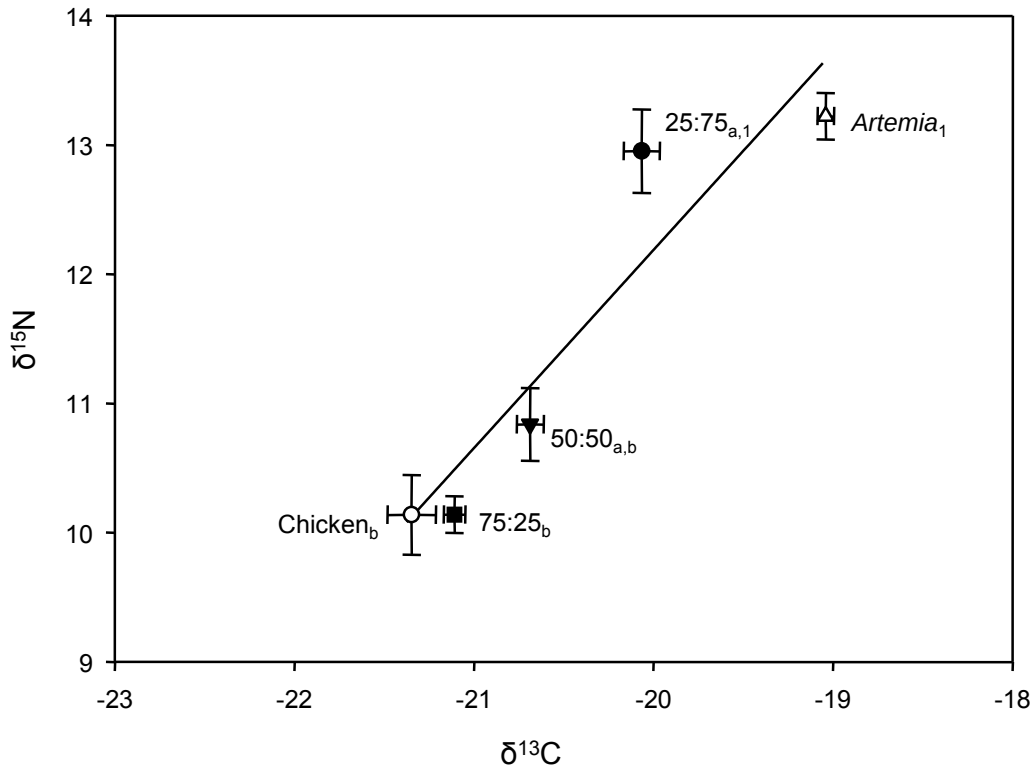

Figure 3.4 Fish muscle $\delta^{15}N$ and $\delta^{13}C$ values (mean ± SE ‰) after 85 days rearing at 24°C for fish fed *Artemia* (A) or chicken (C) or mixtures of the two (C:A; 0:100, 25:75, 50:50, 75:25, 100:0). Note subscript letters denote $\delta^{15}N$ and $\delta^{13}C$ values that are not significantly different; and subscript numbers denote $\delta^{15}N$ values only that are not significantly different. Linear regression of $\delta^{15}N$ against $\delta^{13}C$: $r^2 = 0.88$, $p < 0.05$.

There was a significant effect of tank on $\delta^{13}C$ ($F_{5,49} = 2.88$, $p < 0.05$) and Fulton‟s K ($F_{5,84} = 3.00$, $p < 0.05$) of fish reared for 85 days at 24°C and fed chicken or *Artemia* or a mixture of the two. The pairs of tanks that were significantly different in $\delta^{13}C$ and Fulton‟s K were not the same. The pairs of tanks that were significantly different in Fulton‟s K were the chicken-fed tanks and the difference between tanks was smaller than the difference between other pairs of tanks. The differences in $\delta^{13}C$ of fish tissue and Fulton‟s K among tanks were very small and variance in $\delta^{13}C$ was mostly small within treatment groups (see Fig. 3.4), therefore tanks are presented pooled.

Fish muscle $\delta^{15}N$ decreased with increasing nitrogen in the diet (see Fig. 3.5a), however, the regression is not statistically significant ($F_{1,4} = 6.52$, $p > 0.05$). The $\delta^{13}C$ of fish muscle decreased linearly as the percentage of carbon in the diet increased ($F_{1,4} = 42.17$, $p < 0.01$; Fig. 3.5b).

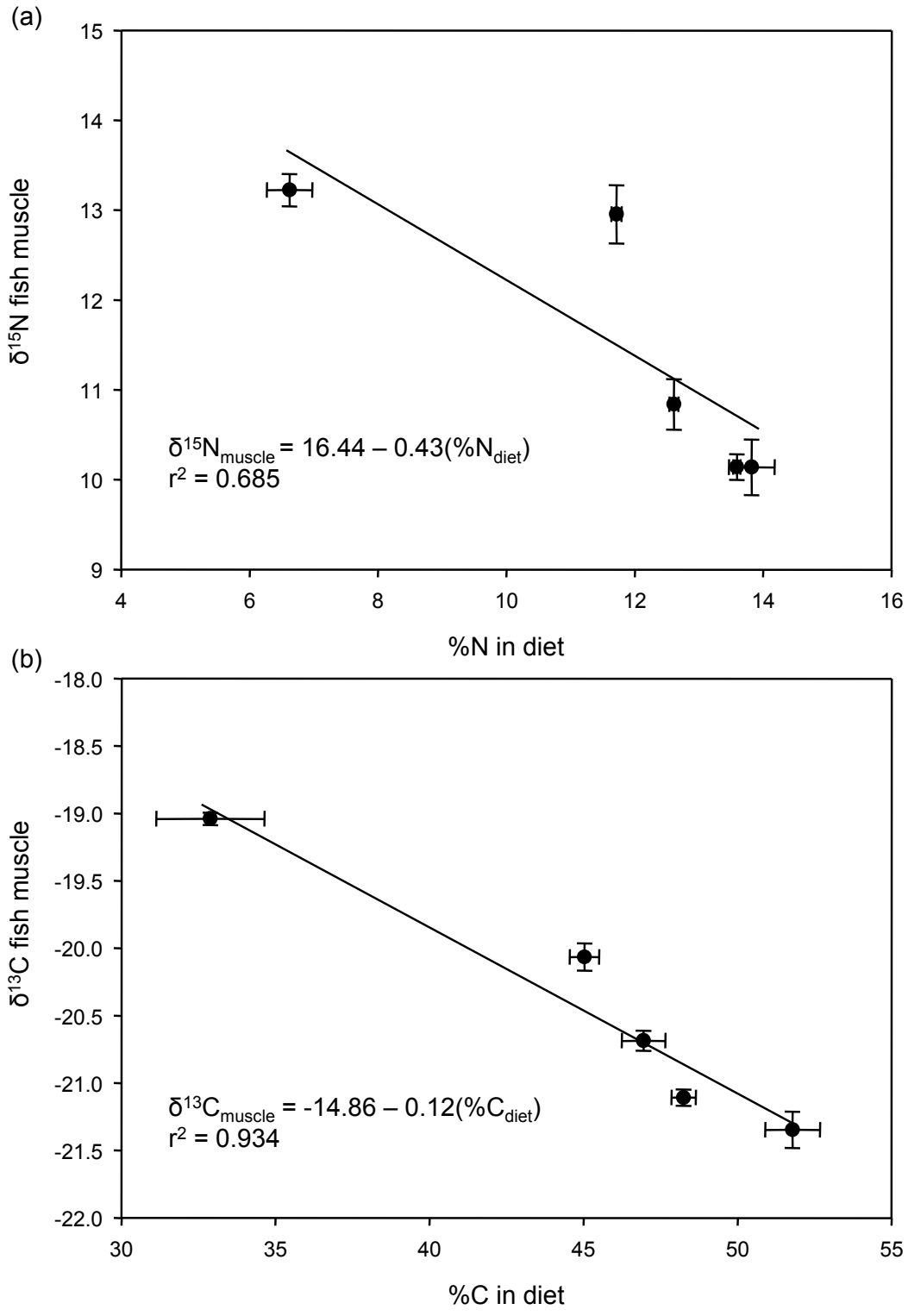

Figure 3.5 Fish muscle (a) $\delta^{15}N$ and (b) $\delta^{13}C$ (mean ± SE ‰) in relation to elemental concentration ((N) or (C) mean ± SE %) of diet after 85 days rearing at 24°C.

Isotope discrimination

There was a significant effect of diet on both $\delta^{13}C$ ($F_{4,49} = 18.64$, $p < 0.01$) and $\delta^{15}N$ ($F_{4,49} = 5.82$, $p < 0.05$) discrimination. For $\delta^{15}N$ discrimination the only significant difference was between *Artemia* and the 75:25 mixed diet (Fig. 3.6a). Fish fed live worms had the smallest $\delta^{15}N$ discrimination, followed by fish fed 100 % *Artemia*, then fish fed 75, 50 and 100 % chicken, with fish fed 25 % chicken having the greatest $\delta^{15}N$ discrimination of all treatments (see Fig. 3.6a). Tissue $\delta^{13}C$ discrimination was similar between fish fed 100 % chicken and fish fed 25, 50 or 75 % chicken, however, discrimination was significantly different between fish fed 25 and 75 % chicken. All other treatments were significantly different from each other (see Fig. 3.6b). Worm-fed fish had the greatest $\delta^{13}C$ discrimination, followed by fish fed 25 and 50 % chicken, fish fed 100 % chicken and fish fed 75 % chicken. Fish fed pure *Artemia* had a negative discrimination of $\delta^{13}C$ (see Fig. 3.6b), as their tissue $\delta^{13}C$ was more negative than the *Artemia* $\delta^{13}C$, although fish tissue $\delta^{13}C$ may still have been increasing towards diet $\delta^{13}C$.

There was a significant effect of temperature on $\delta^{15}N$ discrimination ($F_{1,39} = 41.40$, $p < 0.01$), but not on $\delta^{13}C$ discrimination ($F_{1,39} = 6.13$, $p > 0.05$) for fish fed pure diets of chicken or *Artemia* and reared at 16°C or 24°C. Tissue $\delta^{15}N$ discrimination was greater at 16°C than at 24°C for both chicken and *Artemia* fed fish (see Fig. 3.6a). Tissue $\delta^{13}C$ discrimination was the reverse however, with greater or more positive discrimination at 24°C than at 16°C (see Fig. 3.6b).

Tanks had a significant effect on the discrimination factors of $\delta^{13}C$ ($F_{5,49} = 2.88$, $p < 0.05$), but not $\delta^{15}N$ ($F_{5,49} = 1.93$, $p > 0.05$) for fish fed mixed diets. However the differences detected were not biologically meaningful, similar to when tanks effects were detected for $\delta^{13}C$ of fish fed mixed diets above.

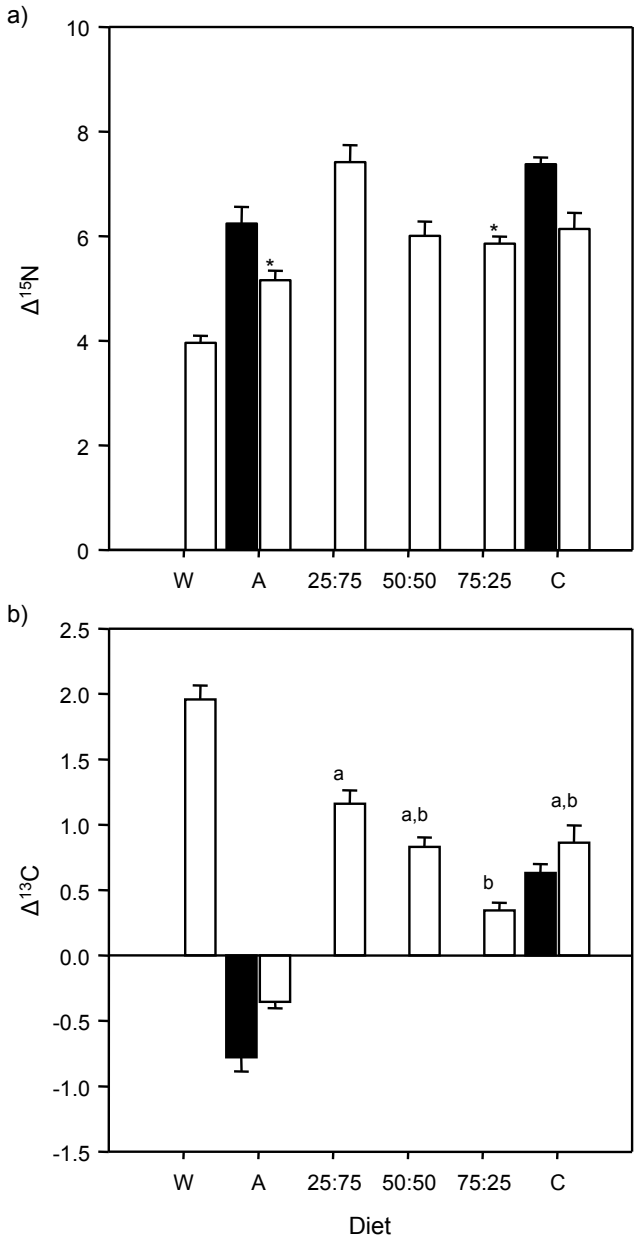

Figure 3.6 Average discrimination factors (Δ ± SE ‰) of (a) $\delta^{15}N$ and (b) $\delta^{13}C$ for fish fed worms (W), *Artemia* (A), chicken (C) or a mixture of chicken and *Artemia* (C:A; 25:75, 50:50, 75:25) after 85 days rearing (116 days for worm-fed fish) at 16°C (filled bars) or 24°C (open bars). Note: * denotes significant differences between diet treatments when no differences were found among other treatments; letters denote groups of similar discrimination factors for diet treatments when all others are significantly different. Temperature significantly affected $\delta^{15}N$ discrimination but not $\delta^{13}C$ discrimination.

Mixing models

The calculated $\delta^{13}C$ and $\delta^{15}N$ of fish muscle for fish fed mixed diets (25:75, 50:50 and 75:25) were more accurate, or closer to the measured values, when elemental concentration was taken into account than when it was not (see Table 3.6). There was one exception where the calculated $\delta^{15}N$ of fish tissue was more accurate when elemental concentration was not taken into account and this was for fish fed 25 % *Artemia* and 75 % chicken. When elemental concentration of diets was accounted for all calculated $\delta^{13}C$ and $\delta^{15}N$ were within one standard deviation of the measured mean values of isotopes of fish muscle. However, when elemental concentration of diets was ignored, calculated $\delta^{13}C$ was outside of one standard deviation but within two standard deviations of the measured mean $\delta^{13}C$ of fish muscle. When elemental concentration of diets was ignored for $\delta^{15}N$, only one diet mix (75:25) was outside of one standard deviation of measured mean $\delta^{15}N$ of fish muscle, but it was within two standard deviations.

Table 3.6 Expected $\delta^{13}C$ and $\delta^{15}N$ of fish muscle calculated with and without taking elemental concentration ((C), (N)) into account, and measured isotopic signatures (mean ± SD) for fish reared for 85 days at 24°C and fed mixed diets of chicken (C) and *Artemia* (A) in varying proportions by mass (C:A; 25:75, 50:50, 75:25). Note we report standard deviation here so that we can discuss the distribution of the data about the sample/measured means.

	$\delta^{13}C$ (‰)			$\delta^{15}N$ (‰)		
Diet	Calculated: without (C)	Calculated: with (C)	Measured	Calculated: without (N)	Calculated: with (N)	Measured
25:75	-19.62	-19.83	-20.06 ± 0.32	12.45	11.96	13.0 ± 1.0
50:50	-20.19	-20.45	-20.68 ± 0.24	11.68	11.14	10.8 ± 0.9
75:25	-20.77	-20.94	-21.11 ± 0.19	10.91	10.56	10.1 ± 0.5

Discussion

Experimental effects of temperature, diet and time

Fish condition, $\delta^{15}N$ and $\delta^{13}C$ of fish muscle changed over time and were affected by diet and temperature. The effect of temperature on fish condition depended on diet. In general, fish reared at 24°C had faster isotope incorporation, as isotopes changed more over the duration of the experiment. However, diet affected isotopic signatures in different ways with $\delta^{13}C$ decreasing for fish fed chicken and increasing for fish fed *Artemia*. Muscle $\delta^{15}N$ decreased over time for fish fed chicken but was variable over time for fish fed *Artemia*.

Muscle $\delta^{15}N$ of fish fed chicken and reared at 24°C showed the classic exponential pattern (e.g. Hesslein et al., 1993; Guelinckx et al., 2007). This exponential pattern likely reflects isotope incorporation by fish muscle during growth periods over the summer time, when food is abundant and the water is warm. The half life was 27.2 days which is similar to the finding of Guelinckx et al. (2007), who found a half life of 27.8 days for $\delta^{15}N$ in muscle tissue of the sand goby *Pomatoschistus minutus* (Pallas, 1770). It is noted that the sizes of the fish in both experiments are also comparable, as are the feed rates (approximately 5 % body weight per feed in this experiment; 3 % body weight in Guelinckx et al. (2007)) and experimental durations. However *P. minutus* were reared at 17°C and *A. forsteri* were reared at 24°C. It would be expected that the half-life would be shorter in this experiment than for *P. minutus*, as isotope turnover is generally faster at warmer temperatures (Chapter 2, Bosley et al., 2002; Bloomfield et al., 2011) and *A. forsteri* were reared in warmer waters. However, the temperatures used in this experiment and that by Guelinckx et al. (2007) represent summer

temperatures for habitats of the respective experimental fishes. The similarities of half lives for $\delta^{15}N$ in muscle between *A. forsteri* and *P. minutus* at different temperatures highlights the need to experimentally determine isotope incorporation rates for individual species at appropriate temperatures.

The change over time in muscle $\delta^{15}N$ of fish fed *Artemia* was smaller than the difference in $\delta^{15}N$ between the worms and *Artemia* feeds. Muscle $\delta^{15}N$ of fish fed *Artemia* varied unpredictably over time and this may be a reflection of variable $\delta^{15}N$ of *Artemia* over time interacting with fish condition. The condition of *Artemia*-fed fish was generally poor and fasting, or poorly nourished, animals are known to increase in $\delta^{15}N$ (Hobson et al., 1993; Gaye-Siessegger et al., 2004b; Kelly and Martínez del Rio, 2010). Therefore the $\delta^{15}N$ of fish fed *Artemia* was potentially being influenced by two opposing factors to create variable $\delta^{15}N$ over time: diet $\delta^{15}N$ (which was variable over time) and poor nourishment (which is likely to increase $\delta^{15}N$). However, Guelinckx et al. (2007) also conducted a starvation experiment on *P. minutus* and found no change in $\delta^{15}N$ of fish muscle after 20 days of starvation. *Aldrichetta forsteri* reared on *Artemia* for 85 days at 16°C were on average very similar in $\delta^{15}N$ to fish fed worms for 116 days, and fish reared at 24°C on *Artemia* were only slightly lower in $\delta^{15}N$. Therefore, these results appear to be supportive of no change in $\delta^{15}N$ of fish muscle during starvation or poor nourishment periods.

Fish muscle $\delta^{13}C$ values were generally higher at warmer temperatures than at cooler temperatures for respective diet treatments. This is consistent with increased metabolism at warmer temperatures, such that fewer lipids are stored or more are metabolised increasing $\delta^{13}C$ values. Tissues with a higher $\delta^{13}C$ value are less likely to have high lipid content than comparable tissues (i.e. reared on the

same diet) because lipids are depleted in ^{13}C (DeNiro and Epstein, 1977). The lower lipid content of fish tissue for fish reared at 24°C is supported by lower C:N ratios, also indicating low lipid content (Post et al., 2007). The temperature effect found here on $\delta^{13}C$ values of muscle reflects increased metabolism at higher temperatures and is supported by differences in condition (Fulton‟s K) and C:N ratios.

There was also an effect of diet on fish muscle $\delta^{13}C$, with fish fed *Artemia* increasing in $\delta^{13}C$ and fish fed chicken decreasing in $\delta^{13}C$ over time. The increase in muscle $\delta^{13}C$ of fish fed *Artemia* was expected as the feed $\delta^{13}C$ of *Artemia* was higher than that of worms. Tissue $\delta^{13}C$ of fish fed chicken decreased and this was not expected as $\delta^{13}C$ of chicken and worms were similar (Fig. 3.1). The decrease in $\delta^{13}C$ over time of chicken-fed fish muscle shows fish were storing lipids on the chicken diet, which is supported by higher C:N ratios and Fulton‟s K. The C:N ratios of fish fed chicken for 85 days were also higher than C:N of fish fed worms for 116 days, indicating that there were more lipids in fish fed chicken (Post et al., 2007). These $\delta^{13}C$ results reflect the nutritional status of the fish to a certain degree, with fish fed chicken storing lipids and decreasing in $\delta^{13}C$. However, fish muscle $\delta^{13}C$ was clearly still changing after 85 days rearing on the two diets, chicken and *Artemia*, and this appears to be related to condition of the fish as well as dietary $\delta^{13}C$ values.

Isotope discrimination factors

Discrimination of $\delta^{13}C$ was not significantly affected by temperature, however, discrimination of $\delta^{15}N$ was affected by temperature and it was 1.15 ‰ less at 24°C than at 16°C. Fractionation, or the difference in isotopic concentration between reactants and products, is smaller at warmer temperatures than at colder

temperatures due to kinetic effects on chemical reactions. Therefore tissue-diet isotope discrimination should also be smaller at warmer temperatures than at colder temperatures, as discrimination is the sum of many chemical reactions. The decrease in $\delta^{15}N$ discrimination with increasing temperature is, therefore, likely to be due to kinetic effects on chemical reactions. The magnitude of the effect of temperature on $\delta^{15}N$ discrimination reported here is similar to that found by Barnes et al. (2007) and Power et al. (2003). Barnes et al. (2007) found a 0.126 ‰ decrease in $\delta^{15}N$ discrimination for every 1°C increase in temperature for European sea bass *Dicentrarchus labrax* (L. 1758). The effect found by Power et al. (2003) was -0.16 ‰ $\delta^{15}N$ discrimination for every 1°C increase in temperature for *Daphnia magna* Straus, 1820. The lack of a significant effect of temperature on $\delta^{13}C$ discrimination may be due to temperature only influencing $\delta^{13}C$ of *Artemia*-fed fish as mentioned above. Barnes et al. (2007) found an effect of temperature on $\delta^{13}C$ discrimination, however, this was accounted for by lipid content of tissues, and C:N ratios of their fish were higher than ours (> 4). It is not possible to draw a numerical conclusion on the magnitude of temperature effects on isotopic discrimination from this experiment because not all groups were in equilibrium with their diets after 85 days. It can be said, however, that the effect of temperature on $\delta^{15}N$ discrimination is less than -0.14 ‰ per 1°C increase in temperature and that there is no effect on $\delta^{13}C$ discrimination.

There was an effect of diet on isotope discrimination, however, the variation in discrimination did not show a linear relationship with feed mixtures but may reflect complex dietary effects. Discrimination of $\delta^{15}N$ was greater for fish fed high proportions of chicken (50, 75, and 100 %) than fish fed pure *Artemia*. The concentration of nitrogen, and probably protein, was higher in diets

with greater chicken content therefore fish may have been catabolising more proteins so that fish tissue was enriched in ^{15}N (Gaye-Siessegger et al., 2004a; Tsahar et al., 2008). However, fish fed the 25:75 mixture had the greatest $\delta^{15}N$ discrimination, although dietary % N was lower. This may be due to fish experiencing protein deficiency in their diet and recycling nitrogen within their bodies with some catabolism of proteins so that ^{15}N is more enriched (Gannes et al., 1998 and references therein; Gaye-Siessegger et al., 2004a). Fish fed high proportions of *Artemia* were in the worst condition and had the lowest C:N ratios, indicating that they were poorly nourished compared to fish fed high proportions of chicken. However, this does not explain the *Artemia*-fed fish having lower $\delta^{15}N$ discrimination; in fact if fish were in a poorer condition then their $\delta^{15}N$ discrimination should have gone up, as it did for fish fed the 25:75 mix. It could be that fish fed pure *Artemia* were sparing their protein such that carbohydrates were being used for energy and proteins were being conserved for growth only (Shiau and Peng, 1993; Kelly and Martínez del Rio, 2010), leading to a lower $\delta^{15}N$ discrimination. The low C:N ratios of *Artemia*-fed fish support this idea, as it appears that fish fed pure *Artemia* were not storing lipids.

Discrimination of $\delta^{13}C$ was less than 1.16 ‰ for experimental diets and there was no significant difference among fish fed any amount of chicken in their diet. Unfortunately, $\delta^{13}C$ of fish fed pure *Artemia* or pure chicken was still changing after 85 days rearing, limiting the conclusions that can be made regarding $\delta^{13}C$ discrimination. The $\delta^{13}C$ discrimination of worm fed fish, which were reared for 116 days, was 1.95 ‰ or approximately double that of fish fed pure chicken. To further confuse the issue, $\delta^{13}C$ of worms and chicken were similar, although worms had 3.69 % less nitrogen than chicken and 1.68 % more

carbon. It could be that there is an interaction occurring between carbon and nitrogen elemental concentration such that fish fed chicken with their higher dietary nitrogen, and consequently protein, catabolise more protein for energy. Catabolising protein allows fish fed chicken to store more lipids and decrease their overall $\delta^{13}C$ signature compared to worm-fed fish. Worm-fed fish may have catabolised less protein for energy and more carbohydrates and have less carbohydrates and lipids stored, leading to greater $\delta^{13}C$ discrimination. Increasing C:N ratios of fish fed increasing proportions of chicken supports the idea that fish fed pure chicken stored more lipids, leading to a lower $\delta^{13}C$. Research into enzyme activity may help unravel variation in isotopic discrimination by indicating which metabolic processes are dominating, and therefore improve dietary back-calculation (Gaye-Siessegger et al., 2005). Until such data are thoroughly tested researchers may need to incorporate more uncertainty regarding $\delta^{13}C$ discrimination to reduce the likelihood of making erroneous conclusions of proportional source estimations.

Elemental concentration

The linear relationships between the isotopic signatures of fish tissue and the elemental concentration of the diet further support the importance of elemental concentration of diet in determining the isotopic signature of consumers. The discrimination factors for diets did not show a linear relationship with proportion of feed or elemental concentration, showing that discrimination factors could not be predicted by diet mixing. However, the accuracy of calculated isotopic signatures for fish tissue using the concentration dependent mixing model showed how important elemental concentration is and that it may be a primary determinant of isotopic signatures of consumers. This supports previous work

where elemental concentration of diet was a primary factor in determining isotopic signatures of consumers (Pearson et al., 2003; Mirón et al., 2006).

Field study implications

The aim of this study was to test and quantify the effects of environmental variability in ambient temperature and elemental concentration of diet sources on isotope discrimination and incorporation rates of $\delta^{15}N$ and $\delta^{13}C$ in *A. forsteri*. Temperature affected isotopic discrimination of $\delta^{15}N$ by less than 0.14 ‰ per 1°C. Although this does not seem to be a large effect size, water temperatures can vary by 10°C or more between summer and winter (e.g. Jones et al., 1996). If temperature effects on $\delta^{15}N$ discrimination are ignored when back-calculating diets erroneous estimates of source contributions may be made, particularly if comparing summer and winter diets. The best estimate of discrimination for $\delta^{15}N$ by *A. forsteri* is 5.19 ± 0.74 ‰ at 24°C (asymptote of chicken fed fish at 24°C minus chicken $\delta^{15}N$ ± 1SD). This value is very similar to that found for *Acanthopagrus butcheri* (5.07 ± 0.66 (1SD) ‰) (Chapter 2, Bloomfield et al., 2011), another omnivorous fish, and it is within a range of estimates for other organisms (Adams and Sterner, 2000; Gaston and Suthers, 2004; Connolly et al., 2005a; Mill et al., 2007). However, this value is higher than the 3.4 ‰ recommended by Post (2002), re-enforcing the need to experimentally quantify discrimination factors for study species whenever possible (Gannes et al., 1997; Martínez del Rio et al., 2009). It is recommended to use 5.19 ± 0.74 (1SD) ‰ at 24°C for $\delta^{15}N$ discrimination by *A. forsteri* in field settings and to account for temperature effects of ambient water by adjusting the discrimination factor by increasing it by 0.14 ‰ per 1°C less than 24°C.

The results for $\delta^{13}C$ discrimination largely agree with that of Post (2002), who recommended applying a 0-1 ‰ discrimination. The estimates of $\delta^{13}C$ discrimination from experimental diets were all below 1.16 ‰ and decreasing. However, the discrimination found for fish fed worms was comparatively large, 1.96 ‰. This result is less than other $\delta^{13}C$ discrimination factors reported in the literature with $\delta^{13}C$ discrimination being recorded as large as 3.7 ‰ for Australian mado *Atypichthys strigatus* (Günther, 1860) (Gaston and Suthers, 2004). Therefore, to include uncertainty in $\delta^{13}C$ discrimination it is recommended to use 1.15 ± 0.67 ‰ (grand mean of fish fed chicken or worms at all temperatures ± SD) for *A. forsteri*. There was no effect of temperature therefore discrimination of $\delta^{13}C$ does not need to be adjusted for temperature differences.

Isotope incorporation rates can be extremely useful for field studies to determine when an animal has arrived in a new habitat that differs in basal isotopic signatures from its previous habitat by interpolating the isotopic data and the rate of change (Herzka et al., 2002). It was found that temperature affected isotopic incorporation rates, however it was only possible to quantify incorporation rates adequately for $\delta^{15}N$ of fish fed chicken at 24°C. Therefore the best estimate of time over which isotopes reflect dietary composition is approximately 54.4 days during summer (at 24°C). Isotopic incorporation rates are known to be affected by other factors as well as temperature; including age, or body size, and ration intake (Herzka and Holt, 2000; Trueman et al., 2005; Carleton and Martínez del Rio, 2010). Therefore these results are likely only applicable to juvenile *A. forsteri* less than 4.5 g or 65 mm SL. More research into how to quantify or adjust for factors that may affect incorporation rates and

isotopic discrimination that cannot directly be measured (such as condition or ration intake) is needed to improve outcomes of field studies.

These results clearly show that elemental concentration should be accounted for in mixing models and that Phillips and Koch‟s (2002) concentration dependent mixing model holds up well when tested against experimental data. However, the regressions of fish muscle isotopes against elemental concentration of diet may not be widely applicable to other situations. The concentration of nitrogen was higher in chicken than in *Artemia*, however the $\delta^{15}N$ was higher in *Artemia* than in chicken and this would more likely be reversed in nature. The reason for the reversal of trends in nitrogen isotopes and elemental concentration in nature is that animal tissue usually has a higher nitrogen concentration than plant matter and animal tissue usually increases in $\delta^{15}N$ up a food web. Therefore animal tissue $\delta^{15}N$ should be positively correlated with concentration of nitrogen in food sources. The negative correlation found here between carbon concentration in food and $\delta^{13}C$ of fish muscle may be applicable to other situations. Animals eating a diet higher in carbon concentration are more likely to store lipids (Gaye-Siessegger et al., 2005) and lipids are known to be depleted in ^{13}C, leading to more negative $\delta^{13}C$ values of tissues. Therefore a negative relationship between carbon concentration of diet and $\delta^{13}C$ of tissues is likely to be found in nature. However, further tests of this theory across a range of elemental concentrations and isotopic signatures are needed before it could be applied to field samples.

This study has shown the importance of using elemental concentration in mixing models and that diet and temperature affect isotopic discrimination and incorporation rates. However, there are still other factors that are known to affect

isotopic discrimination and incorporation rates that cannot easily be measured in the field (e.g. ration intake Barnes et al., 2007). Methods to enable researchers to account for factors that cannot directly be measured need to be further developed to enable the improvement of isotope field studies, such as using lipogenic enzyme activity to adjust for lipid intake (Gaye-Siessegger et al., 2005). It is suggested that future research focus on developing methods to improve isotopic discrimination estimates by analysing key enzyme activity simultaneously with isotopic signatures.

Acknowledgments

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Chapter Four: Stable isotopes allude to separate ecological niches of two omnivorous, estuarine fishes

South West River mouth, Kangaroo Island, October 2008.

Chapter 4 Preamble

This chapter is a co-authored paper, with intention to publish in a peer-reviewed scientific journal. Bronwyn Gillanders and Travis Elsdon are co-authors, therefore it is written in plural.

In this chapter I conceived the sampling design and researched the techniques used. I received intellectual input on field sampling and funding assistance from Bronwyn Gillanders and Travis Elsdon. I collected the samples, with assistance from others (see acknowledgments), and prepared all the samples for analyses. I did all of the statistical analyses and wrote the manuscript with input from co-authors.

I certify that the statement of contribution is accurate

Alexandra Bloomfield (Candidate)

I herby certify that the statement of contribution is accurate and I give permission for the inclusion of the paper in the thesis

Professor Bronwyn Gillanders Dr Travis Elsdon

Stable isotopes allude to separate ecological niches of two omnivorous, estuarine fishes

Abstract

Stable isotopes were used to investigate ecological niches, as isotopes of nitrogen and carbon reflect environmental and dietary attributes of niches. We investigated the isotopic niches of two common, omnivorous fishes that frequently inhabit estuarine areas together: *Acanthopagrus butcheri* and *Aldrichetta forsteri*. We further studied the autotrophic sources that these fishes relied on. Although the fishes relied on similar autotrophic sources in some estuaries, they were feeding at different trophic levels. Isotopic niches of *A. butcheri* and *A. forsteri* did not overlap in any of the estuaries sampled and this is likely due to interspecific competition, potentially causing habitat partitioning. Our results support the theory that no two species can occupy the same ecological niche. Isotopic niches show potential as a tool for a better understanding of ecological niches.

Introduction

Ecological niches are hard to define and difficult to measure, yet they are central to ecological theory. A niche was defined by Hutchinson (1957) as an abstract set of points in multi-dimensional space that define the boundaries within which a species lives. The multiple dimensions or axes represent environmental variables and therefore the set of points define the environmental boundaries in which a species persists. Ecologists have struggled with measuring niches due to the large number of environmental variables that can make up the multiple dimensions. Hutchinson (1978) later made an important distinction between two sorts of

dimensions: scenopoetic and bionomic. Scenopoetic dimensions set the stage of physical and chemical variables in the environment within which an animal lives. Bionomic dimensions relate to resources that an animal uses to sustain its existence. Newsome et al. (2007) suggested that stable isotopes in animals, particularly $\delta^{13}C$ and $\delta^{15}N$, can be used to investigate ecological niches as an animal"s chemical composition is a result of what it has been eating (bionomic) within its environment (scenopoetic).

Stable isotopes of carbon ($\delta^{13}C$) vary among primary producers at the base of the food web due to varying chemical reactions and physical processes that change the ratios of ^{13}C to ^{12}C in molecules (Marshall et al., 2007). There are stark differences in the chemical reactions and physical processes of photosynthesis between C3, C4, and CAM plants that have allowed ecologists and paleoecologists to investigate the relative proportions of these plants in diets of modern and historical animals (see Schwarcz, 1991; Koch, 2007). The $\delta^{13}C$ of algae can vary between benthic and pelagic communities due to boundary effects in the benthos (Fry, 1996; Jennings et al., 1997; Smit, 2001). Planktonic $\delta^{13}C$ can vary spatially due to temperature effects (Wong and Sackett, 1978), causing variation in $\delta^{13}C$ between inshore and offshore food webs (Fry, 1983; Hobson, 1999) and at the larger scale of latitudes (Rau et al., 1982; Johnston and Kennedy, 1998). Carbon isotopic signatures are also known to vary along estuaries with salinity gradients (e.g. Deegan and Garritt, 1997; Leakey et al., 2008; Hoffman et al., 2010). Therefore $\delta^{13}C$ can reflect dietary and environmental aspects of niches.

The heavy stable isotope of nitrogen (^{15}N) is preferentially retained in animals over the lighter isotope (^{14}N) so that animal tissue is enriched in ^{15}N compared to its diet (DeNiro and Epstein, 1981). Enrichment in ^{15}N occurs every

time an animal eats, therefore $\delta^{15}N$ of animal tissue increases as trophic level increases (Minagawa and Wada, 1984). The increase in $\delta^{15}N$ with increasing trophic level has been used to determine trophic position of animals within food webs (e.g. Davenport and Bax, 2002; Jaschinski et al., 2008a) and therefore gives us dietary information. Stable isotopes of nitrogen ($\delta^{15}N$) are also influenced by anthropogenic activities (Heaton, 1986) and have been used to trace sewage inputs through aquatic food webs (e.g. Gaston et al., 2004; Hadwen and Arthington, 2007). Plankton $\delta^{15}N$ signatures are also known to vary spatially (Gruber and Sarmiento, 1997; Popp et al., 2007), therefore $\delta^{15}N$ can also provide environmental information.

It was once thought that no two species could occupy similar ecological niches due to competition for resources (reviewed by Hutchinson, 1978) and this is implicit in Hutchinson"s niche definition (1957). However, niches can overlap to a certain degree when organisms share habitat or occur in similar environmental conditions (e.g. Aguilera and Navarrete, 2011; Silva-Pereira et al., 2011). Two fishes that commonly occur in estuaries in southern Australia are black bream (*Acanthopagrus butcheri*) and yellow-eye mullet (*Aldrichetta forsteri*) (Potter and Hyndes, 1994; Jones et al., 1996; Norriss et al., 2002). Black bream and yellow-eye mullet are both omnivorous (Sarre et al., 2000; Platell et al., 2006), euryhaline fish with a wide distribution across southern Australia (Kailola et al., 1993). They have both been described as opportunistic in their feeding behaviour, suggesting that they eat whatever is readily available (Sarre et al., 2000; Platell et al., 2006). Despite their shared environmental requirements and opportunistic feeding behaviour, black bream and yellow-eye mullet persist together in estuaries as two of the most abundant species. Therefore the ecological

niches of black bream and yellow-eye mullet should be largely different, but may overlap.

Using stable isotopes we aimed to investigate the ecological niches of black bream and yellow-eye mullet and their overlap. We calculated metrics of stable isotopes: 1) the range of $\delta^{15}N$ of a species, and 2) niche width, or area of isotopic variability, per species (Layman et al., 2007; Quevedo et al., 2009). The range of $\delta^{15}N$ of a consumer tells us the range of trophic levels at which the species feeds (i.e. if it is strictly herbivorous or omnivorous). Niche width, or area of isotopic variability, has previously been quantified by total area of the convex hull. Total area of the convex hull refers to the area enclosed within lines drawn between the extreme most values of isotopes per species (Layman et al., 2007). The drawback to these metrics is that they are sample size dependent and can increase with increasing sample size (Jackson et al., 2011). This is particularly problematic for total area of the convex hull as it has been used for niche width analyses among species and populations with varying numbers of samples (e.g. Darimont et al., 2009; Olsson et al., 2009). Jackson et al. (2011) recently described a new method for estimating isotopic niche width, which is not as sensitive to sample size. We analysed the $\delta^{15}N$ range and isotopic niche width using the methods of Jackson et al. (2011) for black bream and yellow-eye mullet in four estuaries.

We aimed to determine the autotrophic sources that black bream and yellow-eye mullet rely on in the estuaries sampled. Black bream and yellow-eye mullet both feed opportunistically and are likely to consume the most abundant or readily available prey. Therefore the diet of these fishes should reflect the autotrophic sources that are contributing the most nutrients and energy to the

ecosystem. However, if black bream and yellow-eye mullet occupy different ecological niches, they may also rely on different autotrophic sources with varying degrees of overlap.

Methods

Estuaries

Four estuaries (Chapman, Harriet, Onkaparinga, and South West River) in South Australia were sampled for black bream, yellow-eye mullet, and autotrophic sources (see Fig. 4.1). The Onkaparinga estuary was the largest sampled in this study and was sampled at two locations 6.5 km apart that varied in salinity (see Table 4.1). The Onkaparinga River was connected to the sea at the time of sampling (see Table 4.1), and the tidal influence can extend 10.5 km inland (Department for Environment and Heritage, 2007a). The Onkaparinga estuary has a main river channel, with tidal flats nearer the sea and saltmarshes fringing the channel. The upper Onkaparinga has some riparian vegetation of trees and shrubs up to the river bank where the river channel is narrow (< 20 m) and can be shallow, although there are some deep holes with fallen branches and rocky sections, creating complex habitat structure. The lower Onkaparinga site had a broader channel (approximately 70 m wide), which was devoid of subtidal structure except for small patches of seagrass. There were waste water sludge lagoons adjacent to the Onkaparinga River at the time of sampling, very close to the lower site, that were known to flood occasionally and spill over into the river. These sludge lagoons were decommissioned not long after field work for this research was completed, however they may still be leaching.

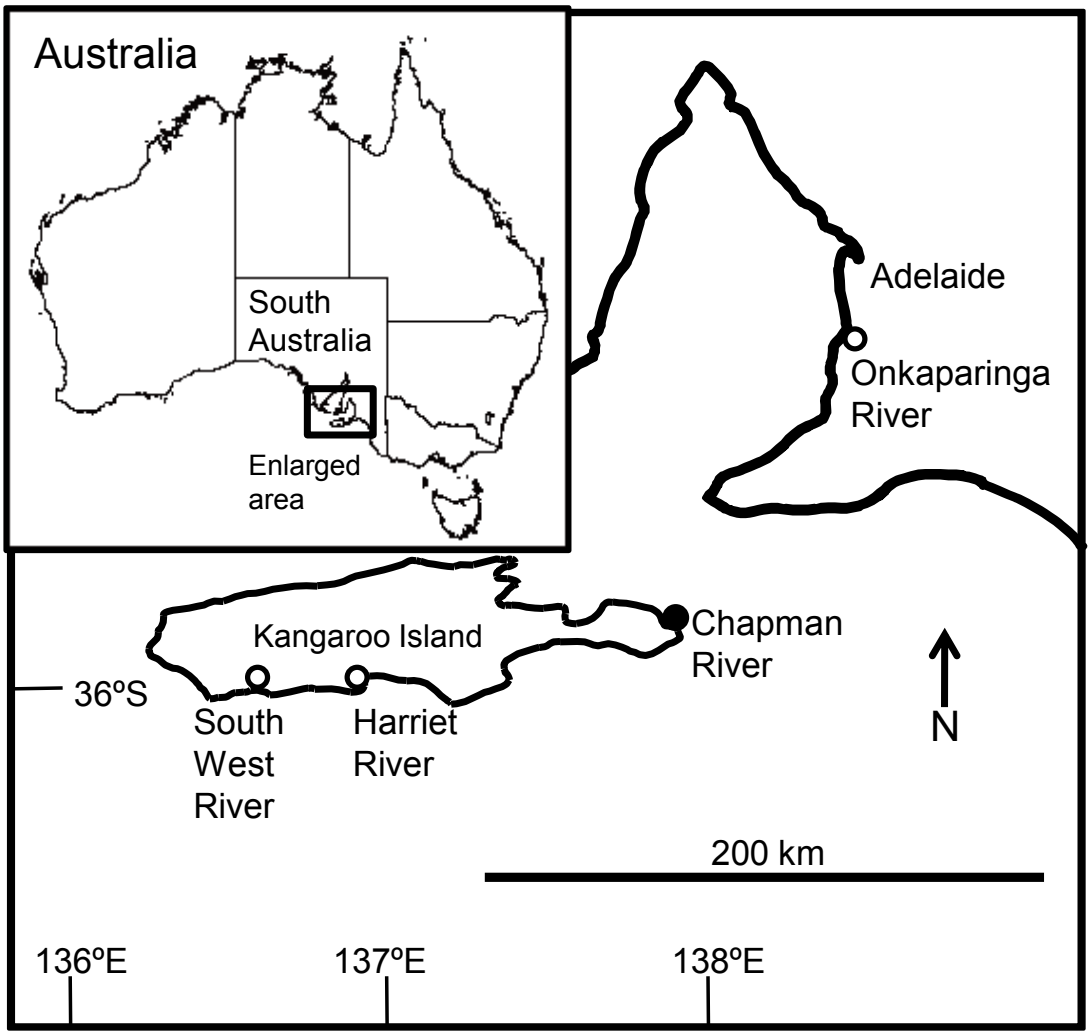

Figure 4.1 Map showing the location of estuaries sampled in South Australia, Australia. Empty circles = open estuaries; filled circle = closed estuary at time of sampling.

Table 4.1 Estuarine and catchment information: size, estuarine type, salinity, temperature and status of estuarine opening when black bream (BB) and yellow-eye mullet (YEM) were collected.

Estuary and site	Length (km)	Channel area (ha)	Catchment area (km^2)	Estuarine type	Salinity (‰)	Temperature (°C)	Status of estuarine connectivity with the sea
Chapman	2.3[2]	6.5[2]	73[1]	Wave dominated[1]	16	18 (BB) 21 (YEM)	Closed
Harriet	1.7[2]	7.8[2]	152[1]	Wave dominated[1]	16 (BB) 6 (YEM)	17 (BB) 20 (YEM)	Open, small connection
Onkaparinga lower	11.0[1]	49.3[2]	554[1]	River dominated, with wave-dominated delta[1]	32	18	Open, large opening, tidally influenced
Onkaparinga upstream					13 (BB) 14 (YEM)	22.5 (BB) 18 (YEM)	
South West	1.6[2]	2.8[2]	155[1]	Wave dominated[1]	3	20	Open, small connection

[1](Department for Environment and Heritage, 2007a, b)

[2](Department of Environment and Natural Resources, 2011)

Chapman River, on Kangaroo Island, is smaller than the Onkaparinga (see Table 4.1) and is surrounded by a conservation park, with riparian vegetation along most of its length. The channel was relatively wide (> 30 m) near the mouth where it was sampled and can be deep (> 2 m), with subtidal structure created by fallen trees and vegetation. There were also shallower sandy sections where yellow-eye mullet were caught. Chapman River seasonally closes to the ocean over summer and it was closed at the time of sampling (see Table 4.1). Harriet and South West Rivers, also on Kangaroo Island, seasonally close to the ocean too; however both were open at the time of sampling (see Table 4.1). Harriet River was the widest estuary sampled with the channel being up to 100 m wide and fringed by riparian vegetation along much of its length, although this vegetation band was quite narrow (< 10 m). The South West River was the shortest estuary sampled and is quite narrow along most of its length (< 25 m) with a shallow pool near the mouth (up to 80 m wide). There was riparian vegetation fringing much of the river as it flowed through a National Park. South West River is also higher above sea level compared to the other estuaries sampled and is known to remain fresher for longer and is generally shallower.

Fish and autotroph collection

Fish and autotroph samples were collected in October 2008 from the four estuaries. Fish were collected by seine net (5-20 m long; 19 mm mesh size) or handline within estuaries. Fish were collected from sites within estuaries where they had previously been sampled as part of other research projects. A minimum of five fish per species per estuary/site were caught and euthanized in an ice water slurry. Fish were kept on ice in the field and frozen on return to the laboratory.

Autotroph samples were collected at the same site and time as fish. Plant samples were collected based on a visual assessment of whether plants were able to directly contribute organic matter to the estuary. Plants were able to directly contribute organic matter to estuarine waters if they grew within the immediate catchment, including within the water body itself. Plants that were able to contribute organic matter to the estuary had samples of leaves or photosynthetic material collected. Triplicate samples per plant species were collected, with individual plants used as replicates where possible. Macroalgae were collected from within seine nets or if found on the shore within estuaries. It was not possible to collect triplicate samples of macroalga species due to the nature of the estuaries sampled, with most estuaries being small with minimal hard substrata for attachment of macroalgae, however samples were analysed in duplicate whenever possible. Terrestrial and aquatic plants, including macroalgae, were identified to lowest taxonomic resolution possible (usually species, but occasionally genus with the exception of saltmarshes). Saltmarshes could only be identified to subfamily (Salicornioideae), as no flowers were present at the time of sampling. Epiphytes and periphyton were collected from plants and other macroalgae, rocks and other hard substrata, and were usually collected as one sample to be analysed in triplicate for stable isotopes. Plant samples were bagged individually and put on ice initially, being frozen later that day.

Particulate organic matter (POM) samples were collected using a plankton-net with 25 μm mesh, ring size 25 cm in diameter, with a cup attached to the end to collect the sample. The net was pulled through 20 m of water (similar to Hadwen et al., 2007) being careful not to disturb sand and mud from the bottom. Three samples were taken over separate 20 m lengths of the estuary

where possible. The net was rinsed so that as much organic matter as possible was washed into the cup. The water in the cup was collected into an opaque container and put on ice until the POM (>25 μm) could be vacuum filtered onto pre-combusted GF/F filter paper later that day.

Microphytobenthos was collected in triplicate when sand or mud was readily available (at all sites except for the upper Onkaparinga where it was very rocky). The top few centimetres of sediment were collected over a 1 m^2 area into an opaque container and kept below 4°C until processing. Water temperature and salinity were measured at each site using a YSI sonde (model 556 MPS).

Sample preparation

Fish were defrosted, weighed (mass, g) and measured (total length, mm) before having dorsal muscle samples taken. Fish muscle samples were freeze-dried before being ground to a powder using an agate mortar and pestle. Plant samples were rinsed with ultrapure water. Plant and filtered POM samples were oven dried at 80°C for 48 hrs. Plant samples were ground using one of three methods (ball mill, coffee grinder or agate mortar and pestle) depending on their volume and fibrous nature. Oven-dried POM was scraped off filters and acidified with 1M HCl in glass vials. Acid was added drop by drop until effervescence ceased (Carabel et al., 2006), after which samples were allowed to dry under a fume hood for several days. Acidified POM was ground with an agate mortar and pestle. Macroalgae samples were not coralline and therefore did not require acidification.

Microphytobenthos (MPB) was sieved sequentially through 1 mm, 500 μm, and 53 μm sieves into a bucket and allowed to stand for several days in a dark cool room until the water was clear (Melville and Connolly, 2003). The supernatant was poured off and the remaining sample was resuspended and mixed

with Ludox TM-50 (colloidal silica) to a density of 1.27 g.mL^{-1} (Hamilton et al., 2005). The mixture was centrifuged at 10 g for 10 mins such that diatoms were suspended in the top layer and detritus was compacted to the bottom. The diatom fraction was extracted and rinsed onto 5 μm fabric. It was then oven-dried for 48 hrs at 80°C. Dried samples were ground with an agate mortar and pestle. Lipids were not extracted from any samples. All samples were weighed into tin capsules for stable isotope analyses.

Stable isotope and elemental concentration analyses

Samples were analysed by a GV Isoprime Mass Spectrometer coupled to a Eurovector elemental analyser 3000 at Griffith University, Queensland, Australia. International and internal laboratory standards (N: Ambient Air, IAEA-305a, C: ANU Sucrose, Acetanilide, Working standards: 'Prawn', „Flour") were run in parallel with fish and plant samples to enable calibration of results. Average precision of the mass spectrometer was 0.06 ‰ for $\delta^{13}C$ and 0.23 ‰ for $\delta^{15}N$ (1SD), with average accuracy of 0.01 ‰ of $\delta^{13}C$ and 0.10 ‰ for $\delta^{15}N$ (average deviation from known value). Average precision of the elemental analyser was 0.62 % for carbon and 0.27 % for nitrogen (1 SD), with average accuracy of 0.24 % for carbon and 0.05 % for nitrogen (average deviation from theoretical value).

Data analysis

Fish condition was calculated using Fulton"s K. Size (length and mass) and condition of black bream and yellow-eye mullet among estuaries/sites were compared in one-way ANOVAs, as size and condition of fish can influence isotopic signatures (Davenport and Bax, 2002; Melville and Connolly, 2003;

Gaye-Siessegger et al., 2007). When significant differences were found, post-hoc comparisons were done using Student-Newman-Keuls (SNK) tests.

Stable isotope values of carbon ($\delta^{13}C$) were not mathematically corrected for lipid content. Post et al. (2007) recommends correcting for lipids in fish samples when C:N ratios are larger than 3.5 and all fish samples had C:N ratios of 3.5 or less (not reported here). It is not logical to correct autotroph samples for lipids as fish, and other potential prey items, ingest whole items without discriminating against lipids, which would be digested and assimilated into fish tissue.

Regression analyses were done to see if there were significant relationships between isotopes and fish sizes, separating the Onkaparinga from other estuaries for $\delta^{15}N$ analyses due to strong ^{15}N enrichment. Isotopic composition ($\delta^{15}N$ and $\delta^{13}C$) of black bream and yellow-eye mullet across estuaries/sites sampled were analysed in a two-factor permutational multivariate analysis of variance (PERMANOVA, Anderson, 2001), with fish species and estuary as fixed factors, to see if isotopic signatures varied between fishes and among estuaries. Data were not transformed, resembled using Euclidean similarity distance matrices, and permutations were unrestricted.

The range of $\delta^{15}N$ values was calculated for each fish species per estuary. Isotopic niche width was calculated using the SIAR package (version 4.1 Parnell and Jackson, 2011) in R (R Development Core Team, 2011). Standard ellipse area, corrected for small sample size (SEAc), was calculated for each species in each estuary (Jackson et al., 2011). SEAc was analysed using ANOVA to test if the isotopic niche width varied between fishes with estuaries as replicates. The Bayesian standard ellipse area (SEAb) was calculated for each fish species per

estuary to obtain a better estimate of isotopic niche width and to estimate which fish species is more likely to have a larger isotopic niche (Jackson et al., 2011). Isotopic niche overlap was also calculated using the SIAR function "overlap", with step = 1.

Proportional contributions of autotrophic sources to fish diets were estimated using SIAR (version 4.1 Parnell and Jackson, 2011). The "siarmcmcdirichletv4" function was used. This function runs a Markov Chain Monte Carlo (MCMC) method on stable isotope data with a Gaussian likelihood assumed for target values and a Dirichlet-distributed prior on the means of sources (Parnell et al., 2010). This function calculates feasible solutions of proportional source contributions, similar to IsoSource outputs (Phillips and Gregg, 2003), however it uses the uncertainty associated with data inputs in the model. It incorporates uncertainty in source (autotroph) and „target" (fish tissue) isotopic signatures as well as uncertainty in discrimination corrections. It is important to incorporate uncertainty in isotopic discrimination (the difference in isotope ratios between a source and consumer), as discrimination is known to vary among and within organisms (Chapters 2 and 3, DeNiro and Epstein, 1978, 1981; Elsdon et al., 2010; Bloomfield et al., 2011) and with environmental factors, such as temperature (Chapters 2 and 3, Bosley et al., 2002; Barnes et al., 2007; Bloomfield et al., 2011). SIAR also allows for the use of elemental concentration of sources in the mixing model, which is known to influence isotopic signatures of animal tissue (Chapter 3, Pearson et al., 2003; Mirón et al., 2006)[5]. We used SIAR

[5] We acknowledge that MPB and POM are concentrated samples and that their elemental concentration does not reflect that consumed in nature. However the elemental concentration of MPB was very small (C% = 0.36 ± 0.38 (mean ± 1SD), N% = 0.035 ± 0.036 (mean ± 1SD)). The elemental concentration of POM was larger, and much more variable (C% = 10 ± 6.7 (mean ± 1SD); N% = 1.2 ± 1.1 (mean ±1SD)) however this was still below the carbon concentration of most other sources sampled (grand mean: C% = 37 ± 9.1 (mean ± 1SD)) although the nitrogen concentration was similar (grand mean: N% = 1.5 ± 0.86 (mean ± 1SD)).

separately for black bream and yellow-eye mullet for each estuary/site, using 400,000 MCMC iterations with 200,000 burn in and thinning by 100 (see Parnell et al., 2010). We report modes with 95 % confidence intervals for variance and we used modes for further data analyses, as recommended by Parnell et al. (2010).

We used different discrimination factors ($\Delta^{13}C$, $\Delta^{15}N$) for black bream and yellow-eye mullet. Black bream and yellow-eye mullet are omnivores, therefore they are likely to feed on both plant and animal matter and may receive nutrients from the same autotrophic source through both plants and animals. As omnivores black bream and yellow-eye mullet are in a trophic position in between herbivores (Trophic level (TL) = 1) and carnivores (TL = 2); approximately TL = 1.5. We used experimentally derived discrimination factors for black bream and yellow-eye mullet (Chapters 2 and 3, Bloomfield et al., 2011) and added half of the average discrimination factor across a range of species (Post, 2002) to account for unquantifiable discrimination by potential prey items. For black bream we used discrimination factor of $\Delta^{13}C = 3.50 \pm 0.73$ (mean ± 1SD) as found in Chapter 2 (Bloomfield et al., 2011) and added half of the average discrimination found by Post (2002) (0.39 ± 1.3 (1SD) /2 = 0.195 ± 1.3; we retained the variation of the full average as dividing the average by two does not improve the accuracy) to give a trophic enrichment factor equivalent to 1.5 trophic levels. On adding the two discrimination factors together we added the variance, as errors were presumed to be additive, to give a $\Delta^{13}C = 3.70 \pm 2.03$ (mean ± 1SD). We did similar additions for $\Delta^{15}N$ for black bream using the discrimination found in Chapter 2 (Bloomfield et al., 2011) to give $\Delta^{15}N = 6.77 \pm 1.64$ (mean ± 1SD).

Discrimination of $\delta^{15}N$ in yellow-eye mullet can be affected by temperature (Chapter 3), therefore we adjusted $\Delta^{15}N$ to the temperature measured

on the day of collection and added half of the average discrimination factor found by Post (2002). We acknowledge that the temperature of the water that fish live in would have varied over the preceding time period that isotopic signatures of tissue were incorporated (approx. 54.4 days for yellow-eye mullet; Chapter 3). However, we did not quantify temperature variation prior to collection and the variance of the discrimination factor was already reasonably large. We adjusted $\Delta^{15}N$ by 0.14 ‰ per 1°C as per the findings in Chapter 3. This resulted in $\Delta^{15}N$ ranging from 7.31 (Chapman) to 7.73 (Onkaparinga) ± 1.72 ‰ (1SD). No affect of temperature was found on $\Delta^{13}C$ (Chapter 3), therefore no temperature adjustments were made and the recommended value of 1.15 ± 0.67 (1SD) (Chapter 3) was added to half of Post‟s average to derive $\Delta^{13}C = 1.35 \pm 1.97$ ‰, which was applied across all estuaries/sites for yellow-eye mullet in the SIAR analyses.

SIAR does not cope well with sources that are too similar in isotopic composition, as it cannot separate their contributions (Parnell et al., 2010; Bond and Diamond, 2011). Several species of marine macroalgae that were collected in the Harriet and South West Rivers were similar in isotopic composition so they were pooled to make one (Harriet) or two (South West River) autotrophic signatures (see Figs 4.3b & d). Some terrestrial shrubs and reeds (*Ficinia nodusa*, *Disticus disticus* and *Carpobrotus rossi*) in the South West River were also similar in isotopic composition and thus were pooled into a „shrubs‟ autotrophic source. Two grasses were also pooled in the South West River to give one isotopic signature.

There were two analyses where isotopes of yellow-eye mullet did not fit well within the mixing polygon for that estuary (the polygon including all sources from that estuary, accounting for variance (1SD) in isotopic signatures and

discrimination): in the Chapman River and at the Onkaparinga lower site. The analyses for yellow-eye mullet in the Chapman River required fish isotopes to be further trophically corrected such that yellow-eye mullet were feeding at a trophic level of two. To correct yellow-eye mullet isotopes for two trophic levels we added the full average of isotopic discrimination across a range of species, as found by Post (2002), to the experimentally derived and temperature corrected trophic discrimination for yellow-eye mullet (Chapter 3). In the Onkaparinga lower analysis, yellow-eye mullet isotopes were over corrected by applying 1.5 TL discrimination. Therefore we used the experimentally derived and temperature corrected trophic discrimination for yellow-eye mullet alone, without adding anything, such that fish were feeding at a trophic level of one.

To determine how similar autotroph relative importance was between black bream and yellow-eye mullet within an estuary, modes from SIAR outputs were used to determine Bray-Curtis similarity indices without transforming data.

Results

Fish size and condition

There was a significant difference in the size (length and mass) of fish among estuaries (black bream total length: $F_{4,24} = 6.06$, $p = 0.003$; mass: $F_{4,24} = 3.68$, $p = 0.03$; yellow-eye mullet total length: $F_{4,24} = 36.58$, $p = 0.001$; mass: $F_{4,24} = 25.80$, $p = 0.001$). Black bream caught in the upper reaches of the Onkaparinga were smaller (length and mass) than black bream caught in all other estuaries/sites (Figs 4.2a & b). Black bream caught in the South West River were significantly shorter than black bream caught in the Harriet River (Fig. 4.2b). There were no other significant differences in size among estuary/site pairs for

black bream. Yellow-eye mullet caught in the Harriet River were significantly larger (length and mass) than yellow-eye mullet caught in all other estuaries/sites (Figs 4.2a & b). Yellow-eye mullet caught in the South West and Chapman Rivers were similar in size (Figs 4.2a & b). Yellow-eye mullet caught in the Onkaparinga did not differ in size between upper and lower sites (Figs 4.2a & b). Yellow-eye mullet size differed significantly among all other pairs of estuaries/sites (Figs 4.2a & b). Despite the size differences among estuaries no significant relationships between fish size (length and mass) and $\delta^{13}C$ or $\delta^{15}N$ were found ($r^2 < 0.5$) for either species.

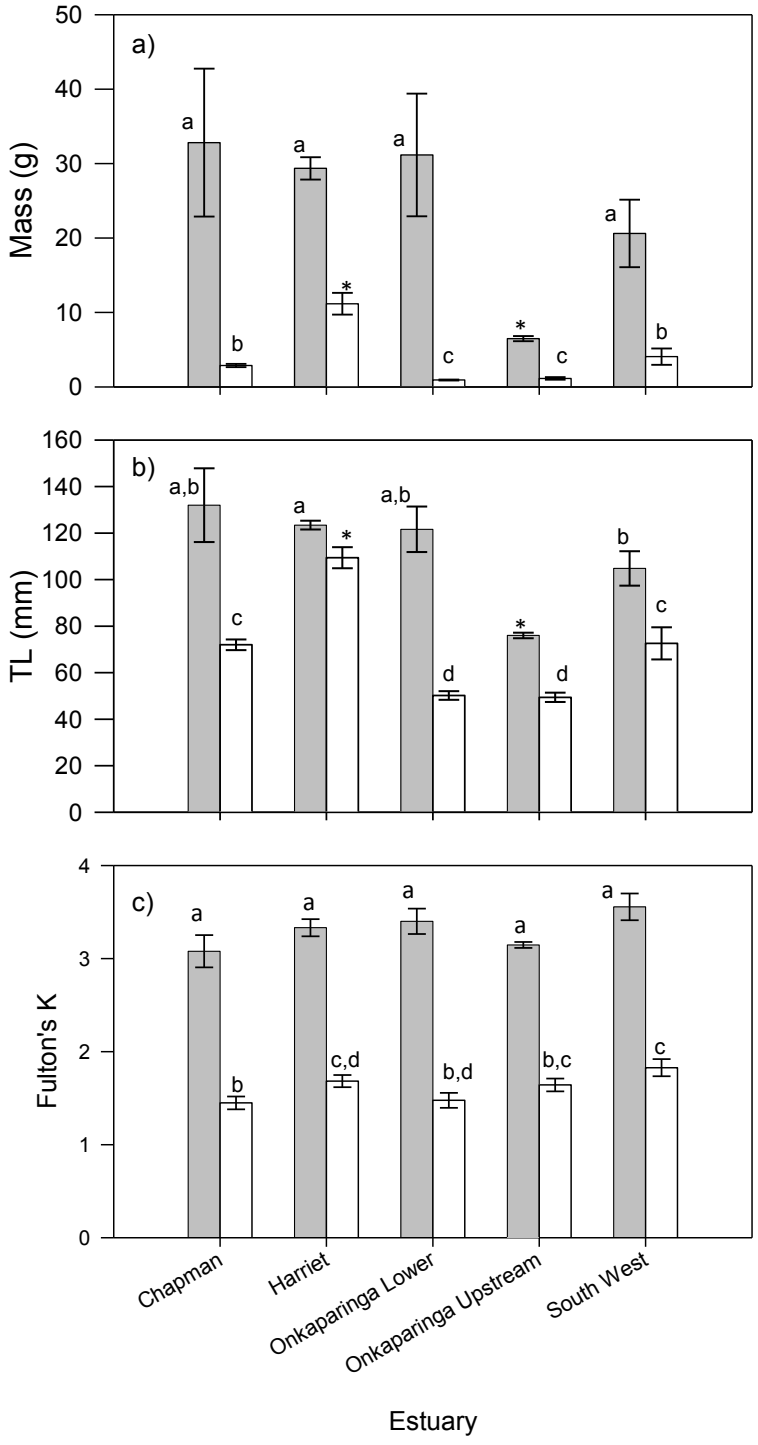

Figure 4.2 Fish a) mass (g), b) total length (TL, mm), and c) condition (Fulton‟s K) of black bream (grey bars) and yellow-eye mullet (white bars) collected in estuaries/sites. Note: letters denote groups of estuaries where fish sizes and condition were not significantly different per species; * denotes sites with significantly different fish sizes from all other sites per species.

The condition (Fulton‟s K) of black bream did not differ among estuaries/sites ($F_{4,24} = 2.21$, $p = 0.11$; Fig. 4.2c). The condition of yellow-eye mullet, however, did differ among estuaries/sites ($F_{4,24} = 4.18$, $p = 0.01$; Fig. 4.2c). Yellow-eye mullet caught in the South West River were in significantly better condition that yellow-eye mullet caught in the Chapman River and Onkaparinga lower site (Fig. 4.2c). Yellow-eye mullet caught in the Harriet River were in significantly better condition that yellow-eye mullet caught in the Chapman River. Fish of both species caught in the Chapman River were in the poorest condition and fish caught in the South West River were in the best condition.

Fish and autotroph isotopes

A significant interaction between fish species and estuary was found for isotopic composition ($\delta^{13}C$ and $\delta^{15}N$) of fishes, with significant differences in isotopic composition between black bream and yellow-eye mullet in each estuary (Table 4.2; Fig. 4.3). Yellow-eye mullet were enriched in carbon and nitrogen isotopes in the Chapman, Harriet, and South West Rivers relative to black bream. However, black bream were enriched in carbon and nitrogen isotopes compared to yellow-eye mullet in both upper and lower sites in the Onkaparinga River. Carbon and nitrogen isotopes of yellow-eye mullet caught in the Chapman, Harriet, and South West Rivers were similar. Isotopes of black bream were similar between Harriet and South West Rivers and between Chapman and South West Rivers. Carbon and nitrogen isotopes of fishes caught in the upper and lower Onkaparinga were significantly different from other estuaries, as well as between the two sites.

Table 4.2 Two factor permutational multivariate analysis of variance (PERMANOVA) of isotopes ($\delta^{13}C$ and $\delta^{15}N$) for black bream and yellow-eye mullet among estuaries. Bolded numbers indicate significant effects ($p < 0.05$).

Source of variation	df	MS	p
Estuary	4	77.75	**0.001**
Fish sp.	1	0.44	0.698
Estuary x Fish sp.	4	25.20	**0.001**
Residual	40	1.23	

There were two terrestrial plants (*Suaeda australis* and saltmarshes) that were sampled in both the upper and lower sites of the Onkaparinga River, as were epiphytes/periphyton and POM. The $\delta^{15}N$ of *S. australis*, saltmarshes, and epiphytes/periphyton was higher at the downstream site than the upstream site (Fig. 4.3c). In comparison, the $\delta^{15}N$ of POM was slightly lower at the downstream site. The $\delta^{13}C$ of *S. australis*, saltmarshes, epiphytes/periphyton, and POM were all higher at the downstream site (Fig. 4.3c). Black bream and yellow-eye mullet were more enriched in ^{13}C at the downstream site, however their $\delta^{15}N$ values did not change between the two sites sampled.

Isotopic niche

The average $\delta^{15}N$ range of black bream was 1.5 ± 0.8 (SD) ‰ with the largest range being found in the Chapman River (see Table 4.3). The average $\delta^{15}N$ range for yellow-eye mullet was smaller at 1.2 ± 0.3 ‰, conversely the smallest range was found in the Chapman River (see Table 4.3). The average isotopic niche width (standard ellipse area, small sample size corrected, SEAc) of black bream across estuaries sampled was 1.5 ± 0.8 (SD) $‰^2$ and the average for yellow-eye mullet was 1.9 ± 1.4 (SD) $‰^2$. The mean of the Bayesian estimate of ellipse area (SEAb) was larger for each estuary than the small sample size corrected ellipse area (SEAc) except for yellow-eye mullet in the Harriet River where SEAb was smaller (see Table 4.3). The isotopic niche width (measured by SEAc and SEAb) of black bream was larger than yellow-eye mullet in the Chapman, Onkaparinga lower and South West Rivers. The isotopic niche width (SEAc and SEAb) of yellow-eye mullet was larger than that of black bream in the Harriet River and the Onkaparinga upper site. However, there was no significant difference in standard ellipse area (SEAc) between black bream and yellow-eye mullet across estuaries ($F_{1,9} = 0.23$, $p = 0.66$). There was also no overlap between black bream and yellow-eye mullet isotopic niches in any of the estuaries/sites sampled ($p\_overlap < 0.001$).

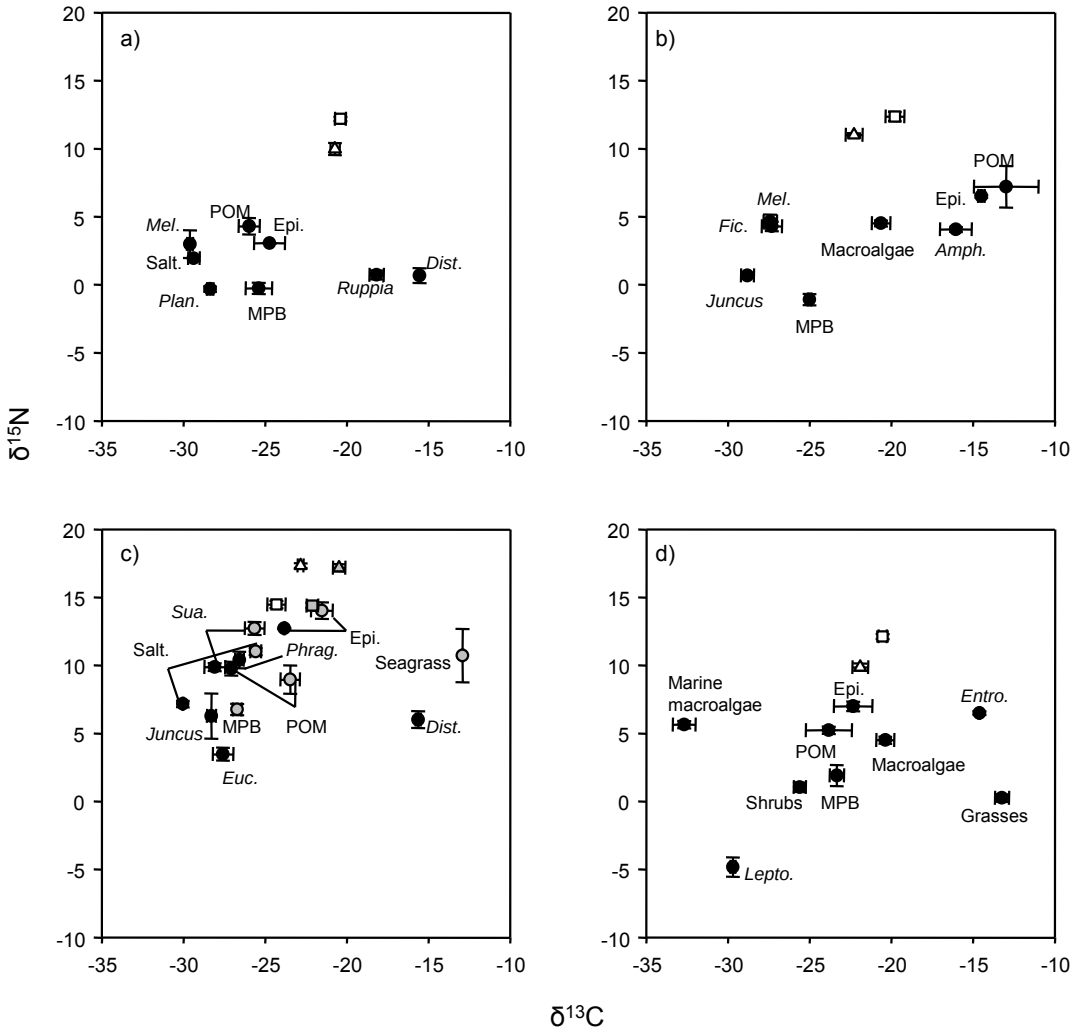
a)
b)
c)
d)
δ15N
δ13C
POM
Epi.
Mel.
Salt.
Plan.
MPB
Ruppia
Dist.
Fic.
Macroalgae
Amph.
Juncus
Sua.
Phrag.
Seagrass
Euc.
Marine macroalgae
Entro.
Shrubs
Grasses
Lepto.

Figure 4.3 Average $\delta^{15}N$ and $\delta^{13}C$ (‰ ± SE) of fish muscle and autotrophic samples from a) Chapman, b) Harriet, c) Onkaparinga and d) South West rivers. Δ = black bream; □ = yellow-eye mullet; grey fish samples are from the lower Onkaparinga; ● = autotroph; grey circles are from the lower Onkaparinga. *Amph*. = *Amphibolis antarctica*; *Dist*. = *Disticus disticus*; *Entro*. = *Enteromorpha* sp.; Epi. = epiphytes/periphyton; *Euc*. = *Eucalyptus* sp.; *Fic*. = *Ficinia nodusa*; Grasses = combined signature of two grasses; *Juncus* = *Juncus* sp.; *Lepto*. = *Leptospermum myrsinoides*; Marine macroaglae/ Macroalgae = combined signatures of several macroalga; *Mel*. = *Melaleuca halmaturorum*; MPB = microphytobenthos; *Phrag*. = *Phragmities australis*; *Plan*. = *Plantago coronopus*; POM = particulate organic matter; *Ruppia* = *Ruppia* sp.; Salt. = saltmarshes; Seagrass = combined signature of *Ruppia* sp. and *Zostera* sp.; Shrubs = combined signature of *F. nodusa, D.disticus* and *Carptrobrotus rossi*; *Sua*. = *Suaeda australis*.

Table 4.3 Isotopic niche data for black bream (BB) and yellow-eye mullet (YEM) in estuaries sampled in South Australia. $\delta^{15}N$ range is the difference between the smallest and largest values of $\delta^{15}N$ for each species per estuary; SEAc is the standard ellipse area corrected for small sample sizes; SEAb is the Bayesian estimate of the ellipse area calculated as per Jackson et al. (2011).

	$\delta^{15}N$ range (‰)		SEAc ($‰^2$)		SEAb (mean $‰^2$ ± SD)	
Estuary	BB	YEM	BB	YEM	BB	YEM
Chapman	2.8	0.7	2.8	0.9	3.5 ± 1.8	2.2 ± 1.1
Harriet	0.7	1.5	1.0	4.1	2.6 ± 1.4	4.0 ± 2.0
Onkaparinga lower	1.7	1.1	1.8	1.1	2.8 ± 1.4	2.4 ± 1.2
Onkaparinga upstream	1.0	1.5	0.7	2.3	1.9 ± 1.0	3.4 ±1.7
South West	1.4	1.0	1.5	1.0	2.9 ± 1.5	2.0 ± 1.0

Autotrophic sources

Chapman River

Black bream in the Chapman River appear to rely most heavily on POM as a source of nutrients, followed by epiphytes/periphyton, *Melaleuca halmaturorum*, MPB, *Disticus disticus*, and saltmarshes (Fig. 4.4a). *Plantago coronopus* and *Ruppia* sp. contributed very little nutrients to black bream diets.

As mentioned in the methods, the isotopic signatures of yellow-eye mullet in the Chapman River did not fit well within the mixing polygon of sources when trophically corrected at 1.5 TLs. Running SIAR with yellow-eye mullet at a trophic level of two gave a better modelled result, although results were similar to those obtained when using 1.5 trophic levels. Similar to black bream, yellow-eye mullet, had high proportions of nutrients coming from *D. disticus*, POM, epiphytes/periphyton, and MPB but with a much larger contribution from *Ruppia* sp. (Fig. 4.4a). The autotrophs that contributed very little to yellow-eye mullet dietary sources were *P. coronopus*, saltmarshes and *M. halmaturorum*.

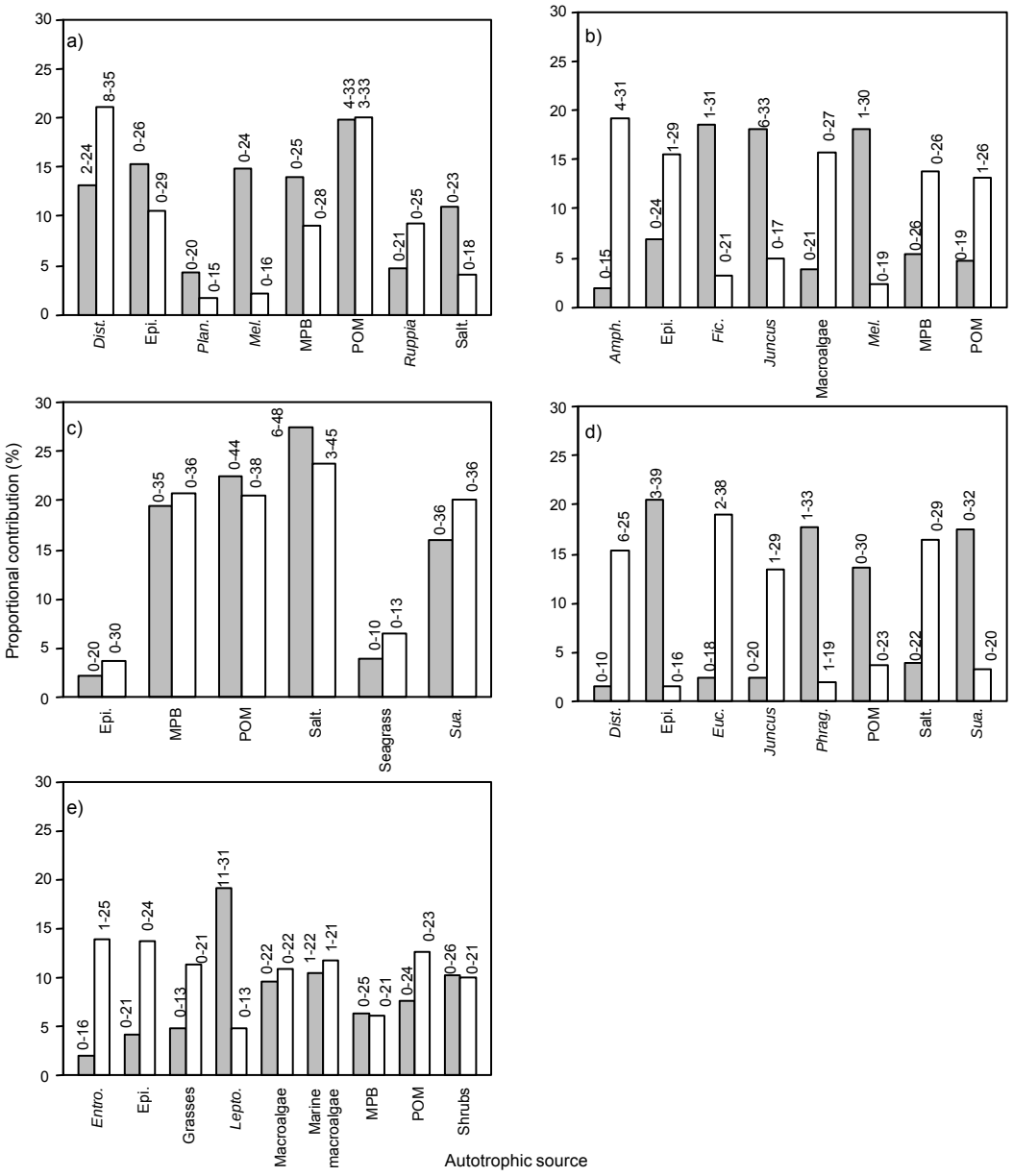
a)
2-24
8-35
0-26
0-29
0-20
0-15
0-24
0-16
0-25
0-28
4-33
3-33
0-21
0-25
0-23
0-18
Dist.
Epi.
Plan.
Mel.
MPB
POM
Ruppia
Salt.
b)
0-15
4-31
0-24
1-29
1-31
0-21
6-33
0-17
0-21
0-27
1-30
0-19
0-26
0-26
0-19
1-26
Amph.
Epi.
Fic.
Juncus
Macroalgae
Mel.
MPB
POM
c)
0-20
0-30
0-35
0-36
0-44
0-38
6-48
3-45
0-10
0-13
0-36
0-36
Epi.
MPB
POM
Salt.
Seagrass
Sua.
Proportional contribution (%)
d)
0-10
6-25
3-39
0-16
0-18
2-38
0-20
1-29
1-33
1-19
0-30
0-23
0-22
0-29
0-32
0-20
Dist.
Epi.
Euc.
Juncus
Phrag.
POM
Salt.
Sua.
e)
0-16
1-25
0-21
0-24
0-13
0-21
11-31
0-13
0-22
0-22
1-22
1-21
0-25
0-21
0-24
0-23
0-26
0-21
Entro.
Epi.
Grasses
Lepto.
Macroalgae
Marine macroalgae
MPB
POM
Shrubs
Autotrophic source

Figure 4.4 Proportional contributions of autotrophs to black bream (grey bars) and yellow-eye mullet (white bars) diets (mode (95 % confidence intervals shown above bars)) from SIAR analyses in the a) Chapman, b) Harriet, c) Onkaparinga lower, d) Onkaparinga upstream, and e) South West rivers. See Fig. 4.3 for autotrophic source groupings and abbreviations. Note: yellow-eye mullet results for the Chapman River are from analyses with fish at a trophic level of two and in the lower Onkaparinga yellow-eye mullet were analysed at a trophic level of one. All other results are for yellow-eye mullet and black bream analysed at a trophic level of 1.5 (see Results for further details).

Harriet River

In the Harriet River, reeds (*Juncus* sp. and *Ficinia nodusa*) and *M. halmaturorum* contributed relatively high amounts of nutrients to black bream diets (modes > 18 %) (Fig. 4.4b). Conversely epiphytes/periphyton, MPB, POM, macroalgae, and *Amphibolis antartica* all contributed very small amounts to black bream diets. Yellow-eye mullet diets showed the opposite pattern with low proportions of reeds (*Juncus* sp. and *F. nodusa*) and *M. halmaturorum*, but with high proportions of *A. antartica*, epiphytes/periphyton, MPB, POM, and macroalgae (Fig. 4.4b).

Onkaparinga River

As mentioned in the methods, isotopic signatures from yellow-eye mullet in the lower Onkaparinga did not fit well within the mixing polygon using 1.5 TLs. However, running SIAR with yellow-eye mullet at TL = 1 gave similar results to TL = 1.5. Although SIAR still flagged the results of the 1-TL analysis as potentially being problematic, SIAR rated the problem as mild and the variance associated with the model (SD2) went down from the model for 1.5-TL. We believe we had sampled all available autotrophic sources in the area and as the variance went down with only 1-TL, we present results from the 1-TL analysis.

In the lower reaches of the Onkaparinga black bream and yellow-eye mullet rely on similar sources of nutrients. Most of their nutrients came from saltmarshes, POM, MPB and *S. australis*, with very little nutrients coming from epiphytes/periphyton and seagrass (Fig. 4.4c).

Upstream in the Onkaparinga epiphytes/periphyton were a major source of nutrients for black bream, along with *Phragmities autralis*, *S. australis* and POM (Fig. 4.4d). Saltmarshes, *Juncus* sp., *Eucalyptus* sp. and *D. disticus* contributed relatively small amounts of nutrients to black bream diets. Yellow-eye mullet

nutrient sources were quite different from black bream in the upper Onkaparinga, with large proportions of nutrients coming from *Eucalyptus* sp., saltmarshes, *D. disticus* and *Juncus* sp. (Fig. 4.4d). Small fractions of yellow-eye mullet dietary nutrients came from POM, *S. australis, P. australis* and epiphytes/periphyton.

South West River

In the South West River, black bream received most of their nutrients from *Leptospermum myrsinoides*, macroalgae, and „shrubs" (Fig. 4.4e). Nutrients from *Enteromorpha* sp., epiphytes/periphyton, MPB and POM contributed comparatively smaller amounts to black bream diets. Although *L. myrsinoides* contributed large amounts to black bream diets, it contributed small amounts to yellow-eye mullet diets. Yellow-eye mullet diets in the South West River consisted of similar proportions of most sources sampled (Fig. 4.4e).

Similarity in autotroph reliance between black bream and yellow-eye mullet

Black bream and yellow-eye mullet similarity of autotrophic reliance was more than 50 % similar in the Chapman (Bray-Curtis similarity = 75 %), the lower Onkaparinga (92 %) and South West rivers (70 %). The similarity of autotrophic reliance in the Harriet River was much lower (40 %) and it was lowest in the upper Onkaparinga (27 %).

Discussion

It was expected that isotopic niches of black bream and yellow-eye mullet might overlap due to their shared environmental tolerances and omnivory. However, the isotopic niches of black bream and yellow-eye mullet did not overlap in any of the

estuaries sampled. Although the autotrophs they rely on within these estuaries were similar in some cases, they were quite different in other estuaries. The significant difference between black bream and yellow-eye mullet isotopic signatures in all estuaries reinforces that these two fishes have different isotopic niches and potentially ecological niches.

The environmental dimensions of black bream and yellow-eye mullet niches were expected to overlap as they are commonly found in the same estuaries. If $\delta^{13}C$ and $\delta^{15}N$ adequately reflect environmental dimensions of habitat use there should have been some overlap of isotopic niches between black bream and yellow-eye mullet, yet our analyses found none. This may be due to the small number of samples collected (n = 5), however Jackson et al. (2011) propose that the methods we used to quantify isotopic niches (SEAc and SEAb) and their overlap are not greatly affected by sample size. Black bream and yellow-eye mullet were often caught in different areas within estuaries, suggesting that there may be habitat separation or partitioning on a small spatial scale (Flaherty and Ben-David, 2010; Pita et al., 2011) and stable isotope signatures may be reflecting this (Janjua and Gerdeaux, 2011). Persistent habitat partitioning would be supported by small movement of fish within an estuary and we found evidence to support this. In the Onkaparinga River the difference in $\delta^{13}C$ between upper and lower sites suggested limited movement by fishes in this estuary, as consumer signatures closely tracked that of autotrophs (Melville and Connolly, 2003; Rasmussen et al., 2009). However, other researchers have found black bream to move over much greater distances (Sakabe and Lyle, 2009), although these fish were much larger than those sampled here (> 250 mm fork length). Also the sites where black bream and yellow-eye mullet were caught together (lower

Onkaparinga and South West River) were sites where fishes relied on similar autotrophic sources, suggesting their habitats overlapped. Therefore the isotopic niche separation between black bream and yellow-eye mullet may reflect spatial separation of these two species within some estuaries on a small scale. This could be tested by acoustically tagging individual fish and quantifying movements simultaneously for both species, although the size of fish caught in this study may be prohibitively small to attach tags to. If the drivers behind habitat partitioning on such a small scale were to be ascertained, considerable work would need to be done (e.g. determining distributions of each species at a scale relevant to habitat characteristics, preference for different habitats, and determining if either species defends habitats (Pita et al., 2011; Whitney et al., 2011)) and this would be very challenging in the estuaries sampled.

The separate isotopic niches of black bream and yellow-eye mullet are also likely to reflect differences in diets. Both fish species are known to be omnivorous and to feed opportunistically on the most abundant prey (Sarre et al., 2000; Platell et al., 2006), however if this were the case the two species would be in direct competition with one another. It has been suggested that niche overlap will be smallest when competition is most intense (Pianka, 1974). It could be that competition is intense between black bream and yellow-eye mullet and this is why we found no isotopic niche overlap. Indeed both black bream and yellow-eye mullet are known to feed within and on the substratum as well as throughout the water column (Sarre et al., 2000; Platell et al., 2006), suggesting that they would be in direct competition with one another for food. Previous studies have found little evidence of dietary overlap between black bream and yellow-eye mullet

(Branden et al., 1974; Harbison, 1974, although these studies were in only one estuary), further supporting the niche separation of the two species.

Although, we did not measure competition between black bream and yellow-eye mullet we can analyse the sizes of the isotopic niches and use them as an indicator to show how successful the fishes are as competitors (Olsson et al., 2009). Black bream had a larger isotopic niche than yellow-eye mullet in three out of the five estuaries/sites analysed suggesting that they are the better competitors. However, yellow-eye mullet had a larger isotopic niche in two sites: the Harriet River and the upper Onkaparinga. Both of these sites had different sizes of fish; the Harriet River had larger yellow-eye mullet and the upper Onkaparinga had smaller black bream than other estuaries. The larger yellow-eye mullet would be able to take larger prey items (Platell et al., 2006; Stouffer et al., 2011) potentially increasing their niche width. Conversely the smaller black bream in the upper Onkaparinga would be restricted to smaller prey items and a smaller niche. Therefore the ability of black bream and yellow-eye mullet to compete with one another may be size dependent and isotopic niches may change with ontogenetic changes in diet, as would their ecological niches (e.g. Hjelm et al., 2000; de la Moriniere et al., 2003; Stouffer et al., 2011). Further research across a range of fish sizes would be beneficial as ontogenetic changes in diets have been found from stomach contents of black bream and yellow-eye mullet (Sarre et al., 2000; Platell et al., 2006) and $\delta^{15}N$ of a congener of black bream (*A. australis*) has been found to vary with fish size (Melville and Connolly, 2003).

The range of $\delta^{15}N$ for black bream and yellow-eye mullet was not greater than the average isotopic discrimination for one trophic level (3.4 ‰, Post, 2002), suggesting that the fishes are feeding within a trophic level and not spanning two.

However, black bream had a $\delta^{15}N$ range greater than half of the average trophic discrimination (1.7 ‰) in two estuaries/sites. This range of half a trophic level suggests that black bream range from being herbivores to omnivores in the lower Onkaparinga and from omnivores to carnivores in the Chapman River. In the lower Onkaparinga, there is a smaller difference between fish $\delta^{15}N$ and autotroph $\delta^{15}N$ suggesting fish may be consuming more plant matter. Conversely in the Chapman River there is a greater difference between fish $\delta^{15}N$ and autotroph $\delta^{15}N$ suggesting black bream could be feeding at a higher trophic level. The largest range in $\delta^{15}N$ was recorded for black bream in the Chapman River (2.8 ‰) and this was also the site of greatest size variation for black bream. Indeed there was a positive correlation between variation in fish size and $\delta^{15}N$ range for black bream, but not for yellow-eye mullet. This is further evidence that more research is needed into the relationship between fish size and $\delta^{15}N$ of wild black bream in particular.

Considering the isotopic niches of black bream and yellow-eye mullet were different from one another and did not overlap: how similar was their reliance on autotrophic sources? At the Onkaparinga lower site the similarity between black bream and yellow-eye mullet autotroph reliance was at its highest (92 %). The high similarity at this location may be due to the fact that the estuary here is simplified into a relatively straight channel with little complex subtidal habitat to partition between the two species. As fish are unlikely to partition this habitat they are forced to compete for resources and yellow-eye mullet were feeding at a lower trophic level than black bream and thus filling a different niche. Black bream and yellow-eye mullet are being supported by similar autotrophs in

the lower Onkaparinga as they are feeding at different trophic levels within the food web.

Yellow-eye mullet in the Chapman River were feeding at a different trophic level to black bream and this again may explain the high similarity in autotroph reliance. Fishes in the South West River also had relatively high similarity in autotroph reliance. The South West River was the only river that was actually flowing at the time of sampling and therefore there may have been ample nutrients available for both black bream and yellow-eye mullet such that there was little need to compete (Milbrink et al., 2008; Chen et al., 2011). Further evidence of lack of competition in the South West River is found in the condition of fishes sampled. Fish collected in the South West River were in the best condition of all fish for both species, suggesting a lack of competition (Milbrink et al., 2008). Conversely fish were in the poorest condition in the Chapman River, where competition appears to be high as black bream and yellow-eye mullet were feeding at different trophic levels. The Chapman River is frequently bar-blocked so competition in this estuary may be higher as there is little in-flow of nutrients to stimulate productivity.

Low similarity of autotrophic sources between black bream and yellow-eye mullet was found in the upper Onkaparinga (27 %). This part of the estuary has a lot of structure to create microhabitats, where black bream and yellow-eye mullet could partition habitats more readily. There had been little rain and freshwater coming into the estuary prior to sampling, potentially making nutrients less available and therefore forcing the two fishes to compete and utilise different autotrophic sources/nutrients (Milbrink et al., 2008). In the Harriet River black bream were caught in the lower layer of a salt-wedge incursion into the river and

yellow-eye mullet were caught in the upper, fresher layer. This may explain why the autotrophic sources the two fishes relied on were quite different, as they were occupying different water bodies (Hjelm et al., 2000; Janjua and Gerdeaux, 2011). The autotrophs that black bream relied on in the Harriet River were dominated by terrestrially derived sources, suggesting that black bream were receiving nutrients through detrital pathways in the lower layer of water. Yellow-eye mullet received nutrients predominantly from marine and aquatic sources in the littoral zone, suggesting they are using different resources and occupying different habitats to black bream.

The data collected for this study represents only a snapshot in time and repeated sampling over longer time periods, particularly over different seasons, may provide insight into niche changes through time and with nutrient fluxes from freshwater inputs. Alternatively sampling of multiple tissues from the same individuals may elicit similar information, although discrimination factors for tissues other than muscle for these two species have not yet been experimentally quantified.

The results of this study show that two omnivorous fishes (black bream and yellow-eye mullet), which are commonly found in the same estuaries, occupy different isotopic niches and likely ecological niches. Previously, ecologists have undertaken complex studies of numerous attributes to understand the different niches co-habiting animals fill (e.g. Douglas and Matthews, 1992). Stable isotopes may provide a more efficient means to study ecological niches and their potential overlap, particularly with the advent of more advanced statistical approaches (Jackson et al., 2011). Our data also shows that within a food web two omnivores may receive nutrients from different autotrophs when competing with one

another, but may rely on similar autotrophs when feeding at different trophic levels.

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Chapter Five: Fish abundance and recruitment show a subsidy-stress response to nutrient concentrations in estuaries

Western River opening to the sea beyond, Kangaroo Island, November 2011.

Chapter 5 Preamble

This chapter is a co-authored paper, with intention to publish in a peer-reviewed scientific journal. Bronwyn Gillanders and Travis Elsdon are co-authors, therefore it is written in plural.

In this chapter Travis Elsdon conceived the sampling design and secured most of the funding. Bronwyn Gillanders assisted with funds. I assisted in collecting the samples and prepared samples for stable isotope analyses. Travis Elsdon prepared and analysed the otoliths and did the statistical analyses on otolith data. I did the quantile regression spline analyses and wrote the manuscript with input from Travis Elsdon and Bronwyn Gillanders.

I certify that the statement of contribution is accurate

Alexandra Bloomfield (Candidate)

I herby certify that the statement of contribution is accurate and I give permission for the inclusion of the paper in the thesis

Professor Bronwyn Gillanders Dr Travis Elsdon

Fish abundance and recruitment show a subsidy-stress response to nutrient concentrations in estuaries

Abstract

Increased nutrients from human activities can have large effects on aquatic ecosystems and may act in a subsidy-stress relationship with fish productivity. A subsidy-stress affect may occur when low additions of nutrients increase phytoplankton and subsequently fish production, due to increased food availability. However, at high additions of nutrients fish production may decrease due to degradation of habitat, such as seagrass and macroalgae. We assessed the hypothesis that fish productivity will show a subsidy-stress response to nutrient concentrations in estuaries by quantifying abundance and recruitment as productivity measures. We measured abundance and recruitment of black bream, *Acanthopagrus butcheri*, in estuaries in South Australia that varied in nutrient concentrations. To determine recruitment, young-of-year fish were collected in estuaries and their chemical otolith signatures determined. Subsequent year classes (1^+ and 2^+ year old fish), originating from the young-of-year cohorts, were assigned to their juvenile estuary from otolith analyses to determine recruitment success. Nutrient concentrations of ammonia, oxidised nitrogen and orthophosphorus were measured for each estuary in conjunction with fish abundance. Stable nitrogen isotopes of fish muscle were analysed to determine potential uptake of anthropogenic nutrients. Abundance and recruitment showed subsidy-stress responses to nutrient concentrations, with peaks in black bream abundance and recruitment occurring at low levels of nutrients. Fish with enriched

nitrogen isotopes were found in estuaries with high ammonia concentrations. Black bream abundance and recruitment peaked in estuaries with low anthropogenic inputs of nutrients. Our observations are a basis on which future work can build on to elucidate the mechanisms causing the subsidy-stress response of black bream abundance and recruitment.

Introduction

Anthropogenic additions of nutrients to coastal waterways and ecosystems is a global problem (Vitousek et al., 1997; Carpenter et al., 1998). Human-derived additions of nitrogen and phosphorus lead to increased phytoplankton productivity and decreased biomass of long lived aquatic plants and slow-growing macroalgae (Cloern, 2001; Rabalais, 2002). This is a particular problem in estuaries as they contain complex biogenic habitats of aquatic plants and macroalgae that are known to support increased abundance of fish and invertebrates (e.g. Beck et al., 2001; Bloomfield and Gillanders, 2005; Payne and Gillanders, 2009). Estuaries and their complex biogenic habitats act as nurseries for juvenile fish (Beck et al., 2001) where fish have increased growth and survival within complex habitats (Heck et al., 2003; Minello et al., 2003). Estuaries often receive high concentrations of nutrients through river systems that service large catchments. Therefore estuaries, and their complex biogenic habitats, are important for fish productivity but may be greatly affected by anthropogenic additions of nutrients.

Increases in nutrient concentration in aquatic systems may not always have detrimental effects on fish productivity. Increased nutrients can „fertilise" aquatic systems, stimulating primary production, particularly phytoplankton (Cloern, 2001; Capriulo et al., 2002; Rabalais, 2002). Increased primary production can lead to increased availability of food for fish, which can result in higher growth

rates and better fish condition (Keller et al., 1990; Bundy et al., 2003; Milbrink et al., 2008; González et al., 2010). Therefore some addition of nutrients may lead to increased fish productivity through increased food availability. However, excessive addition of nutrients to estuaries may lead to decreased fish productivity when biogenic nursery habitats are lost or when water quality deteriorates, leading to anoxia (Rabalais, 2002). This is an example of a subsidy-stress situation (Odum et al., 1979) where increasing inputs of nutrients can lead to increasing outputs of fish productivity from low to moderate levels (i.e. fish productivity is subsidised by additional nutrients). However, at high levels of nutrients fish productivity is diminished as biogenic habitats are lost and water quality deteriorates, detrimentally affecting fish growth and survival (i.e. the system is stressed).

Fisheries productivity is commonly measured by biomass, however the number of individual fish present and the proportion of fish that recruit successfully (those that survive to be counted in following years) are also suitable measures of fish productivity. Fish abundance and recruitment are measures of productivity because they reflect survival and growth. We predict that if high numbers of fish are present in estuaries with high nutrients it suggests that fish productivity is stimulated by high nutrients, through increased growth and survival. Conversely, if low numbers of individual fish are present in estuaries with high nutrients it suggests that fish productivity has been suppressed due to low survival and growth. If high nutrients detrimentally affects the proportion of fish surviving to recruit into subsequent year classes (0 to 1^+ or 2^+ year old fish) it will also have a detrimental effect on fish numbers in the future, and therefore productivity. If high concentration of nutrients enhances recruitment it will also enhance fish abundance and productivity in the future. Therefore measuring fish

abundance and determining recruitment success among estuaries with different nutrient concentrations will allow an assessment of the impacts of nutrients on fish productivity.

Stable isotopes of nitrogen can be used to directly link anthropogenic sources of nitrogen to fish and their food. Stable isotope ratios of nitrogen are affected by human activities, with elevated ratios of ^{15}N to ^{14}N in compounds formed by human activities compared to naturally occurring compounds that have lower ratios (Heaton, 1986). The increase in $\delta^{15}N$ (the ratio of ^{15}N to ^{14}N expressed in relation to air as a standard) has been used to trace sewage inputs through aquatic food webs (e.g. Gaston et al., 2004; Hadwen and Arthington, 2007). When an animal consumes a food item it assimilates the molecules from that food item into its cells and takes on the $\delta^{15}N$ of its food; thus the adage „you are what you eat" (DeNiro and Epstein, 1981). The incorporation of nitrogen stable isotopes from food sources into animal cells allows tracking of anthropogenic nitrogen sources through elevated $\delta^{15}N$ throughout food webs. However, the heavy isotope of nitrogen, ^{15}N, is preferentially retained over ^{14}N within animals during deamination of amino acids (Martínez del Rio et al., 2009 and references therein). Therefore $\delta^{15}N$ also increases with increasing trophic level in a food web (Minagawa and Wada, 1984) and the phrase has been adjusted to „you are what you eat, plus a few parts per thousand" (the units of measure of stable isotopes) (Fry, 2006). Stable isotopes of nitrogen present in fish tissue can be used to determine trophic level and if anthropogenic inputs of nitrogen are being taken up in a food web.

Black bream, *Acanthopagrus butcheri*, is a common estuarine sparid that completes its life cycle entirely within estuaries in southern Australia (Potter and

Hyndes, 1999; Norriss et al., 2002 and references therein). While there is evidence of a subsidy-stress situation of freshwater flows and salinity stratification affecting black bream spawning and recruitment success (Jenkins et al., 2010; Sakabe et al., 2011), the research was undertaken in only two estuaries and there has been no research into the effects of nutrients on black bream recruitment and abundance. It is acknowledged that freshwater flows and nutrient concentration are linked, however the processes by which they affect black bream recruitment may be quite different. Salinity, as a result of freshwater flows, affects black bream recruitment success directly through physiology, particularly eggs and larvae (Haddy and Pankhurst, 2000). However, nutrient concentration may affect black bream indirectly through food and habitat availability, which in turn affects survival and growth. Larvae and juvenile black bream feed primarily on copepods (Norriss et al., 2002 and references therein), which may have increased abundance under high nutrient concentrations (Capriulo et al., 2002; Bundy et al., 2003). Therefore productivity of black bream may be subsidised by anthropogenic additions of nutrients through increased food availability for larvae and juvenile fish. Juvenile black bream are known to have high abundance in seagrass and macroalgal beds (Butcher, 1945; Norriss et al., 2002) where they may benefit from increased growth or survival. However, seagrass and macroalgal cover may be reduced under high nutrient conditions (Rabalais, 2002 and references therein) potentially reducing black bream survival or growth. Therefore productivity of black bream may be reduced under high or stressful additions of nutrients.

Although black bream complete their entire life cycle within estuaries they can migrate among neighbouring estuaries (Butcher and Ling, 1962; Burridge et al., 2004; Burridge and Versace, 2007), which is thought to be associated with

heavy freshwater flows (Chaplin et al., 1998; Potter and Hyndes, 1999). Therefore black bream may form a metapopulation among neighbouring estuaries. Otolith chemistry can be used to determine successful recruitment from individual estuaries within a metapopulation by tracking individual fish to their juvenile estuary (Elsdon et al., 2008). Otoliths are paired calcium carbonate ($CaCO_3$) structures within the inner ear of fish, primarily used for hearing and balance (Popper and Lu, 2000). The ability of otoliths to track fish movements is based on their accretion of new carbonate and protein material onto the outside surface of the otolith on a daily basis (Campana, 1999). Incorporated within the carbonate and protein are elemental impurities. The $CaCO_3$ structure and elements are preserved and are not subject to metabolic absorption, hence otoliths accurately record a chemical chronology over the life-time of a fish. When fish living in different environments incorporate different chemical concentrations into otoliths, irrespective of the mechanism or reason behind the incorporation (Elsdon and Gillanders, 2004; de Vries et al., 2005), then these chemicals may be used as a habitat or location specific tag (Gillanders and Kingsford, 1996; Campana, 2005; Elsdon et al., 2008). Thus, the chemicals in otoliths of black bream can be used to determine recruitment success from different estuaries by tracking fish to juvenile estuaries within a metapopulation (e.g. Gillanders, 2002).

We assessed the hypothesis that black bream productivity will show a subsidy-stress response to nutrient concentrations by measuring abundance and recruitment success of black bream in a cluster of neighbouring estuaries in South Australia, Australia. We determined recruitment success by tracking 1^+ and 2^+ year old fish to juvenile estuaries using otolith chemistry. We measured nutrient concentrations of estuaries to determine if black bream productivity is subsidised

at low to moderate levels of nutrients and stressed at high nutrients. These measures also allowed us to estimate optimal nutrient conditions for black bream abundance and recruitment. The $\delta^{15}N$ of black bream muscle tissue was measured from one cohort of young-of-year fish, in conjunction with nutrient analyses of estuaries, to see if there is a link between anthropogenic additions of nitrogen and fish productivity.

Methods

Study system, species, and collections

We sampled juvenile young-of-year (0^+), 1^+, and 2^+ year old black bream and measured nutrient concentrations in a group of temperate estuaries within South Australia to determine which estuaries were more productive for black bream. Twelve estuaries were sampled on the mainland and on a large offshore island, Kangaroo Island (see Figure 5.1) over a three year period (2007-2009). These estuaries represent a spatially discrete cluster of estuaries within the larger distribution of black bream along the Australian coast, which spans from Murchison River in Western Australia to Myall Lake in New South Wales (Norriss et al., 2002). However, the estuaries sampled represent a group separated by geographical breaks to the west and east of at least 100 km to the nearest open estuary, which can result in genetic isolation due to the reduced migration habits of black bream (Burridge et al., 2004; Burridge and Versace, 2007). We therefore considered these estuaries to be a separate ecological population that may exhibit local mixing of individuals among estuaries via movements of juvenile fish.

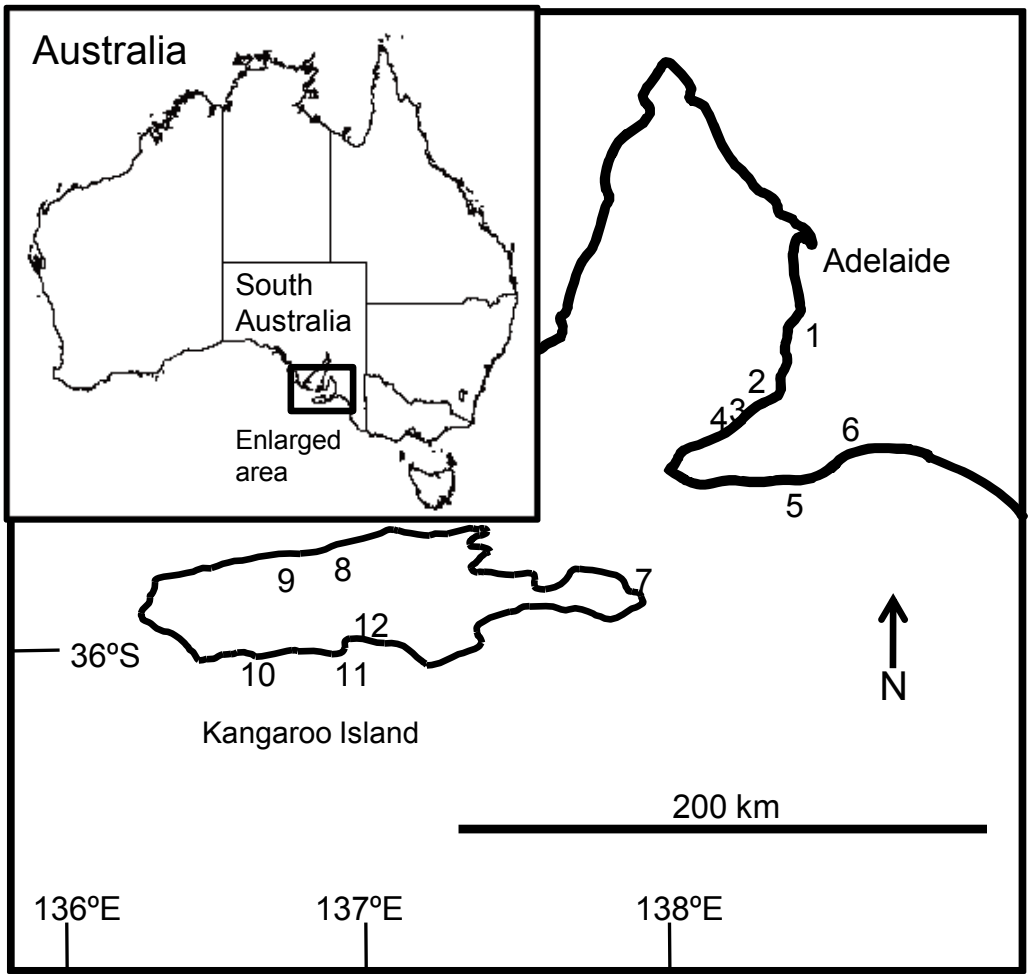

Figure 5.1 Map of location of estuaries sampled in South Australia, Australia.

1 = Onkaparinga, 2 = Myponga, 3 = Carrickalinga, 4 = Bungala, 5 = Waitpinga,

6 = Hindmarsh, 7 = Chapman, 8 = Middle, 9 = Western, 10 = South West,

11 = Harriet, 12 = Eleanor.

To measure black bream abundance collections were taken seasonally from summer/autumn 2007 to summer 2008, along with water samples for nutrient analyses (see *Water Sampling and Analyses* for details). To determine recruitment success fish were sampled in summer/autumn 2007, summer 2008, and summer 2009. Although fish were sampled from all estuaries annually; not all estuaries contained populations of 0^+ fish in 2007. In 2007 no 0^+ fish were caught in the Onkaparinga, Myponga, Carrickalinga, or Hindmarsh estuaries; which suggest either poor spawning, settlement of larvae or poor recruitment of fish. Estuaries in which no 0^+ fish were caught in 2007 contained 1^+ and 2^+ fish from previous years‟ 0^+ population in 2008 and 2009.

Within each estuary, 0^+ fish (n = 16 fish) were collected in 2007 and 2008 and 1^+ and 2^+ fish (n=28-122) were also collected in subsequent years (2008: 1^+, 2009: 1^+ and 2^+) to link back to the 0^+ fish from 2007 and 2008. Fish were collected using seine nets. Collections were done along the entire length of estuaries, and estuaries were not sub-sampled at multiple sites to assess within estuary variation (e.g. Gillanders, 2005) because estuaries were generally small in length (2.5 ± 1.5 km, mean ± SE; range 0.39 -11.02 km) and approximately 5-100 m wide. All fish were aged to confirm they were young-of-year (0^+) fish, 1^+ or 2^+. Recruitment was assessed based on the numbers of 1^+ and 2^+ fish found in the metapopulation in subsequent years from estuaries with characterised otolith signatures for 0^+ fish.

Otolith preparation and analysis

Sagittal otoliths of fish were dissected, washed, and cleaned in deionised water, and allowed to air dry in microcentrifuge tubes under a laminar flow cabinet. One otolith from each fish was embedded in epofix resin (Struers) that had been spiked

with 40 ppm indium to allow for discrimination between the otolith matrix and resin upon analysis. Otoliths were sectioned transversely through the focus (centre section) using a low-speed diamond saw (Buehler Isomet) and polished to 200 - 300 μm thickness using lapping film (Elsdon and Gillanders, 2002). Polished sections were fixed to glass slides with thermoplastic glue that was spiked with indium resin (CrystalBond 509) and allowed to dry. Once the glue was dry slides were sonicated in ultrapure water and dried again before analysis. Slides were stored individually in plastic bags.

The concentrations of elements (Sr, Ba, Ca, Mn, Mg, Li, and Zn) in otolith samples were determined using a New Wave 213 nm UV laser connected to an Agilent 7500cs inductively coupled plasma-mass spectrometer (ICP-MS). Laser ablations occurred inside a sealed chamber with the sample gas being extracted to the ICP-MS via a smoothing manifold in the presence of argon and helium gas. The chamber was purged for approximately 10 s after each ablation to remove background gas from previous ablations (Lahaye et al., 1997). The laser operating conditions were similar to Munro et al. (2008): frequency, 5 Hz; laser spot size, 30 μm; laser power, 65 %; beam energy ~ 0.08 – 0.12 mJ; carrier gas, Ar (0.87 L min^{-1}); with ICP-MS operating conditions of: optional gas, He (57.5 %), dwell times, Ca^{43} (50 ms), Sr^{88}, Ba^{138}, Mn^{55}, Mg^{24}, Li^{7}, Zn^{66} (all 200 ms), In^{115} (100 ms). Background concentrations of elements within the chamber were measured before each ablation (for 25 s), to allow for correction of sample concentrations.

A spot on the outside edge of otoliths of 0^{+} fish was analysed in order to quantify chemical concentrations of newly incorporated material from juvenile estuaries. A spot on the edge of the first growth band of otoliths from 1^{+} and 2^{+}

fish, which corresponded to the same location as that analysed for 0^+ fish, was also analysed to determine juvenile chemical signals. Otoliths were analysed in several sampling sessions with two reference standards to allow for comparisons among sampling sessions. A reference standard (National Institute of Standards and Technology, NIST 612) was analysed after every 12 otolith ablations to correct for machine drift (Ludden et al., 1995). The concentrations of elements in otoliths were corrected using a fish otolith standard (a 32 mm pressed powdered disk of finely ground otolith, similar to that described by Fallon et al., 1999 for coral), which was analysed at the beginning and end of each sample session. Analytical accuracy determined from the concentrations of the NIST standards and averaged across all samples was 100 % for Ca, Sr, 101 % for Ba, Mg, Mn, Li, and 103 % for Zn. Detection limits were assessed as 3 standard deviations above the blanks that were run during analyses, and were < 0.0005 $\mu mol.mol^{-1}$ for Sr, Ba, Mg, Mn, Li, Zn, and < 0.004 $\mu mol.mol^{-1}$ for Ca. All otolith values were above detection limits. All data reduction was done off-line in spreadsheets and consisted of smoothing, background subtracting, standardising to NIST 612, normalising to calcium (internal standard for ablation yield), and correcting to the fish standard.

Water sampling and analyses

Water samples were collected seasonally from summer/autumn 2007 to summer 2008 from estuaries on the mainland of Australia (Onkaparinga, Myponga, Carrickalinga, Bungala, Waitpinga and Hindmarsh Rivers, see Fig. 5.1). Water samples were also collected from estuaries on Kangaroo Island (Chapman, Middle, Western, South West, Harriet, and Eleanor Rivers, see Fig. 5.1) in winter 2007 and summer 2008. Black bream were collected for abundance measures at

these times, to enable black bream abundance to be related to nutrient concentrations. Two replicate water samples were taken from nine sites along each estuary with three sites in three sections of estuaries; the upper, middle and lower sections of each estuary. As estuaries varied in length it was necessary to divide estuaries into relevant sections, with the upper section being at the headwaters, or area of lowest salinity; the lower sections being at the estuary mouth or closest to the sea; and the middle section being halfway between the upper and lower sections. Sites within sections were separated by at least 30 m, with replicate water samples taken approximately 5 m apart. Black bream were also sampled for abundance at these sites using a seine net over an area of approximately 25 m^2.

Water samples were collected using a 25 mL syringe, which was submerged to the depth of the syringe (10 cm) so that samples represented surface water samples. Water was then filtered through a 0.45 μm membrane filter into a 15 mL polypropylene screw-top vial. Samples were stored on ice in the field, and immediately frozen at − 20°C until analysis. Prior to samples being analysed they were defrosted (within 2 h of analysis) and placed in racks on the auto analyser stage. Nutrients were analysed using an Automated Ion Analyser (QuickChem 8500 FIA), using standard methods of oxidised nitrogen ($NO^-_{2/3}$: nitrite/nitrate (NO_x); QuickChem® Method 31-107-04-1-A), orthophosphate (PO_4^{3-}; Method 31-115-01-1-I), and ammonia (NH_3; Method 31-107-06-1-B). The lower limits of oxidised nitrogen concentration measures were considered to be 0.001 mg N/L.

Stable isotope analysis

The $\delta^{15}N$ of fish muscle was measured to determine if anthropogenic sources of nutrients were being taken up into the food web and potentially affecting black

bream productivity. Dorsal muscle samples were taken from YOY fish collected in 2008, as YOY black bream were caught in all estuaries that year. Muscle samples from ten fish, where possible (all estuaries except Myponga), from each estuary were freeze-dried and ground into a powder using an agate mortar and pestle. Samples were weighed into tin capsules for $\delta^{15}N$ analysis. Samples were analysed by a GV Isoprime Mass Spectrometer coupled to a Eurovector elemental analyser 3000 at Griffith University, Queensland, Australia. International and internal laboratory standards (Ambient Air, IAEA-305a, Acetanilide, Working standard: 'Prawn') were run in parallel with fish muscle samples to enable calibration of results. Average precision of the mass spectrometer was 0.18 ‰ (1SD), with average accuracy of 0.18 ‰ (average deviation from known value).

Statistical analyses

Otolith chemistry analysis and classification

To determine whether there were significant differences of multi-element signatures among estuaries for 0^+ fish in each of 2007 and 2008 permutational multivariate analysis of variance (PERMANOVA, Anderson, 2001) was used. Data were $\ln(x+1)$ transformed and resembled using Euclidean distance similarity matrices. Permutations were done on residuals under the reduced model. Post-hoc comparisons, (Anderson, 2001) were used to determine which estuaries differed in otolith signatures.

Quadratic Discriminant Function Analysis (QDFA) does not assume homogeneity of covariance matrices and tolerates modest deviations from normality (McGarigal, 2000). Therefore QDFA was used, with a jackknife cross-validation procedure to determine classification accuracy. QDFA was used to determine classification success of fish to their estuary of known origin for 0^+

fish. Classification tests were done for individual estuaries, and when chemical tags of fish were similar among estuaries classification tests were done using a combination of individual estuaries and groups of estuaries.

To determine the proportion of recruitment to the metapopulation from each estuary elemental signatures of otoliths for 0^+, 1^+, and 2^+ yr old fish from 2007, 2008 and 2009 respectively were compared. Elemental signatures of otoliths from 0^+ and 1^+ fish from 2008 and 2009 respectively were also compared using the Maximum Likelihood Estimation (MLE) program HISEA (Millar, 1990). HISEA computes the likelihood of observing the measurements made on an individual given that it is from a particular group: i.e. the likelihood of a fish with measured elemental signatures (1^+ and 2^+ yr old fish) coming from an estuary with characterised elemental signatures (0^+ fish of the respective year). HISEA was run in bootstrap mode without re-sampling of standards (n = 16 fish/estuary), although mixtures were re-sampled with varying numbers of fish caught in each year/age group (2008 1^+ fish n = 28; 2009 2^+ fish n = 39; 2009 1^+ fish n = 122). The program calculated standard deviations of the contribution of each estuary by re-sampling the mixed stock data 100 times with replacement.

Recruitment, abundance and nutrients

We analysed the relationship of black bream productivity (abundance and recruitment) with nutrient concentrations (ammonia (NH_3), oxidised nitrgoen (NO_x), orthophosphorus (P)) using quantile spline regressions (quantile spline regressions encapsulate a set proportion of data points below the line (Koenker, 2005)) to find the nutrient concentrations at which recruitment and abundance peaked. Black bream abundance data were single measures of abundance taken at the same time as water samples (see above). If no black bream were recorded at

sites where water samples were taken, points were omitted as the absence of black bream may be due to factors other than nutrient concentrations. Black bream recruitment data were the percent classification of 1^+ and 2^+ fish back to juvenile estuaries. We therefore had to average nutrient data over the entire estuary to relate nutrient concentrations during juvenile development to estuarine-scale recruitment.

Two sets of analyses were done to relate nutrient concentrations of estuaries to black bream recruitment, as there were no water samples collected from estuaries on Kangaroo Island in summer/autumn 2007. One set of analyses used recruitment data from estuaries on the mainland for fish that were young-of-year in 2007 (2008 1^+, 2009 2^+), paired with nutrient data from summer/autumn 2007. Recruitment data from all estuaries for 2008 0^+ (2009 1^+) fish were also used in these analyses, pairing them with nutrient data from summer 2008. These data sets were paired together as fish would have experienced these nutrient concentrations before high winter rainfall and freshwater flows when they may leave the estuary. The second set of analyses were done assuming no inter-annual variation in nutrient concentrations within estuaries and used summer 2008 nutrient data with all corresponding recruitment contributions per estuary. Doing this enabled us to use all the recruitment data gathered. We acknowledge that no inter-annual variation in nutrient concentrations is an assumption, however, we believe it is feasible due to the local climate being relatively consistent across years. Summer rainfall is extremely low in South Australia, therefore minimal nutrients from surrounding land would be washed into estuaries over this time period. Groundwater tables in summer should also be fairly stable, or decreasing as it is dry. Similar levels of nutrients were found between summer/autumn 2007

and summer 2008 in estuaries that we had data for. The only differences in some estuaries were increased ammonia concentrations in the summer and decreased oxidised nitrogen and orthophosphorus compared to autumn.

Quantile regression spline models were done to encapsulate 95 % of the data below the spline and were done in the R statistical environment (R Development Core Team, 2011). The function "rq" was used (as part of the "quantreg" package Koenker, 2007) and combined with "bs" (part of the "splines" package, see Hastie, 1992) to fit piecewise polynomials of specified degrees (similar to Anderson, 2008). The small sample correction version of Akaike‟s information criterion (AICc) was used to determine the appropriate number of parameters for the piecewise polynomials. The model with the lowest AICc value having polynomial degree = 3, 4 or 5 was chosen as the best fit for the data. If two models had AICc values within 2 units of each other (and so could be deemed the same due to parsimony (Burnham and Anderson, 2002)), the model with the better visual fit or more accurate confidence intervals for peaks was chosen. The nutrient concentrations at which black bream abundance and recruitment peaked were determined from the regressions. To determine the 95 % confidence intervals of peaks, we calculated bias corrected percentiles by re-applying the model to each of 10,000 bootstrapped sample pairs using the appropriate polynomial per data set and restricting the range of nutrients to the first peak of the regression curve.

We further investigated the relationship of $\delta^{15}N$ values of black bream muscle and nutrient concentrations (NH_3 and NO_x only). We expected that high concentrations of ammonia and oxidised nitrogen would be due to anthropogenic influences and therefore $\delta^{15}N$ of black bream muscle would also be high, suggesting a positive correlation. We also graphed black bream abundance and

recruitment against $\delta^{15}N$ to show that elevated nutrient inputs from anthropogenic sources were affecting black bream abundance and recruitment.

Results

Otolith chemistry and classification to juvenile estuary

Elemental signatures of otoliths differed among most estuaries for the two years of 0^+ fish sampled (PERMANOVA: 2007 $F_{7,112} = 7.704$, $p = 0.0002$; 2008 $F_{10,154} = 10.576$, $p = 0.0002$). In 2007, eight estuaries contained 0^+ black bream. Of those estuaries, all fish except those from Harriet and South West estuaries had different otolith signatures. By grouping the Harriet and South West estuaries together, the minimum classification accuracy was 73 % (increased from 67 % when estuaries were separate), and the average classification success was 85.1 % (Table 5.1).

Table 5.1 Summary of classification accuracies of young-of-year (0^+) black bream back to their juvenile estuary from Quadratic Discriminant Function Analysis for fish collected in 2007 and 2008. Justification for groupings of estuaries is based upon PERMANOVA results.

	Classification success of 0^+ black bream to juvenile estuary	
Estuary/Grouping	2007	2008
Onkaparinga	no fish	100 %
Myponga	no fish	too few fish to classify
Carrickalinga	no fish	80 %
Bungala	87 %	77 %*
Waitpinga	93 %	93 %
Hindmarsh	no fish	*
Chapman	93 %	93 %
Middle River	80 %	80 %
Western River	73 %	76 %#
South West	90 %†	#
Harriet	†	80 %
Eleanor	80 %	#
Groupings	† = South West/ Harriet	* = Bungala/ Hindmarsh # = Western River/South West/Eleanor

In 2008, a total of 11 estuaries contained 0^+ black bream that could be used in analyses. Although Myponga estuary was sampled extensively in 2008, only two 0^+ black bream could be found and were therefore excluded from analyses. Of the 11 estuaries, there were two groupings that had similar otolith signatures: the Bungala and Hindmarsh; and the Western River, Eleanor, and South West. The remaining estuaries all differed in elemental signatures (Table 5.1). Classification accuracies for estuaries/groups ranged from 100 % in Onkaparinga to 76 % in the Western River/Eleanor/South West group with average classification success of 84.9 % (an increase from 79.3 % when ungrouped; Table 5.1).

Of the estuaries that had 0^+ fish collected from them in 2007, the Eleanor River and South West/Harriet group contributed by far the greatest proportions of recruits to 1^+ and 2^+ age groups (Table 5.2). The Bungala, Waitpinga, and Chapman estuaries contributed small proportions of recruits to the overall metapopulation ($\leq$ 15 %), with no individuals classified to the Middle or Western River. The Western River/South West/Eleanor group contributed the most recruits of 1^+ fish to the metapopulation of 2009 (see Table 5.2). The Bungala/Hindmarsh group contributed the second highest proportion of recruits to the metapopulation, with very small proportions (< 8 %) being identified from other estuaries.

Table 5.2 Proportional contribution of recruitment to the metapopulation estimated for 1^+ and 2^+ year old black bream per estuary/group based on otolith chemistry. Maximum Likelihood Classification estimator results: average % (± SD).

	2007 0^+ fish		2008 0^+ fish
Estuary/Grouping	2008 1^+ fish	2009 2^+ fish	2009 1^+ fish
Onkaparinga	-	-	3 % (± 2)
Myponga	-	-	-
Carrickalinga	-	-	1.2 % (± 1)
Bungala	6.7 % (± 6)	6 % (± 6)	20.3 %* (± 5)
Waitpinga	0 % (± 0)	2 % (± 3)	2.6 % (± 2)
Hindmarsh	-	-	*
Chapman	0 % (± 0)	15 % (± 7)	2.7 % (± 2)
Middle River	0 % (± 0)	0 % (± 0)	1.8 % (± 2)
Western River	0 % (± 0)	0 % (± 0)	#
South West	38.2 %† (± 8)	44 %† (± 6)	60.8 %# (± 5)
Harriet	†	†	7.6 % (± 4)
Eleanor	55.1 % (± 12)	33 % (± 9)	#
Total	100 %	100 %	100 %
Groupings	† = South West/ Harriet		* = Bungala/ Hindmarsh # = Western River/South West/Eleanor

Black bream abundance and recruitment related to nutrient concentrations

Black bream abundance and recruitment showed subsidy-stress responses to increased concentrations of ammonia, oxidised nitrogen and orthophosphorus (Fig. 5.2). Black bream abundance and recruitment increased with increasing ammonia and orthophosphorus concentrations at low levels (Figs 5.2 a, c, d, f, g and i). However, at high levels of ammonia black bream abundance and recruitment were reduced. At high levels of orthophosphorus black bream abundance was reduced, however recruitment appeared to be increasing again, although this is driven by few data points. Black bream abundance and recruitment peaked at very low levels of oxidised nitrogen concentrations and decreased sharply thereafter (Figs 5.2 b, e and h). Both nutrient data sets showed similar trends for recruitment analyses (Figs 5.2 d to i), with accuracy of peaks in recruitment and nutrient concentrations varying between data sets (Table 5.3).

Black bream abundance peaked at ammonia concentrations of 0.012 mg N/L, with recruitment peaking at 0.031-0.036 mg N/L (depending on which data set was used; see Figs 5.2a, d, g, and Table 5.3). Black bream abundance peaked at the lower limits of oxidised nitrogen concentration detection (0.001 mg N/L) and recruitment peaked at approximately 0.01 mg N/L (see Table 5.3). Black bream abundance and recruitment also peaked at low levels of orthophosphorus concentration (approximately 0.01 mg P/L).

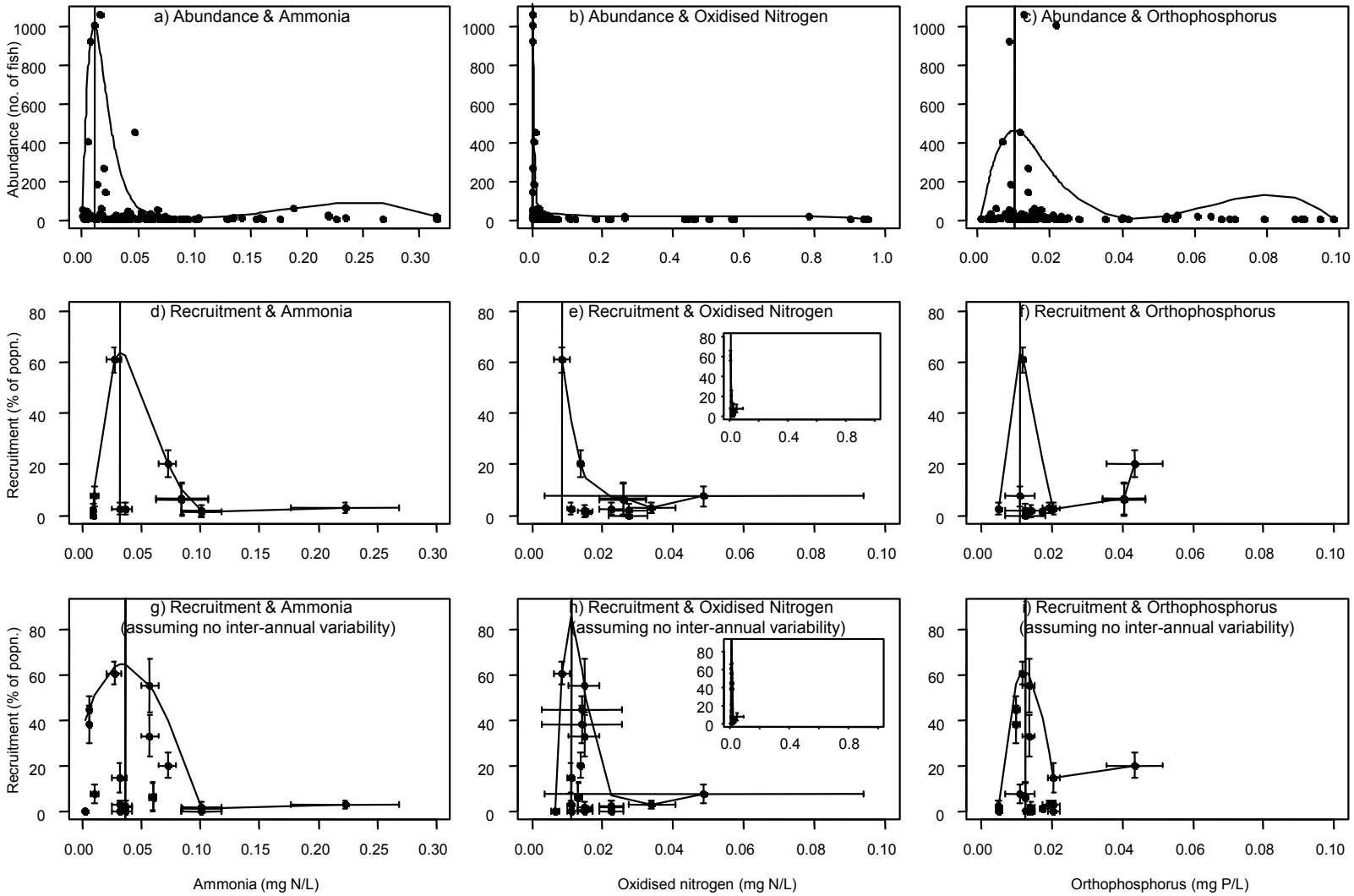
a) Abundance & Ammonia
b) Abundance & Oxidised Nitrogen
c) Abundance & Orthophosphorus
d) Recruitment & Ammonia
e) Recruitment & Oxidised Nitrogen
f) Recruitment & Orthophosphorus
g) Recruitment & Ammonia
(assuming no inter-annual variability)
h) Recruitment & Oxidised Nitrogen
(assuming no inter-annual variability)
i) Recruitment & Orthophosphorus
(assuming no inter-annual variability)
Abundance (no. of fish)
Recruitment (% of popn.)
Ammonia (mg N/L)
Oxidised nitrogen (mg N/L)
Orthophosphorus (mg P/L)

Figure 5.2 Abundance (number of fish caught in seines over approx. 25 m^2; a), b), and c)) and recruitment (% of population; d), e), f). g), h), and i)) of black bream related to concentration of nutrients: ammonia (mg N/L; a), d), and g)), oxidised nitrogen (nitrite/nitrate; mg N/L; b), e), and h)), and orthophosphorus (mg P/L; c), f), and i)). Nutrient measures paired with abundance data are averages of two water samples taken at the same location as black bream, collected seasonally over a year along estuaries (data are not included when black bream were absent). Nutrient measures for recruitment graphs are averages of water samples taken along the length of the estuary, or group of estuaries, for mainland estuaries in summer/autumn 2007 and all estuaries in summer 2008 (d), e), and f)) corresponding to cohorts that grew in those estuaries as young of year; and using nutrient samples collected in summer 2008 only (g), h), and i)). Recruitment estimates were calculated by Maximum Likelihood Estimation of which estuary 1^+ and 2^+ year old fish recruited from using otolith chemistry data. Quantile spline regressions are shown for the 95^{th} percentile with peaks in black bream abundance and recruitment, as calculated from the models, indicated by vertical lines (see Table 5.3 for specifics of regression analyses). Note the scale of graphs e) and h) are one tenth of that of b), shown in full as small inset graphs.

Table 5.3 Estimated optimal nutrient concentrations (ammonia = NH_3, oxidised nitrogen (nitrite/nitrate) = NO_x, orthophosphorus = P) for black bream abundance and recruitment (and 95 % confidence intervals) from quantile regression splines (see Fig. 5.2), with polynomial degree indicated. Nutrient data set used are noted. Note: *Rounded up to 0 as the lower limit; **Lower detection limit of machine.

Productivity measure	Nutrients data set	Nutrient	Polynomial degree	Estimated optimal nutrient concentration (mg N or P per L)	95 % CI
Abundance	Seasonal water samples collected from summer/autumn 2007 to summer 2008	NH_3	5	0.012	(0*, 0.026)
		NO_x	5	0.001**	(0*, 0.330)
		P	5	0.010	(0.008, 0.038)
Recruitment	Summer/autumn 2007 for mainland estuaries and summer 2008 data for all estuaries	NH_3	4	0.031	(0.005, 0.079)
		NO_x	4	0.008	(0.006, 0.020)
		P	4	0.011	(0.002, 0.022)
	Summer 2008 data for all estuaries	NH_3	3	0.036	(0*, 0.074)
		NO_x	4	0.011	(0.004, 0.034)
		P	3	0.013	(0.005, 0.025)

Nitrogen stable isotopes of fish tissue and nutrient concentrations

There was a positive linear relationship between $\delta^{15}N$ of black bream muscle and ammonia concentration of estuaries (r^2=0.56; see Fig. 5.3a), with the slope being significantly different from zero ($p < 0.001$). However there was no relationship between $\delta^{15}N$ of black bream muscle and oxidised nitrogen concentration of estuaries (r^2=0.22; see Fig. 5.3b), with the slope being not significantly different from zero ($p = 0.13$). The positive relationship between $\delta^{15}N$ of black bream muscle and ammonia concentration was strongly influenced by high values of both in the Onkaparinga.

Black bream abundance and recruitment peaked in estuaries with low $\delta^{15}N$ of black bream muscle (see Fig. 5.4), this relationship appears to be strongly driven by data from the Onkaparinga. The peaks in black bream abundance and recruitment are shown for illustrative purposes only in Fig. 5.4 as the data sets were too small to obtain sensible confidence intervals.

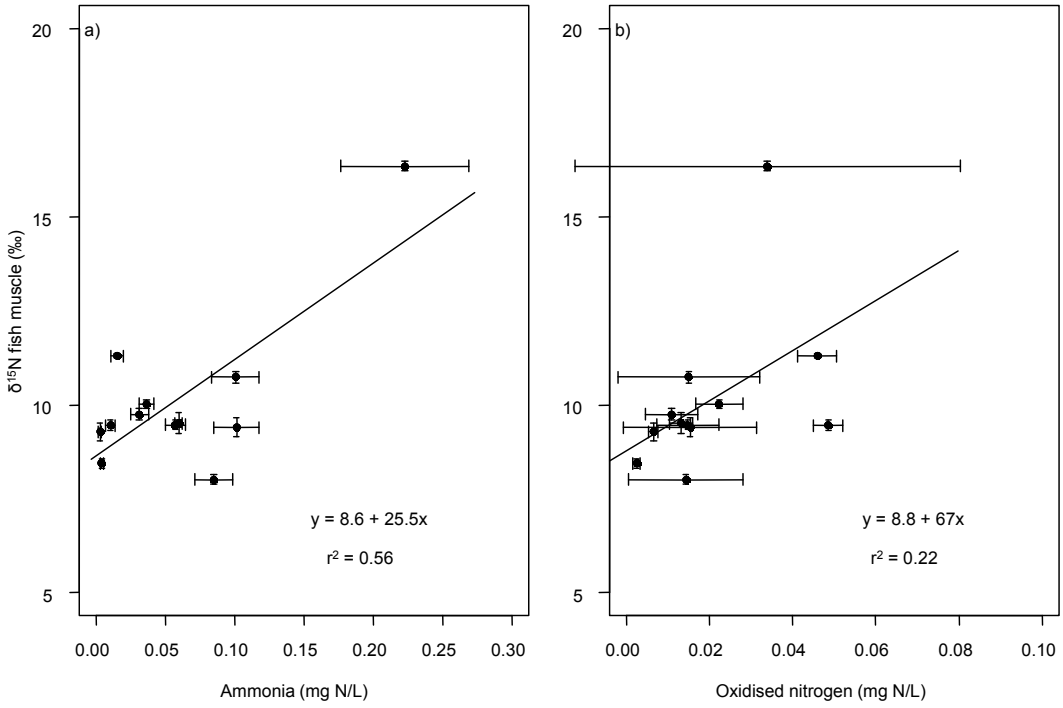

Figure 5.3 $\delta^{15}N$ of young of year (0^{+}) black bream muscle (‰; mean ± SE) and a) mean ammonia and b) mean oxidised nitrogen concentration of estuarine waters (mean mg N/L ± SE) for twelve estuaries sampled in summer 2008. Linear regression lines shown with equations and r^2 details given.

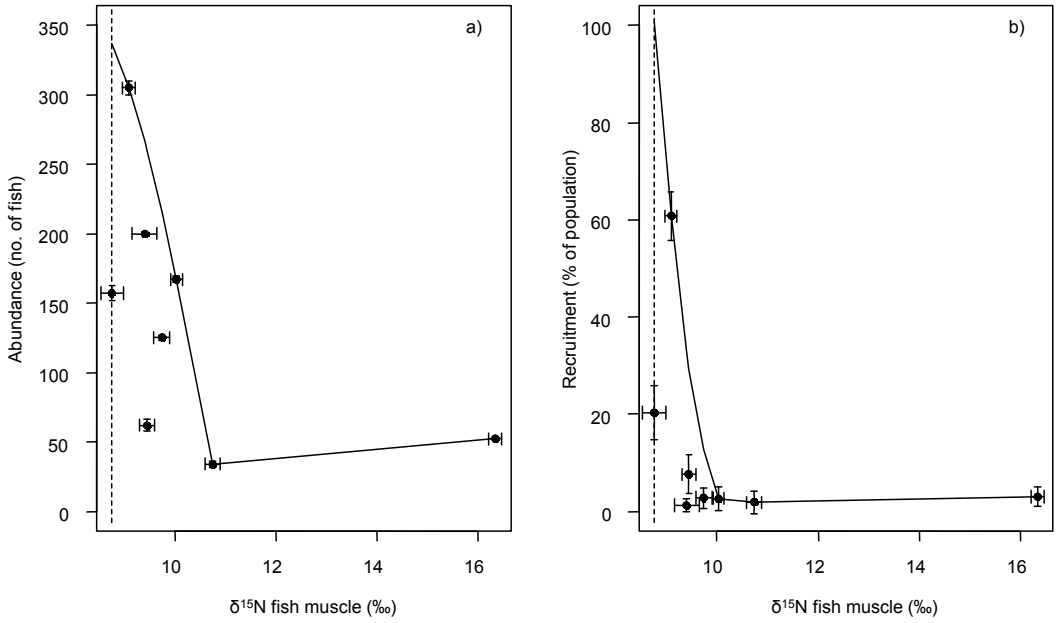

Figure 5.4 $\delta^{15}N$ of black bream muscle tissue (‰; mean ± SE) from young of year (0^{+}) fish caught in summer 2008 and a) abundance of black bream in estuaries in summer 2008 (total number of fish caught per estuary) and b) recruitment per estuary/group of estuaries (% of population tracked to different estuaries/groups) of fish caught in summer 2009 (1^{+} yr old fish). Solid lines show quantile regression splines of degree three. Peaks in black bream abundance and recruitment occur at $\delta^{15}N$ = 8.76 ‰ shown by dashed lines. Confidence intervals for peaks in abundance and recruitment could not be obtained due to too few data points.

Discussion

Black bream productivity showed a subsidy-stress response to nutrient concentrations in estuaries. Black bream abundance and recruitment increased with increasing nutrient concentrations at low levels of ammonia, oxidised nitrogen and orthophosphorus. However, at medium to high levels of nutrients black bream abundance and recruitment were detrimentally affected and decreased dramatically. The high levels of ammonia in estuaries appear to be caused by anthropogenic influences as $\delta^{15}N$ of fish tissue increased with increasing ammonia concentration of estuaries.

Significant differences in otolith chemistry were found among most estuaries for juvenile young-of-year baseline otolith signatures. Some estuaries had to be grouped together, however, which was required as it increased classification accuracies of 0^+ fish and was subsequently needed for classification of 1^+ and 2^+ fish to juvenile estuaries. Estuaries with similar otolith signatures either shared adjacent watersheds or had similar geology. South West and Harriet Rivers both have deposits of sandstone along the estuary and had similar otolith signatures in 2007. However, in 2008 their otolith signatures were significantly different, with South West River grouping out with Western and Eleanor Rivers. This may be due to environmental factors influencing otolith chemistry, such as salinity and temperature (Elsdon and Gillanders, 2002), which can vary due to rainfall and bar-blocking of estuaries. Unfortunately we do not have detailed climatic data available on estuarine flows, rainfall, or bar-blocking over the sampling period to correlate with groupings of otolith signatures. Bungala and Hindmarsh estuaries had similar otolith signatures in 2008, and although they are spatially separated, both drain glacial and fluvioglacial deposits. The similarity of

otolith chemistry signatures among estuaries may be due to similar geology and/or similar environmental conditions (Barnett-Johnson et al., 2008).

The proportion of the metapopulation that each estuary contributed varied between years, although some estuaries contributed small numbers of black bream to both cohorts (< 5 %: Onkaparinga, Carrickalinga, Waitpinga, and Middle Rivers). The estuaries that were grouped together contributed the largest proportions of recruits to the metapopulation (Harriet & South West Rivers, 2007 0^{+}; Western, South West, & Eleanor Rivers, Bungala & Hindmarsh Rivers, 2008 0^{+}). The grouping of estuaries for otolith signatures may have encompassed more variability in otolith chemistry allowing more fish to be classified to the groups. However, South West, Eleanor, and Harriet Rivers were always among the groups or individual estuaries contributing high proportions of recruits. These high contributions of recruits indicate the importance of these estuaries as spawning and nursery grounds for black bream and show that they act as sources of black bream for the metapopulation (Gillanders, 2002; Hamer et al., 2005).

The strong recruitment of black bream from groups of estuaries drove the subsidy-stress responses observed for recruitment and nutrient concentrations. Regardless of which nutrient data set was used for the recruitment analyses, both sets showed similar subsidy-stress responses with peaks found at similar nutrient concentrations. Therefore we believe that assuming no inter-annual variation in nutrient concentrations was reasonable. There were also subsidy-stress responses observed for black bream abundance and nutrient concentrations. These subsidy-stress responses of black bream abundance and recruitment suggest that small increases in nutrient concentrations may increase growth and survivorship. Increased survivorship of black bream is shown by the high numbers of fish

collected in estuaries with low additions of nutrients, as well as high recruitment from those estuaries. Increased growth at low nutrient concentrations may be reflected by increased recruitment of fish to the metapopulation from those estuaries with low nutrient additions. If additions of nutrients lead to increased food abundance at low levels, fish may grow faster and larger in these estuaries (Keller et al., 1990; Bundy et al., 2003). Body size has been found to be a major influencing factor on an individual‟s ability to successfully disperse (Benard and McCauley, 2008). Therefore if fish can grow larger and faster in estuaries with low additions of nutrients they can disperse further, and potentially sooner, and be more numerous in the metapopulation than fish that grow in estuaries with high additions of nutrients, where growth rates may be slower. Growth rates may be slower in estuaries with high nutrient additions for several reasons; one of which is that other planktivorous fishes may dominate the fish assemblage and out-compete black bream leading to slower growth and decreased survival. Comparing the growth rates of black bream and the amount of food available to fish among estuaries was beyond this study, but is needed to further our understanding of the mechanisms causing the peaks in abundance and recruitment at low nutrient concentrations.

Black bream abundance and recruitment were suppressed at high nutrient concentrations. High additions of nutrients can lead to decreased biomass of long lived aquatic plants and slow-growing macroalgae (Cloern, 2001; Rabalais, 2002), suggesting that black bream may need these biogenic habitats as juvenile areas. There is some evidence of higher abundance of black bream in seagrass and macroalgae (Butcher, 1945; Norriss et al., 2002), however we did not quantify the extent of these habitats within the estuaries studied and therefore can only

speculate that the aerial extent of these habitats may vary among estuaries. Although it seems likely that black bream may have increased survival and growth in biogenic habitats (Heck et al., 2003) this has not specifically been quantified for this species. More extensive research is needed to understand the mechanisms that are causing black bream abundance and recruitment to be suppressed at high nutrient concentrations.

We found a positive relationship between ammonia concentration of estuarine waters and $\delta^{15}N$ of muscle from fish living in those waters. The relationship is strongly influenced by high values of both ammonia and $\delta^{15}N$ from the Onkaparinga. The Onkaparinga was the only urban estuary sampled, with the remainder being within rural catchments. There were waste water sludge lagoons situated next to the Onkaparinga at the time of sampling that were known to flood into the Onkaparinga occasionally and were possibly leaching into the estuary. Therefore the high ammonia concentration is likely caused by human influences, which also causes high $\delta^{15}N$ of nitrogen compounds (Heaton, 1986). Although we did not measure the $\delta^{15}N$ of ammonia or dissolved inorganic nitrogen in estuarine waters, the ^{15}N of ammonia in the Onkaparinga is probably enriched (Heaton, 1986). As the ^{15}N-enriched ammonia, and other anthropogenically derived nitrogen containing compounds, are taken up by plants and algae the entire food web is enriched in ^{15}N. Indeed enriched ^{15}N of plants and algae has been recorded in the Onkaparinga (Chapter 4). This scenario is much more realistic than juvenile black bream feeding at a higher trophic level in the Onkaparinga, as all fish analysed for $\delta^{15}N$ were the same age and a similar size. We also found that sites with high fish abundance and recruitment had low $\delta^{15}N$ of fish muscle. This

indicates that the estuaries with high black bream abundance and recruitment were also estuaries with low human impacts.

Estuaries are naturally variable, and perhaps stressful, environments. It has been suggested that due to the high variability of estuarine environments organisms that live in estuaries are particularly well adapted to environmental variability (Elliott and Quintino, 2007). Elliott and Quintino (2007) further argued that we should assess functional characteristics of estuaries instead of structural characteristics, such as biodiversity, because high environmental variability is likely to lead to decreased biodiversity and structure of ecosystems. Here we have assessed the function of black bream recruitment from estuaries to a metapopulation and it has shown a subsidy-stress response with nutrient concentrations. The subsidy-stress response of this function further supports the hypothesis that organisms inhabiting estuaries are well adapted to environmental variability, as black bream recruitment occurred even at high nutrient concentrations although it was somewhat diminished. The adaptability of black bream and other estuarine organisms may obscure our ability to detect anthropogenic impacts in estuaries, as they can adapt and persist in highly variably environments (Elliott and Quintino, 2007).

Conclusion

We found that black bream productivity showed a subsidy-stress relationship with nutrient concentrations and that the increase in nutrient concentrations is probably due to human influences. However, as we have only focused on nutrient concentrations we cannot rule out the affects of other water quality parameters, such as dissolved oxygen, salinity, and heavy metals, and their effects on black bream recruitment and abundance (Breitburg et al., 1999a; Breitburg et al.,

1999b). Our observations suggest that the mechanisms behind the subsidy-stress response of black bream abundance and recruitment to nutrient concentrations warrant further investigation.

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Chapter Six: General Discussion

Aquarium set up for feeding experiments on yellow-eye mullet.

Darling Aquarium room, University of Adelaide.

General Discussion

Stable isotopes have become a commonly used tool in ecological research. They can help us decipher food web interactions, migratory paths, and track our impact on the environment. However, the discrimination of stable isotopes varies among animals and tissue types and this may lead to erroneous results of field studies. Environmental influences, such as temperature and diet composition, can also affect discrimination and these effects need to be accounted for to aid interpretations. Consequently there have been calls for experimental determination of discrimination factors for individual species and further investigations into the causes of variation in discrimination. This thesis investigated variation in discrimination factors and applied experimentally derived discrimination factors to field investigations to improve determination of autotrophic sources.

Temperature effects

Most organisms experience temperature variation throughout their life and seasonal variation in temperature can have large affects on organisms. Temperature affected both tissue turnover rates and discrimination factors of $\delta^{13}C$ and $\delta^{15}N$ (Chapters 2 and 3). Fish reared at warmer temperatures generally had faster tissue turnover rates and smaller discrimination values than fish reared at colder temperatures. This was largely due to kinetic effects on chemical reactions and diffusion, where molecules with the heavier isotope move slower at colder temperatures and so are less involved in chemical reactions (Dawson and Brooks, 2001). This results in fewer molecules with the heavier isotope being taken up

into animal tissue from the diet at colder temperatures. These results largely agree with other published studies (e.g. Bosley et al., 2002; Witting et al., 2004).

Tissue turnover of isotopes occurs through both growth and metabolism, which are affected by temperature (Chapters 2 and 3, Fry and Arnold, 1982; Hesslein et al., 1993; Herzka et al., 2002). Growth is generally considered to be the main process contributing to isotope turnover of muscle in growing poikilotherms (Fry and Arnold, 1982; Bosley et al., 2002; Witting et al., 2004; Trueman et al., 2005; Carleton and Martínez del Rio, 2010) and fish reared at warmer temperatures generally grew faster than fish reared at colder temperatures (Chapters 2 and 3). As an animal grows and accretes new tissue it uses nutrients from recently consumed food to build that new tissue. Through metabolism, absorbed food is catabolised for energy and some is used for tissue replacement and maintenance. Therefore, in growing animals, growth will contribute more to changes in isotope ratios through dilution effects than metabolism where most food is burnt for energy (Karasov and Martínez del Rio, 2007). Results from Chapter 2 further support this. In Chapter 2, $\delta^{15}N$ of black bream muscle did not change greatly over 42 days for fish reared at 16°C, but they did change for fish reared at 23°C which were growing faster. These results suggest that metabolism alone may be responsible for tissue turnover at colder temperatures as fish reached equilibrium sooner at 16°C than at 23°C (Chapter 2). Considering the variable growth rates of fish with temperature, isotopic signatures of fish may reflect their diets only during warmer growth periods (Chapters 2 and 3, Perga and Gerdeaux, 2005; Carleton and Martínez del Rio, 2010).

Discrimination factors were larger at colder temperatures than at warmer temperatures for $\delta^{15}N$ of both black bream and yellow-eye mullet (Chapters 2

and 3). However, the effect of temperature on discrimination of $\delta^{13}C$ was dependent on the diet fish were fed in both experiments. The diets with lower C:N ratios (fish-meal feed and chicken) had higher proportions of protein and fish fed these diets had larger $\delta^{13}C$ discrimination at warmer temperatures than at colder temperatures. Fish fed diets with higher protein content may have catabolised more protein from their diet for energy, compared with those fed a diet with lower protein content, allowing fish to store more lipids (Karasov and Martínez del Rio, 2007). However, at warmer temperatures these lipids are also metabolised or not formed through increased metabolism. Conversely at colder temperatures fish store lipids and do not metabolise them causing fish $\delta^{13}C$ to be more negative with increasing lipid content (DeNiro and Epstein, 1977; Post et al., 2007). Fish muscle C:N ratios support this (Chapter 3). Animal condition and C:N ratios of tissue are closely related (Kaufman and Johnston, 2007) and other research has found discrimination to vary with ration intake (Barnes et al., 2007), which also likely influences animal condition.

Diet effects

They say "you are what you eat" and to a certain degree „isotopically" fish are, however, what constitutes their diet may have complex affects on isotopic signatures. Diet quality, or C:N ratios, appears to have a strong influence on tissue turnover rates and discrimination. In Chapter 3, I found that the poor diet quality of *Artemia* restricted the growth of yellow-eye mullet, which may have resulted in little change in $\delta^{15}N$ of muscle tissue. This may be due to fish metabolising lipids during short starvation periods instead of using protein, as others have found starvation caused $\delta^{15}N$ to increase (Hobson et al., 1993; Gaye-Siessegger et al., 2004b; Kelly and Martínez del Rio, 2010). Starvation/fasting can be divided into

three phases (Karasov and Martínez del Rio, 2007). In phases one and two lipids are used for energy and protein catabolism is reduced. However in stage three proteins are catabolised (Karasov and Martínez del Rio, 2007) and this is probably when $\delta^{15}N$ increases, as ^{14}N is preferentially excreted as a product of protein catabolism and no new nitrogen is consumed. Therefore, fish fed the low quality diet of *Artemia* were likely sparing their dietary protein leading to lower discrimination of $\delta^{15}N$ and little change in $\delta^{15}N$ over time (Chapter 3, Guelinckx et al., 2007).

Further evidence to support the idea of protein being spared from catabolism on poor quality diets is found in the $\delta^{13}C$ of tissues. In Chapter 3, although $\delta^{15}N$ did not change greatly for yellow-eye mullet fed *Artemia* over time, $\delta^{13}C$ did change. Yellow-eye mullet fed *Artemia* increased in $\delta^{13}C$ over time. This may be due to *Artemia* having a higher $\delta^{13}C$ signature than worms, but it also may be confounded by fish burning ^{13}C-depleted lipids leading to a further increase in $\delta^{13}C$. Fish fed *Artemia* were in poor condition with those reared at 24°C being in the worst condition, and having the lowest C:N ratios and the highest $\delta^{13}C$, suggesting they have burnt off lipids and are using all carbohydrates consumed for metabolism (Karasov and Martínez del Rio, 2007).

Turnover of $\delta^{15}N$ was affected by the magnitude of difference in $\delta^{15}N$ between the swapped diets. The differences in $\delta^{15}N$ between the hatchery diet and the fish-meal feed (Chapter 2), and between worms and *Artemia* (Chapter 3) were smaller than the differences between the baseline feeds (hatchery diet and worms) and the other diets used (vegetable feed and chicken) in the experiments. This created different turnover rates between diets (Chapters 2 and 3). In both experiments fish changed from diets with high $\delta^{15}N$ to diets with lower $\delta^{15}N$ and

this may have shown a slower turnover or elimination of ^{15}N within fish tissue; as opposed to switching from a low-$\delta^{15}N$-diet to high-$\delta^{15}N$-diet (uptake). If ^{15}N is already incorporated into tissue protein, then it may be more difficult to eliminate as ^{15}N forms stronger molecular bonds than ^{14}N, leading to slower elimination/turnover rates (MacNeil et al., 2006). However, if an animal has been fed a low-$\delta^{15}N$-diet and is then switched to a high-$\delta^{15}N$-diet, it may take ^{15}N up faster as ^{15}N may be more readily able to displace ^{14}N.

Although compound-specific isotope analyses can provide us with insights into animal nutrition, physiology, and ecology (e.g. Chapter 2, Chikaraishi et al., 2007; Hannides et al., 2009; Lorrain et al., 2009) the analyses themselves are relatively expensive and time consuming and therefore may be restricted in their application to field studies. Elemental concentration is easily measured and is routinely provided when analysing stable isotopes, with most mass spectrometers having elemental analysers attached to them. In Chapter 3, I investigated the importance of elemental concentration in determining isotopic signatures of animal tissue. Indeed, accounting for elemental concentration improved predictions of muscle tissue isotopic signatures when using mixing models (Chapter 3). Correlations between isotopic signatures of fish muscle and elemental concentration of diet were also found. However, no relationship was found between isotopic discrimination and elemental concentration or C:N ratios, in contrast to others (Adams and Sterner, 2000; Kelly and Martínez del Rio, 2010). Adams and Sterner (2000) found a positive correlation between $\delta^{15}N$ of *Daphnia magna* and C:N ratios of its diet (*Scenedesmus acutus*). They also found that the discrimination of $\delta^{15}N$ by *D. magna* was positively correlated to the C:N ratios of its diet. The pivotal differences between their experiment and Chapter 3

is that they used the one diet source and manipulated the C:N ratios of the algae, analysing whole *D. magna.* In contrast, two different diet sources were mixed in Chapter 3 to obtain different C:N ratios and muscle tissue was analysed. Therefore in the experiment by Adams and Sterner (2000) the *D. magna* would have fed on a diet of similar constituents (in terms of amino acids, lipids, and other essential nutrients), however, in Chapter 3 yellow-eye mullet diets likely varied in constituents as well as C:N ratios and this may have resulted in isotopic routing (Kelly and Martínez del Rio, 2010).

Ecological applications

Stable isotopes of carbon and nitrogen can provide useful insights into the ecology of systems that are more challenging to study traditionally, such as estuaries. Estuaries are complex ecosystems, often comprised of various habitats, and can be difficult to study due to water turbidity, among other factors. Estuaries are also one of the most heavily impacted environments in the world (Kennish, 2002). Black bream abundance and recruitment showed subsidy-stress responses to increased concentrations of nutrients, with peaks in abundance and recruitment occurring at very low nutrient concentrations (Chapter 5). Human impacts may be traced by anthropogenic enriched nitrogen isotopes, particularly sewage impacts, through to black bream in estuaries (Gaston and Suthers, 2004; Hadwen and Arthington, 2007). Animal wastes and sewage mainly contain nitrogen in the form of urea which is hydrolysed to ammonia. Some of the ammonia escapes as gas and this gas is strongly depleted in ^{15}N, leaving behind an enriched ^{15}N ammonia in solution (Heaton, 1986). Therefore water bodies with sewage inputs will have high ammonia concentrations and high $\delta^{15}N$. A positive linear relationship between ammonia concentration of estuarine waters and $\delta^{15}N$ of black bream

muscle tissue was found (Chapter 5), showing that ammonia was taken up into the food web. This relationship was strongly influenced by data from the Onkaparinga River, where high values of both ammonia concentration and $\delta^{15}N$ of black bream muscle were recorded. The Onkaparinga had sewage sludge lagoons adjacent to it at the time of field sampling. These sludge lagoons were known to flood into the river occasionally and may still be leaching contaminants through ground water inputs, even though they have since been decommissioned. Chapter 5 showed that these sludge lagoons were likely having an impact on the estuarine ecosystem of the Onkaparinga as black bream abundance and recruitment were suppressed, although the mechanisms leading to lower abundance and recruitment need further investigation.

It has been suggested that niche overlap will be smallest when competition is most intense (Pianka, 1974). In the lower Onkaparinga I found high similarity in autotroph reliance between black bream and yellow-eye mullet, despite no overlap in niches. However, fish were caught in the same area, where habitat was simplified and potentially somewhat difficult to defend (Chapter 4). It is possible that in the lower Onkaparinga competition between black bream and yellow-eye mullet was intense and that this had forced yellow-eye mullet to feed at a lower trophic level than black bream, thus occupying a different niche. The anthropogenic influences in this estuary may have simplified the ecosystem somewhat, causing more competition between fish (González-Ortegón et al., 2010). In the South West River, which is mostly surrounded by National Park, high similarity in autotrophs between the fishes and no niche overlap were also found. In contrast to the Onkaparinga, I suggested that sufficient food was available for both fishes in the South West River such that competition was

reduced. Both species were in good condition in the South West River, the best condition of all estuaries sampled, suggesting competition was reduced and that the ecosystem was thriving (Chapter 4, Milbrink et al., 2008; Chen et al., 2011). The finding of no overlap in isotopic niches, and potentially ecological niches, of black bream and yellow-eye mullet could be because competition within estuaries has resulted in black bream and yellow-eye mullet occupying separate niches and this may be influenced by anthropogenic impacts on the entire ecosystem.

Black bream were enriched in ^{15}N over yellow-eye mullet in the Onkaparinga whereas they were ^{15}N-depleted in all other estuaries (Chapter 4). The potentially high concentration of ^{15}N-enriched ammonia in the Onkaparinga may be taken up by black bream directly, increasing $\delta^{15}N$ of fish muscle. Moeri et al. (2003) found that both an ammonotelic and ureotelic fish took up ^{15}N-enriched ammonia at the cellular level, although at different rates, when held in ^{15}N-enriched ammonia solution. In their experiment the ureotelic fish was not as enriched in ^{15}N as the ammonotelic fish and they suggested this may be due to its active ornithine-urea cycle which enables it to rapidly sequester ammonia away from the circulatory system to the liver, reducing the exchange of ^{15}N-enriched ammonia with muscle tissue (Moeri et al., 2003). Although most bony fishes are ammonotelic, some can be ureotelic (McDonald et al., 2006). It may be that yellow-eye mullet can be ureotelic and therefore are able to reduce the amount of ammonia being taken up from the water at the cellular level. In contrast, black bream are likely ammonotelic and not able to prevent uptake of ammonia, resulting in black bream being more ^{15}N enriched than yellow-eye mullet in the Onkaparinga. This is an alternative explanation, as opposed to feeding at a higher

trophic level, as to why black bream were enriched in ^{15}N over yellow-eye mullet in the Onkaparinga (Chapter 4).

If black bream are ammonotelic and yellow-eye mullet are ureotelic, it may also explain the differences in turnover rates of $\delta^{15}N$ between the two species (Chapters 2 and 3). Experimental fish were not reared in flow through systems and therefore excreted ammonia was held in tanks for up to 48 hrs. If black bream are ammonotelic, they may have taken up excreted ammonia $\delta^{15}N$ from within tanks and therefore shown slower turnover rates. Conversely, if yellow-eye mullet are ureotelic they would not have taken up dissolved ammonia from within tanks and would have had faster turnover rates. The differences in physiology of fishes may have affects on experimental and food web interpretations using $\delta^{15}N$ if the physiology of fish (i.e. ammonotelic or ureotelic) is not known and ^{15}N-enriched ammonia is present.

Future directions

Since the initial call for more experiments on stable isotopes in animals (Gannes et al., 1997) the field of stable isotope research has progressed somewhat, but new innovations are needed. The most popular application of stable isotopes is to determine diets, however diet quality can affect discrimination and subsequently isotopic signatures. Without being able to determine the relationships between diet quality (Chapters 2 and 3), ration intake (Barnes et al., 2007), nutritional status of animals (Hobson et al., 1993; Gaye-Siessegger et al., 2004b), and the consequences on stable isotope signatures we may come to erroneous conclusions when it comes to dietary and food web studies. Future research into relationships between animal condition and stable isotopes may benefit field studies as we cannot always quantify diet quality or ration intake in

the field. Research into enzyme activity may also help us understand variation in isotopic discrimination and nutritional status of wild animals, by indicating which metabolic processes are dominating, and therefore improve dietary back-calculation (Gaye-Siessegger et al., 2005).

There may be generalities in tissue turnover rates for animals within taxonomic groupings that are similar in size or growth pattern. I found a similar muscle tissue turnover rate of $\delta^{15}N$ for yellow-eye mullet (27.2 days half life) to that of the sand goby *Pomatoschistus minutus* (27.8 days half life, Guelinckx et al., 2007). The fish in both studies were of similar size and tissue turnover rates were quantified for the respective fish"s ambient summer temperatures (24°C for yellow-eye mullet and 17°C for the sand goby). Therefore a review of the literature may discover patterns in tissue turnover rates with animal size and temperatures experienced by animals in nature.

Although only elimination rates of isotopes were quantified in this thesis, others have quantified uptake and elimination of $\delta^{15}N$ (MacNeil et al., 2006). MacNeil et al. (2006) found variable uptake and elimination of $\delta^{15}N$ in a stingray, *Potamotrygon motoro*, with the initial uptake of ^{15}N being much faster than elimination in several tissue types (liver, blood, cartilage, and muscle). In nature it is more likely that an animal will switch from a diet low in $\delta^{15}N$ to one that is higher, as it grows and moves up the food chain, because ^{15}N is enriched with every trophic level (Minagawa and Wada, 1984). Thus, it would make more sense to aim to quantify the uptake rates of ^{15}N in the future to obtain more relevant turnover rates.

To improve field studies using stable isotopes it may be better to analyse homogenised samples of entire animals in the future, so as to eliminate the

possibility of isotopic routing skewing results. It is apparent that particular constituents of diets are routed or directed to certain tissues and therefore the isotopic signatures of those tissues are more similar to the particular diet constituents (e.g. bone apatite $\delta^{13}C$ reflects that of food catabolised for energy Ambrose and Norr, 1993; muscle tissue more closely reflects $\delta^{13}C$ of dietary protein Kelly and Martínez del Rio, 2010). Kelly and Martínez del Rio (2010) point out that mixing models do not account for isotopic routing, and indeed to do so would be extremely complicated. Therefore, it may be more realistic to aim to analyse homogenised samples of entire animals (where appropriate) than to account for isotopic routing in field studies.

Although I did not find a correlation between fish size and $\delta^{15}N$ for black bream or yellow-eye mullet directly, there was evidence that size of black bream may influence $\delta^{15}N$. There was a positive correlation between $\delta^{15}N$ range and fish size variation for black bream, but not for yellow-eye mullet (Chapter 4). Within any one estuary the range of yellow-eye mullet size was small, and this is probably because it is a schooling species. Therefore finding a similar relationship between $\delta^{15}N$ range and fish size variation for yellow-eye mullet was unlikely in Chapter 4, although it may occur in nature. Future studies should try to sample a larger range in fish sizes per estuary to determine if there are relationships of fish size and niche width with $\delta^{15}N$ of fish tissue.

To further investigate habitat partitioning between black bream and yellow-eye mullet, fish abundance needs to be quantified at different spatial scales and surveys repeated at different places within estuaries over time. However, finding adequate sampling gear and methodologies may be challenging in the estuaries sampled due to complex habitats, making it difficult to catch fish

without influencing abundance measures. Acoustic tagging of both fishes within the same area may also help determine if habitat partitioning is occurring, although this method may only be applicable to fish larger than those collected in this study due to the size of the tags.

Conclusion

Using stable isotopes in ecology often involves accounting for discrimination, however discrimination factors applied in field studies are often grand means across many species or are from related organisms. These bulk discrimination factors fail to acknowledge that discrimination can vary among animals, as well as within animals. Here I have begun to answer the calls for more experiments on the causes of variation in discrimination factors and tissue turnover rates (Gannes et al., 1997; Robbins et al., 2005; Martínez del Rio et al., 2009). I found that temperature and diet affected discrimination factors and tissue turnover rates. However our ability to predict temperature conditions and diet quality experienced by fish in the wild prior to collection is limited. Future research into relationships of fish condition and enzyme activity with stable isotopes may help improve estimates of discrimination and consequently field study interpretations. I found evidence to suggest that ammonia was being taken up at the cellular level, by black bream in particular, and this may affect experimental and field data interpretations. Stable isotopes of carbon and nitrogen will continue to be used in ecology and although some progress has been made, new innovations in experimental research are needed.

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Appendix B: Supplementary data for Chapter Two

Average $\delta^{15}N \pm SE$ ‰ of individual amino acids for all treatment groups analysed.

Diet	Hatchery feed			Vegetable feed				Fish-meal feed
Temperature	16°C			23°C		16°C	23°C	16°C
Day	0	7	14	28	42	42	42	42
Alanine	26.96 ± 0.76	27.77 ± 1.00	26.38 ± 1.32	25.51 ± 0.96	25.06 ± 0.55	24.90 ± 0.77	25.81 ± 0.74	25.30 ± 0.75
Glycine	6.46 ± 0.81	7.73 ± 0.33	6.02 ± 0.62	5.18 ± 0.68	5.02 ± 0.20	4.54 ± 0.71	4.82 ± 0.67	4.81 ± 0.67
Threonine	-12.94 ± 1.03	-11.37 ± 1.13	-11.69 ± 1.18	-11.82 ± 1.53	-12.20 ± 1.19	-14.55 ± 1.17	-13.87 ± 0.91	-14.03 ± 0.85
Valine	7.47 ± 0.79	9.03 ± 0.81	7.69 ± 0.33	7.40 ± 1.27	6.33 ± 0.30	5.81 ± 0.17	6.62 ± 0.97	5.39 ± 1.07
Serine	22.26 ± 1.37	23.64 ± 1.41	23.38 ± 1.13	22.37 ± 0.88	23.07 ± 0.70	20.66 ± 0.10	22.94 ± 0.84	23.64 ± 1.97
Leucine	25.71 ± 1.06	25.43 ± 0.88	24.78 ± 1.17	23.54 ± 1.50	23.56 ± 0.73	24.59 ± 0.23	24.95 ± 0.14	25.62 ± 1.08
Isoleucine	24.98 ± 1.10	25.14 ± 0.82	25.40 ± 1.11	25.85 ± 0.46	23.69 ± 1.03	24.84 ± 0.76	23.95 ± 0.62	24.58 ± 1.14
Proline	21.82 ± 0.51	20.90 ± 0.78	21.12 ± 0.62	19.95 ± 0.87	20.16 ± 0.90	20.22 ± 1.58	20.09 ± 0.45	21.21 ± 0.41
Aspartic acid	24.13 ± 0.78	22.34 ± 0.78	22.33 ± 0.80	21.16 ± 2.25	21.76 ± 0.88	22.69 ± 0.49	22.41 ± 0.18	23.19 ± 0.64
Glutamic acid	26.21 ± 0.95	25.48 ± 0.89	26.06 ± 0.64	24.29 ± 1.41	23.88 ± 0.93	24.58 ± 0.28	25.54 ± 0.19	26.26 ± 0.62
Phenylalanine	8.94 ± 0.56	7.93 ± 0.89	8.62 ± 0.90	10.34 ± 0.66	8.52 ± 0.67	9.13 ± 1.71	8.38 ± 0.38	9.64 ± 1.00
Lysine	9.75 ± 0.53	9.22 ± 0.52	9.67 ± 0.34	7.56 ± 2.39	8.91 ± 0.69	8.90 ± 0.80	8.60 ± 0.33	9.13 ± 0.28

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