The organic flight recorder:

Lifetime connectivity and habitat use reconstructions with fish otolith amino acids

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The only wisdom is in knowing you know nothing

Socrates

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Summary

With increasing disturbances to fish habitats, it is becoming essential to understand how fish species use and move between different habitats during their lifetime. This will lead to a better comprehension of how environmental changes will impact the population and how to mitigate these effects. Fish otoliths have often been used to investigate habitat connectivity and use. The calcareous structures grow and retain environmental chemistry during the lifetime of a fish, enabling the reconstruction of an individual's lifetime movement. The otoliths proteinaceous matrix could provide a direct connection to the environment and facilitates the extraction of lifetime resource utilization, trophic position and migration. The application is still hampered by the inability to extract low sample volumes from otolith increments and interpret the isotopic analysis of amino acids (AAs). Thus, this thesis aims to investigate the application of lifetime connectivity and habitat use reconstruction with compound specific isotope analysis of $\delta^{13}C$ and $\delta^{15}N$ values in amino acids of *Cynoscion acoupa* otoliths.

Previous applications of $\delta^{13}C$ measurements of AAs in biogenic carbonates have not yet managed to analyse material from small biogenic carbonates and their growth rings. To enhance the ability to analyse small calcareous sample volumes, it was shown how a simple derivatization method of methoxycarbonyl esterification (MOC) avoids decalcification procedures, which can lead to samples losses and unknown isotopic fractionation. The comparison to N-acetyl methyl esterification (NACME) without a decalcification procedure displayed that extra non-AA compounds were present in the AA chromatography. These extra compounds are a result of the reaction of water retained in the calcareous matrix with water sensitive chemicals used in the NACME derivatization procedure. As the NACME method and chemicals are highly similar to other methods of trifluoroacetic anhydride and pivaloylchloride esterification, MOC is the preferred method for sampling small volumes from biogenic carbonates.

The increasingly enriched $\delta^{13}C$ measurements of AAs in otolith edges of Amazonian *C. acoupa* with increasing length, referred to as baseline $\delta^{13}C_{EAA}$ values, accurately described their estuarine to coastal shelf distribution. Moreover, the differences between individual AAs, as source diagnostic $\delta^{13}C_{EAA}$ patterns, overcome spatial isotopic variations of resources and indicated that estuarine algal resources support the food web of the juvenile *C. acoupa* population. Otolith core and edge $\delta^{13}C_{EAA}$ patterns however, showed that the use of estuarine and coastal shelf resources in juvenile and adult stages differ between *C. acoupa* individuals.

The $\delta^{15}N$-AA analysis of juvenile and adult sections in large adult (~100 cm) otoliths showed that these *C. acoupa* life stages in the Amazon have a distinct habitat use and feed on significantly different trophic positions (2.8 vs. 3.5 on average). Compared to muscle tissue, reconstructed TPs were identical to those from otoliths, although differed in source $\delta^{15}N$ AA values due to muscle tissue representing only the dry season nitrogen dynamics. In the semi-arid Rio Grande do Norte, however, the same *C. acoupa* life stages overlap both in habitat and trophic position (3.1 vs. 3.6 on average), indicating an earlier migration towards the adult marine habitat due to low estuarine productivity. In both locations, trophic positions of juvenile stages were very similar compared to adult stages, which could vary by one trophic position. Adult *C. acoupa* stages with the highest trophic positions also displayed the lowest source $\delta^{15}N$ AA values that suggest less productive oceanic environments. Thus, it could show that individual adult *C. acoupa* diet can change according to the coastal distance.

In conclusion, this thesis shows that otolith AAs give insight into the life history of individual *C. acoupa* in terms of resource utilization, TP and migration. This can aid in providing more holistic information for fisheries management and conservation, and for predicting the impact of environmental disturbances on the population.

Zussamenfassung

Zunehmende Beeinträchtigungen der Lebensräume von Fischen erfordern ein tieferes Verständnis über Nutzungsverhalten und Wanderbewegungen von Fischarten und ihren Lebenstadien in verschiedenen Lebensräumen. Dabei ist ein Verständnis dafür, wie Umweltveränderungen die Population negativ beeinflussen und wie negative Effekte eingeschränkt werden können, essentiell. Fischotolithen werden oft dafür benutzt, die Konnektivität zwischen Lebensräumen und deren Nutzung zu untersuchen. Während des Wachstums werden Spurenelemente entsprechend der chemischen Umweltparameter in diese kalkigen Strukturen eingebaut und ermöglichen somit eine Rekonstruktion der individuellen Bewegungsmuster über die gesamte Lebenszeit des Fisches. Es hat sich gezeigt, dass die proteinhaltige Matrix des Otolithen die Umweltbedingungen noch genauer widerspiegeln kann als die Karbonatmatrix und einen weiteren Informationsgewinn über die lebenszeitliche Ressourcennutzung, trophische Position (TP) und Wanderbewegungen der Individuen ermöglicht. In der praktischen Anwendung begrenzen die zur Verfügung stehenden geringen Probenmengen die Isotopenanalyse und Interpretation der Aminosäuren (AS). Deshalb zielt diese Arbeit darauf ab, anhand von AS von *Cynoscion acoupa*-Otolithen die lebenszeitliche Konnektivität und die Nutzung von Lebensräumen mittels komponentenspezifischer Isotopenanalyse von $\delta^{13}C$ und $\delta^{15}N$ im AS zu rekonstruieren.

Mit früheren Methoden der $\delta^{13}C$ Messung von AS biogener Karbonate war es bisher nicht möglich, Material von kleinvolumigen kalkhaltigen Proben zu analysieren. Um die Analysemöglichkeit kleinvolumiger kalkhaltiger Proben zu verbessern wurde gezeigt, wie eine einfache Derivatisierungsmethode von Methoxycarbonyl Veresterung (MOC) eine Dekalzifizierung verhindern kann, was sonst zu Probenverlust und unbekannter Isotopenfraktionierung führen kann. Der Vergleich zur N-acetyl Methyl Veresterung (NACME) ohne spezielle Dekalzifizierungsmethode zeigte dabei, dass zusätzliche nicht-AS Bestandteile im AS Chromatogramm vorhanden sind. Diese zusätzlichen Bestandteile sind ein Ergebnis der Reaktion von gebundenem Wasser der kalkhaltigen Matrix mit wassersensitiven Chemikalien, die in der NACME Derivatisierungsmethode verwendet werden. Da die NACME Methode und verwendete Chemikalien große Ähnlichkeit mit anderen Methoden wie TFAA und Pivaloylchlorid haben, wurde die MOC Methode hier für kleinvolumige Proben von biogenen Karbonaten bevorzugt.

Die Anreicherung von $\delta^{13}C$ in AS in Otolithen-Rändern des amazonischen *C. acoupa* mit zunehmender Länge, hier bezeichnet als $\delta^{13}C_{EAA}$, bildet die Verbreitungsmuster in den Mündungsregionen und küstennahen Schelfgebieten sehr gut ab. Darüber hinaus überlagern die Unterschiede zwischen individuellen AS, als Quelle diagnostischer $\delta^{13}C_{EAA}$ Muster, gebietsbezogene Isotopenvariationen von Nahrungsressourcen und deuten darauf hin, dass mündungsnahe Algenvorkommen Bestandteil des Nahrungsspektrum junveniler *C. acoupa* Populationen sind. $\delta^{13}C_{EAA}$ Muster von Otolithen-Kernen und Rändern zeigen jedoch auch, dass der Umfang der Nutzung mündungsnaher Nahrungsressourcen bei *C. acoupa* Individuen im Jugendstadium unterschiedlich ausgeprägt sein kann und auch Nahrungsquellen des küstennahen Schelfbereichs mit einbezogen werden.

$\delta^{15}N$-AS Analysen juveniler und adulter Wachstumsringe in großen adulten (~100 cm) Individuen zeigen, dass die einzelnen Lebensstadien von *C. acoupa* im Amazonasgebiet einen definierten Lebensraum nutzen und sich aus signifikant unterschiedlichen TP ernähren (durchschnittlich 2.8 vs. 3.5). Ein Vergleich von Muskelgewebe und Otolithen zeigte, dass die rekonstruierten TP identisch sind, jedoch besitzen die $\delta^{15}N$-AS unterschiedliche Quellen, da Muskelgewebe nur die trockenzeitliche Stickstoffdynamik widerspiegelt. Im semiariden Rio Grande do Norte überschnitten sich die Lebensstadien jedoch in ihrer Lebensraumnutzung und TP (durchschnittlich 3.1 vs. 3.6), was auf Grund von niedriger Produktivität im mündungsnahen Bereich auf eine frühe Migration der adulten Fische in den marinen Lebensraum hindeutet. In beiden Gebieten waren die TPs der Juvenilen sehr ähnlich derer adulter Fische, was bis zu einer TP variieren kann. Adulte *C. acoupa* Individuen mit der höchsten TP hatten die niedrigsten $\delta^{15}N$-AS Messwerte, was marine Umweltbedingungen niedriger Produktivität anzeigt. Dies könnte darauf hinweisen, dass die Nahrungszusammensetzung von adulten *C. acoupa* Individuen im direkten Bezug zu einer zunehmenden Entfernung vom küstennahen Bereich steht.

Zusammenfassend zeigt diese Arbeit, dass Otolithen-AS einen Einblick in die Lebensweise von *C. acoupa* bezogen auf Resourcennutzung, TP, Wanderbewegungen und in Stickstoffzyklen ermöglichen. Dies kann mit dazu beitragen, genauere und umfassendere Informationen für Fischereimanagement und Umweltschutz zu gewinnen.

Chapter 1. Introduction

Understanding connectivity for better fisheries management

In the vast expanses of the aquatic realm, fish can migrate over various distances in search of food, shelter and reproductive mates (Dingle and Drake 2007). Their lifetime migrations take them through multiple habitats, which provide the necessary requirements during their different life stages. Thus, migrations can take place due to distinct ontogenetic needs and necessitate connectivity between habitats. Especially juvenile stages of fish are highly reliant on their habitat to provide an ample food supply for growth and protection from predators. The state of their habitat, hence, also determines the recruitment to the adult population (Caddy 2014). Over the past decades, habitat loss and alteration, environmental pollution, climate change, and overexploitation, amongst others, have severely decimated fish populations (Jackson 2001b; Halpern et al. 2008; Breitburg et al. 2009; Pauly and Zeller 2015). Assessment and mitigation of the causes and effects of declining fish populations is a complicated endeavour for fisheries managers and conservationists (Caddy 1999; Crook et al. 2015). This is also due to a lack of knowledge on the biology and ecology of many fish species, especially in terms of how various habitats are utilized during different life stages and the migrations that fish make between them. Such information is valuable for making predictions on how fish populations will react to environmental and habitat changes and how to mitigate these effects. Protecting these essential habitats, for example through the establishment of marine reserves, requires the precise knowledge of which habitats a species uses during their lifetime (Nagelkerken et al. 2015b).

Obtaining information on lifetime habitat use and migration by fish, however, is a complicated process. The large spatial scales over which fish can move and seasonal changes in their behaviour necessitate long term observational studies over distances (Beck et al. 2001; Gillanders et al. 2003; Dahlgren et al. 2006). Such studies are often not possible due to the limited amount of techniques that can be employed over such large areas and the inadequate amount of time and research funding. The accessible fish otolith chemistry, which can be considered as a lifetime archive of the encountered ambient chemistry by the fish individual, has therefore become a common tool in fisheries management (Landsman et al. 2011; Nelson et al. 2013; Miles et al. 2014).

These calcium carbonate structures with an organic matrix accrete from the early embryonic stage throughout the fish individual's life (Degens et al. 1969; Popper and Lu 2000; Schulz-Mirbach et al. 2014). As part of a sensory organ for auditory and balance purposes, three pairs of otoliths grow in the head of teleost fish; the lapilli, sagittae and asterisci. The most commonly used for research are the largest sized sagittae, which are commonly referred to as otoliths (Payan et al. 2004), Fig. 1A). When sectioned, otoliths frequently display repeated features that can be observed as opaque and translucent areas. The observation that these repeated features were linked to annual and daily growth, lead to the routine use of otoliths in fisheries science to age individual fish and investigate their life history (Pannella 1971; Campana 1999) fig. 2A+B).

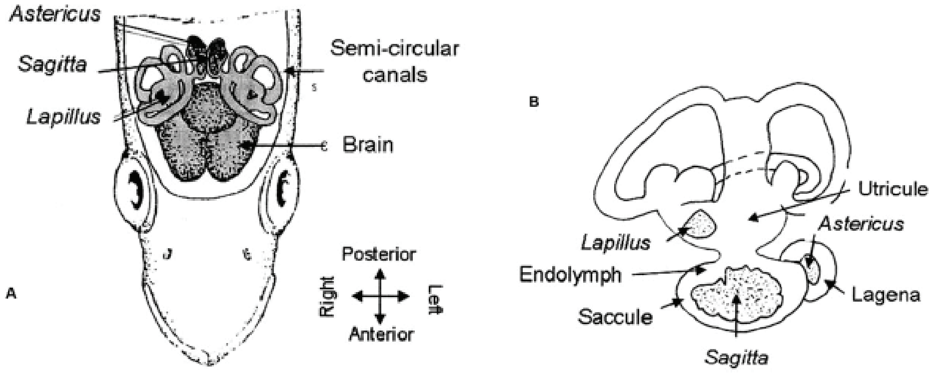

Figure 1. A) Location of the otoliths within the head of a fish as seen from above. B) The structure encapsulating the otoliths (taken from(Payan et al. 2004).

The utilization of the otoliths inorganic chemistry, in terms of elements and their stable isotopes, followed the example of coral skeleton records (Radtke 1985; Radtke et al. 1987; Radtke 1989). As the lifetime otolith accretion occurs without resorption and is therefore metabolically inert (Mugiya and Watabe 1977), the otolith permanently retains its chemistry. This otolith chemistry was hypothesised to be in equilibrium with the environment and thus could be used to reconstruct the ambient chemistry (Devereux 1967; Degens et al. 1969; Mulcahy et al. 1979).

However, the subsequent development of sensitive analytical instrumentation, resulted in the expansion of otolith chemistry studies some 15 years later (Gunn et al. 1992; Campana et al. 1997). Consequently, otolith chemistry was increasingly used to study lifetime migration, population connectivity, stock identification and environmental history such as temperature and salinity (Campana 1999).

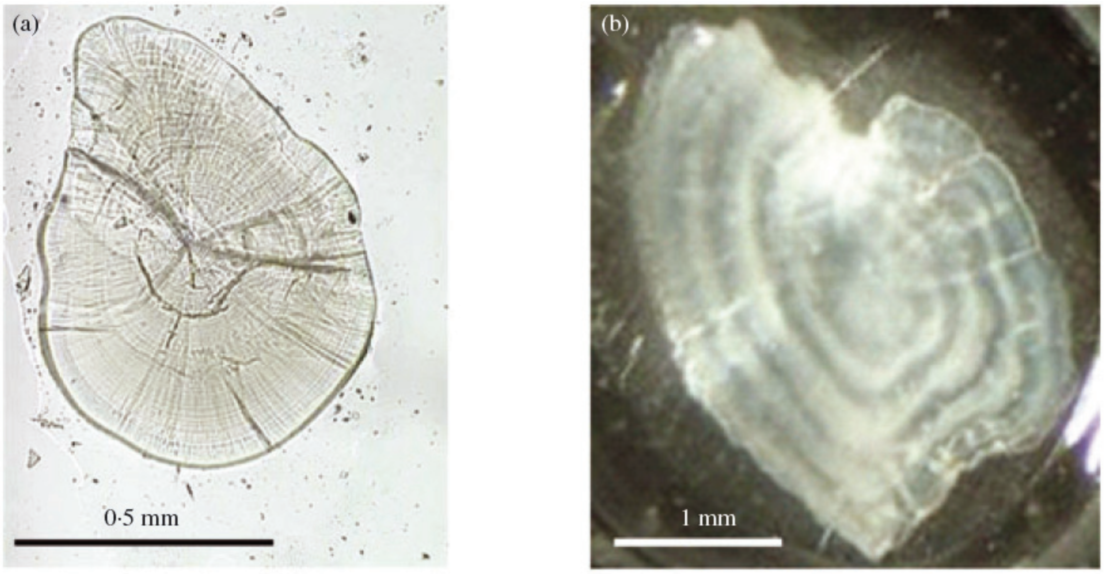

Figure 2. A) Daily increments in an otolith of a juvenile American shad, *Alosa sapidissima*. B) Annual increments in an adult salmon, *Salmo salar*, otolith (taken from (Walther and Limburg 2012).

Otolith elements used as indicators for environmental conditions

Variations in the chemical composition and physical processes of water masses create a geochemical landscape in the aquatic environment that is taken up by fish and eventually incorporated into their otoliths. Thus, the otolith chemistry records the migrations and habitat use by the individual fish, and can in this way be regarded as the piscine equivalent of a flight recorder. These chemical variations within aquatic environments can occur due to elemental weathering of terrestrial rock formations, atmospheric influx, anthropogenic input and seafloor hydrothermal activity (Dasch 1969; Palmer and Edmond 1989; Cutter 1991). This results in trace and minor elements (e.g. Zn, Cu, Sr, Ba, Mn, Mg) distributed in very low concentrations of less than 2-3 ppm in marine environments (Kennish 2001). The most commonly used elements to study fish movement across riverine and oceanic habitats are Sr and Ba (Hamer et al. 2006; Macdonald et al. 2013; Rohtla et al. 2014;

Rohtla and Vetemaa 2016; Fowler et al. 2016). Ba concentrations are relatively depleted compared to Sr concentrations in ocean surface layers (Wolgemuth and Broecker 1970; Capo et al. 1998; Monnin et al. 1999; Paytan and Griffith 2007), with exceptions where upwelling of deeper Ba richer water can cause Ba levels to increase locally in oceanic areas (Lea et al. 1989; Montaggioni et al. 2006). Consequently, these elements have a strong relationship with salinity and several studies confirmed that the Sr:Ca and Ba:Ca ratios in otoliths were correlated with changes in salinity and ambient concentrations (Secor et al. 1995; Kawakami et al. 1998; Bath et al. 2000; Elsdon and Gillanders 2002; Reis-Santos et al. 2013b). Also hypoxic conditions in the water column are thought to increase Mn concentrations in the water and post primordium incorporation into the otolith (Limburg et al. 2011; Mohan et al. 2014; Limburg et al. 2014). Most studies, however, use a combination of elements in otoliths to describe the location of origin of individual fish. Analysing the elements in whole otoliths or across increments of sectioned otoliths indicates the lifetime residence in different water masses and thus could identify subpopulations (Campana et al. 1994; 2000; Tanner et al. 2012). Sampling the cores of adult otoliths and comparing them to juvenile otoliths of different nursery areas has been used to backtrack connectivity between juvenile and adult habitats (Thorrold 2001; Reis-Santos et al. 2013a).

Individual elements that exist with different atomic masses and do not decay into other elements are called stable isotopes. The most commonly used elemental stable isotopes in otolith inorganic matrix studies exist in light atomic mass (e.g. ^{12}C, ^{16}O, ^{86}Sr) and a heavier counterpart (^{13}C, ^{18}O, ^{87}Sr). The rates at which heavy and light stable isotopes react during physical or chemical reactions differ, and can be connected to physical processes in the environment. Oxygen isotopes in aquatic waters are mainly controlled by evaporation and condensation. Due to condensation resulting in rainwater, rivers or freshwater bodies have overall lower $\delta^{18}O$ values than marine waters where evaporation dominates. Thus, a strong relationship of salinity and temperature with $\delta^{18}O$ values in water bodies exists (Epstein and Mayeda 1953; Dansgaard 1954). Latitudinal, small spatial scale and depth profile differences in aquatic $\delta^{18}O$ values also exist due to spatial temperature variations, ice melts and formation, and upwelling (Bradley 1999; Matta et al. 2013; Huijbers et al. 2013). Oxygen isotopes can therefore be used as a tracer of freshwater input and for reconstructions of temperature (Devereux 1967; Kalish 1991; Thorrold et al. 1997). The determination of Sr isotope values depends on the type and geological age of terrestrial rocks and minerals being weathered and can thus vary across locations (Capo et al. 1998). With a relatively stable signature over time and no physiological effects on the fractionation of Sr isotopes, it can be used as a reliable tracer for distinguishing fish populations (Kennedy et al. 2000; 2002; Hobbs et al. 2010; Muhlfeld et al. 2012; Brennan et al. 2015; Crook et al. 2017).

Variability and bias in the uptake of trace elements into otoliths

The applicability of inorganic otolith chemistry to reconstruct ontogenetic movements of individual fish depends on its correlation with ambient environmental chemistry (Campana 1999). However, this correlation has been under debate for some time and continues to limit the application of otolith chemistry for management purposes (Campana and Thorrold 2001; Veinott et al. 2014). This is mainly due to the metabolic influences affecting the elemental concentration within the fish and interactive environmental factors (Elsdon and Gillanders 2002; Marohn et al. 2011; Barnes and Gillanders 2013). Understanding the route and principles of the incorporation of chemistry into the otolith matrix is crucial for the correct and meaningful application of inorganic otolith chemistry.

Elements from the water enter the fish's body by water passing over the gills, continual water ingestion, and diet (Farrell and Campana 1996). Before elements are incorporated into the otolith matrix, several other physiological barriers, such as the blood vessels and the otolith epithelium, need to be passed and thus otolith elemental concentrations are far lower (< 1ppm) than in blood plasma or ambient water (Campana 1999; Izzo et al. 2016). Calcium like elements (e.g. Sr, Ba) get incorporated into the otolith mineral matrix, while elements with smaller atomic radii than calcium (e.g. Cu, Zn, Mn) bind at otolith proteins. Elements are thus heterogeneously distributed throughout the otolith (Doubleday et al. 2014; Izzo et al. 2016). Furthermore, elements are not incorporated immediately as saturation of the respective element concentrations in the otolith have been observed 12-60 days after exposure to ambient element concentrations (Elsdon and Gillanders 2005;

Yokouchi et al. 2011; Miller 2011). Elemental concentrations in the blood plasma can be altered by physiological influences (e.g. reproductive periods, gender, maturity), indicating that these factors also determine the availability of elements for otolith incorporation (Sturrock et al. 2014). Some studies have shown that diet shifts (Marohn et al. 2009), otolith precipitation rate and genetics (Clarke et al. 2011; Barnes and Gillanders 2013) are also potential interfering factors that influence the otolith elemental chemistry interpretation.

The physiological processes before elemental incorporation into the otolith complicate the extraction of detailed life history information from otolith increments. However, it can be beneficial for distinguishing populations within a species as distinct environments and interspecies physiological variations can create elemental differences in otoliths on a population basis (Chang and Geffen 2013). Although temporal instability and indistinctiveness of ambient elements between locations can be an issue for separating populations based on otolith chemistry only (Gillanders 2005; Reis-Santos et al. 2012). The most robust approach therefore might be application of otolith elemental markers in combination with genetic or artificial markers, otolith shape, area specific parasites or soft tissue isotopes (Reis-Santos et al. 2015; Tanner et al. 2016).

It is thus very important that the correlation between the otolith chemistry and the ambient chemistry is known or unperturbed by physiological processes, and temporally stable in order to reliably reconstruct fish movements, population separation, and environmental history (Walther and Thorrold 2009).

Compound specific isotope analysis of otolith amino acids

Although the main focus of otolith chemistry research has been on the inorganic matrix, its organic matrix has been little researched (McMahon et al. 2011b; a; 2012; Grønkjær et al. 2013). Similar to all biogenic carbonates such as corals and shells, otoliths contain low amounts of proteins that biosynthesise the calcium carbonate matrix and get incorporated in the process (Nagasawa 2014). The saccular epithelium encasing the otoliths produces these proteins *de novo* daily (Payan et al. 1999; Takagi et al. 2005) and it is estimated that only ~1% is deposited within the calcium carbonate structure (Edeyer et al. 2000; Borelli et al. 2001).

The relatively recent developments in compound specific isotope analysis of amino acids (CSIA-AA) for ecological applications have also had interesting implications for the use of otolith proteinogenic AAs. CSIA-AA is based on the finding that the carbon and nitrogen isotopes of certain AAs remain relatively unchanged from diet to consumers (Edgar Hare et al. 1991; McClelland and Montoya 2002; O'Brien et al. 2002; Howland et al. 2003; O'Brien et al. 2005; McMahon et al. 2010). This consistency in carbon isotopes is associated with the essential amino acids (EAAs) for which animals do not have the biosynthetic pathways to produce the carbon skeleton and are thus directly assimilated from their diet (Howland et al. 2003). These EAAs can be biosynthesised by primary producers, as well as funghi and bacteria (also collectively termed source endmembers), which through their distinctive physiological fractionation processes, can create unique isotopic signatures from which they can be identified (Abraham and Hesse 2003; Larsen et al. 2009; 2012; 2013). Thus, the $\delta^{13}C$ values of EAAs in consumer tissues can be utilized to directly determine the source endmembers at the base of the foodweb without trophic fractionation (Arthur et al. 2014; Schiff et al. 2014; McMahon et al. 2015c). The ^{13}C fractionation process of EAAs by the source endmembers is, however, influenced by factors such as the local $\delta^{13}C$ values of dissolved inorganic carbon and temperature. As a result, geographical variations in $\delta^{13}C$ values of EAAs, or baseline $\delta^{13}C_{EAA}$ values, of the same source endmembers can exist. This complicates the identification of resources, as otoliths are an indication of fish movements and resource utilization across space and time. However, it possible to infer biosynthetic origins of EAAs despite changes in the baseline $\delta^{13}C$ values of the growth medium because different taxa of source endmembers display characteristic isotope patterns among their AAs that remain constant regardless of environmental conditions (Larsen et al. 2009). The use of these $\delta^{13}C_{EAA}$ patterns thus might enable the identification of spatial and temporal differences in resource utilization by organisms. Identifying source end members that provide the basis of the food web upon which different fish life stages feed, determines the important nutritional elements within the habitat for the species. The effect of environmental changes on the productivity of source end members or primary producers is often better understood and thus can indicate productivity variations within food web and fish populations (Pauly and Christensen 1995; Beardall et al. 2001).

Nitrogen cycling of AAs is distinct and independent from their carbon counterparts. Generally, baseline $\delta^{15}N$ values are mainly determined by biological processes and anthropogenic influences such as runoff from agricultural fertilization or sewage input (Deutsch et al. 2004; Levin et al. 2015). Two main biological mechanisms play a role in the determination of nitrogen isotopes in the ocean. Nitrogen fixation by prokaryotes or diazotrophs produces bioavailable nitrogen into the ocean with $\delta^{15}N$ values of ~-2 to 0‰ which is close to atmospheric nitrogen (Macko et al. 1987). Contrastingly, denitrification by microbes occurs under suboxic conditions and the use of nitrate in the microbial respiration leads to nitrogen gas production, resulting in lower baseline $\delta^{15}N$ values (Somes et al. 2010). These baseline $\delta^{15}N$ values are recorded in certain source AAs by primary producers and is unrelated to whether AAs are essential or not. Consistent differences in $\delta^{15}N$ values of AAs lead to their classification as source and trophic AAs (Popp et al. 2007). Source AAs remain largely unaffected by metabolic processes and directly indicate the nitrogen baseline as they do not change from primary producers to consumers (McClelland and Montoya 2002; Chikaraishi et al. 2007). Source AAs therefore also reflect nitrogen biogeochemical processes of the ecosystems in which the organism resided (Choy et al. 2012; Lorrain et al. 2015; Hetherington et al. 2016). Trophic AAs are enriched in ^{15}N relative to source AAs due to complex nitrogen cycling in organisms (Chikaraishi et al. 2007; O'Connell 2017). This ^{15}N enrichment with each trophic transfer is thought to occur when trophic AAs undergo transamination, transfer of an amino group to a ketoacid, or deamination by removing amine functional groups (McClelland and Montoya 2002; Chikaraishi et al. 2007; Braun et al. 2014). Importantly, the difference in $\delta^{15}N$ values between trophic and source AAs provide an estimate of the trophic position of the organism that is normalised to baseline isotopic compositions (McClelland and Montoya 2002; Chikaraishi et al. 2009; Nielsen et al. 2015; Bradley et al. 2015).

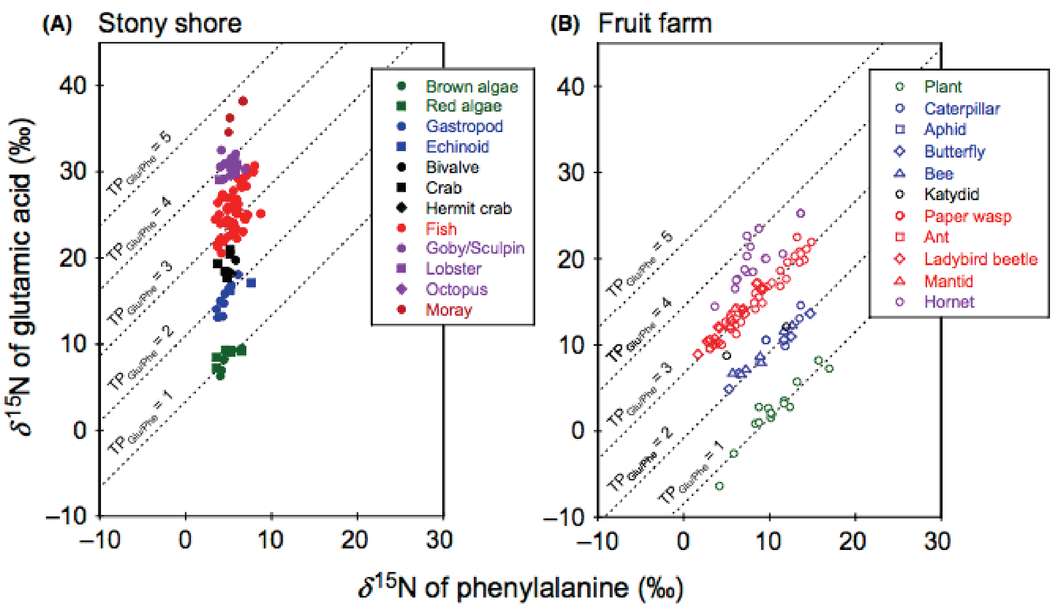

Fig. 3. An example of how δ^{15}N values of trophic AA glutamic acid and source AA phenylalanine indicate trophic positions (TP) of organisms in A) coastal marine and B) terrestrial ecosystems (taken from (Chikaraishi et al. 2014).

Ontogenetic changes in resources provides a means to optimise nutrition for the increasing energy demand due to growth (Olson 1996; Mittelbach and Persson 1998). Indications of individual lifetime trophic position might thus display what kind of prey are consumed by different life stages and how impacts such as fishing and climate change on their food web might affect productivity of the life stages (Jennings et al. 2002; Specziár and Rezsu 2009; Nakazawa 2011).

Analysing the δ^{13}C and δ^{15}N values of AAs in otolith increments could thus provide insight into lifetime resource utilization and trophic position of individual fish, and their environmental nitrogen dynamics. Moreover, since δ^{15}N values of source AAs and δ^{13}C baseline values of EAAs among primary producers can vary across geographical landscapes, ontogenetic fish migrations can be reconstructed (McMahon et al. 2012; Vokhshoori et al. 2014; Madigan et al. 2014; 2016). Assessing the life history migration of fish species can thus indicate how changes in these migration areas (e.g. habitat removal or damming) can impact these necessary movements in search of resources, shelter or mates (Dingle and Drake 2007; Miles et al. 2014; Nagelkerken et al. 2015b; Hegg et al. 2015).

Exploring the CSIA-AA application to *Cynoscion acoupa* otoliths

Research into the use of the otolith organic matrix for habitat use and migration reconstructions remains limited (McMahon et al. 2011b; a; 2012; Grønkjær et al. 2013; Sirot et al. 2017). This is likely due to the limited understanding of how to use the AA approach on otoliths and how to sample these mostly small calcareous structures. Thus, a refinement in the analytical methods and exploration of the CSIA-AA application is needed to enable the reconstruction of lifetime connectivity and habitat use of individual fish with otoliths. The Acoupa weakfish, or *Cynoscion acoupa*, is an ideal study species as it is presumed to make an ontogenetic migration from mangrove estuaries as larvae and juvenile to coastal shelf areas as adults. Changes in resource utilization and trophic position during the lifetime of *C. acoupa* can hence be expected to occur. Moreover, its large adult otolith size (Fig. 4) can accommodate both the analysis of δ^{13}C and δ^{15}N in AAs, as for δ^{15}N analysis a 9-fold increase in sample volume is required relative to δ^{13}C -AA analysis.

Figure 4. *Cynoscion acoupa* otoliths of individuals with increasing standard length from 12 to 120 centimeters. The ruler on the right indicates the otolith size in centimeters.

Little is know about the ecology of the *C. acoupa*, or pescada amarela (yellow fish) in local language (Fig. 5). It is an important commercial species caught mainly by artisanal fishery and sold throughout Brazil for their meat but also for their swimbladders, which are exported mainly to Asian markets (Barletta et al. 1998; Isaac et al. 2009, Tuuli et al. 2016). Even though there are no continuous catch records in Brazil, *C. acoupa* catch declined by more than a third between 2003 and 2005 (see references within Rodrigues et al. 2008). It can thus be expected that high catch rates will be maintained. To date, in most states of Brazil there are no management or conservation strategies in place or enforced to control the *C. acoupa* fisheries. Moreover, climate change, anthropogenic influences such as eutrophication due to sewage and agricultural runoff and overfishing are probably the main threats to the northern *C. acoupa* population (Martinelli et al. 2012; Bustamante et al. 2015, Levine et al. 2016; Zanin et al. 2017). Yet, due to the limited knowledge on the ecology and life history of this species, predictions on how the population will react to these environmental disturbances are highly complicated.

Adult *C. acoupa* are caught up to ~125 cm total length and typically weigh ~16 kg (de Matos and Lucena 2017), although some publications mention 170 cm individuals (Rodrigues et al. 2008). In northern Brazil *C. acoupa* spawns offshore at the onset and during the wet seasons and matures at a length of 40 centimeters (Almeida et al. 2016). Before and after the major rainfalls *C. acoupa* larvae and juveniles can be observed in the mangrove estuaries in large numbers, following the growth cycles of zooplankton and shrimp populations (Barletta-Bergan et al. 2002a; Nóbrega et al. 2013; Lima et al. 2015). Generally it is known that the *C. acoupa* is a demersal fish with a that diet consists of mainly shrimp and fish as well as some crustaceans and polychaetes (Ferreira et al. 2016).

Figure 5. A *Cynoscion acoupa* juvenile of 32 cm total length caught in the Caéte river in Pará, Brazil.

This sciaenid species occurs along the entire coast of Brazil, which encompasses very distinct hydrodynamics and climate (Fig. 6). In northern Brazil it occurs in a high annual precipitation ecosystem of over 2000 mm, which leads to high freshwater discharges by the Amazon river. Consequently, in the wet season a high nutrient load is dispersed several kilometres offshore in a northward direction (Smith and Demaster 1996; Bustamante et al. 2015). The estuarine environment harbours extensive mangrove deltas and is strongly macrotidal with amplitudes of ~4 meters (Schaeffer-Novelli et al. 1990). In northeastern Brazil at the coast of the state Rio Grande do Norte, the *C. acoupa* exists in a semi-arid ecosystem where annual precipitation is ~1250 mm. The coastal areas are characterized by low freshwater runoff with microtidal amplitudes of ~2 meters and low estuarine mangrove coverage (Schaeffer-Novelli et al. 1990). In comparison to the north, a narrow coastal shelf can be found at Rio Grande do Norte and is influenced by a strong North Brazil Current as a branch of the Southern Equatorial Current (Medeiros et al. 1999; Knoppers et al. 1999).

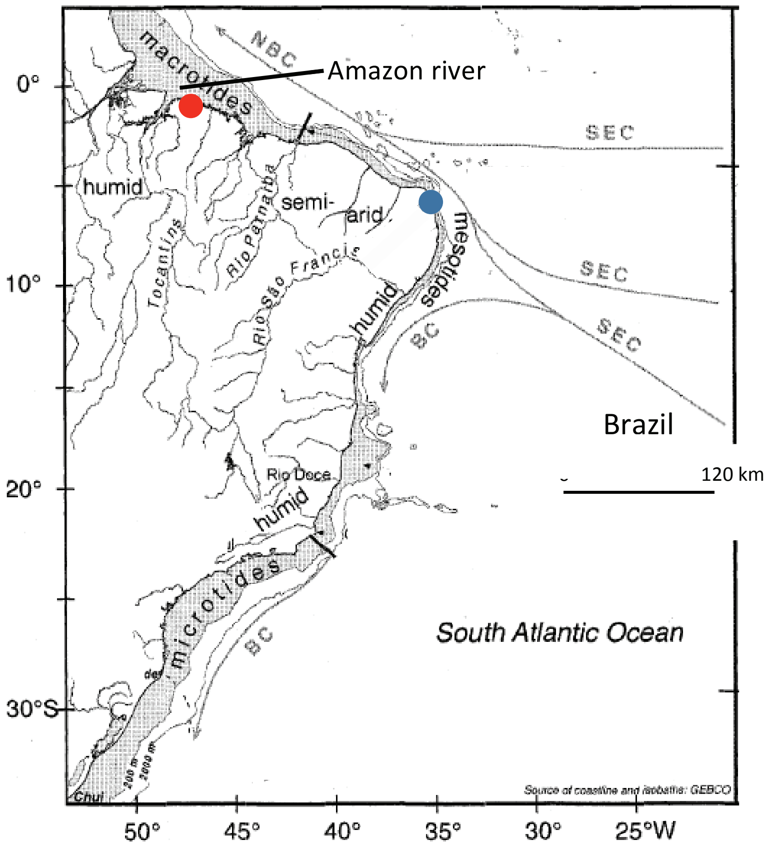

Figure 6. The coast of Brazil and sampling sites in Brazil, where the red dot is Bragança in the state Pará and the blue dot Natal in the state Rio Grande do Norte.

Also currents are indicated; Southern Equitorial Current (SEC), North Brazil Current (NBC) and (BC) Brazil Current (adapted from Knoppers et al. 1999)

Thesis objective and outline

CSIA-AA in fish otoliths could be a valuable tool for fisheries management, providing insights that are not possible or limited with inorganic otolith chemistry. The extraction of life history parameters such as resource utilization and trophic position could contribute to a deeper understanding of fish habitat connectivity and usage. This might strengthen predictions into how fish populations might react to environmental changes and enable a holistic approach to their management and conservation. Thus, this thesis aims to refine the methodological approach to the AA application for identifying lifetime resource utilization and tropic position with $\delta^{13}C$ and $\delta^{15}N$ values in AAs in *Cynoscion acoupa* otoliths. It is among the first to identify how CSIA-AA can be applied to long-term biogenic carbonate records such as fish otoliths in a technical aspect and the use of CSIA-AA principles for identifying lifetime resource utilization and trophic position. Moreover, the life history information acquired from the *C. acoupa* otoliths can aid in a preliminary understanding of how the population might be impacted by environmental disturbances. The key questions this thesis aims to answer are organised in the following chapters.

Chapter 2. How can small volume sampling in biogenic carbonates be achieved methodologically?

Until now relatively high amounts were extracted from biogenic carbonates for the analysis of $\delta^{13}C$ values in EAAs. This limits the potential of reconstructing lifetime resource utilization by sampling down to the small growth bands of biogenic carbonates. To assess whether choosing a particular derivatization method can reduce sampling amount, this study compares two approaches; N-acetyl methyl (NACME) and methoxycarbonyl (MOC) esterification. Differences in chromatography after using both methods without preceding decalcification are evaluated when applied to otoliths, coral skeleton and bivalve shell. In particular, the elution behaviour of MOC AAs in otoliths was assessed on two different gas chromatography columns, the non-polar DB5 and the highly polar VF-WAXms. Moreover, MOC AA derivative stability over two months is evaluated.

Vane K., Scholz-Böttcher B.M., Kopke B., Westphal H., Ekau W., Enabling high temporal resolution sampling in biogenic carbonates for $\delta^{13}C$ measurement of amino acids with methoxycarbonyl esterification, to be re-submitted to Limnology and Oceanography: Methods

Chapter 3. How can lifetime resource utilization and migration be reconstructed from *C. acoupa* otoliths with δ^{13}C values of EAAs?

The ability to assess and identify lifetime resource utilization and migration of fish can provide insight into how the increasing influences of habitat alterations and removal, climate change and pollution will affect the fish population. As δ^{13}C values in EAAs can be directly related back to the sources supporting the food web of fish, the organic matrix in otolith increments can potentially identify these resources during different life stages of fish.

Juvenile *C. acoupa* are distributed in mangrove estuaries and in coastal shelf areas as adults, implying an ontogenetic migration between the habitats. How these resource utilization and migration records can be extracted from *C. acoupa* otoliths is investigated by comparing bulk inorganic δ^{13}C values with mean δ^{13}C values of EAAs. Furthermore, δ^{13}C of EAA values in otolith edges of individuals across different sizes are used to detect a pattern of lifetime resource utilization and distribution from estuary to coastal shelf. Core and edge δ^{13}C values of EAAs in individual otoliths were also analysed to see if it reflects the expected estuarine to coastal shelf migration of *C. acoupa*.

Vane K., Larsen T., Scholz-Böttcher B.M., Kopke B., Ekau W, Ontogenetic resource utilization and migration reconstruction with $\delta^{13}C$ values of essential amino acids in the Cynoscion acoupa otolith, submitted to Ecology and Evolution

Chapter 4. What do $\delta^{15}N$ values of otolith AAs from *C. acoupa* otoliths indicate about lifetime trophic position and migration in two distinct Brazilian ecosystems?

Nitrogen isotopes of AAs reflect the nitrogen biogeochemical processes of the ecosystems in which the fish resided and can be used to reconstruct trophic position of the fish in the food web. Measured in the otolith, this could give information on lifetime trophic position and migration. Otoliths of *C. acoupa* and muscle tissue were collected simultaneously in the dry season of the Amazon state Pará, Brazil, to indicate whether source AA $\delta^{15}N$ values, and trophic position of juvenile and adult life stages extracted from both tissues are similar. Differences in life history trophic position and migration are determined by analysing $\delta^{15}N$ values of AAs in juvenile and adult stages of *C. acoupa* otoliths from the wet Amazon area of Pará and the semi-arid area of Rio Grande do Norte (Fig. 6).

Vane K., Wallsgrove N.J., Ekau W., Popp B.N., Reconstructing lifetime nitrogen baselines and trophic position of Cynoscion acoupa from $\delta^{15}N$ values of amino acids in otoliths, under review at Marine Ecology Progress Series

Author contribution

	Chapter 2	Chapter 3	Chapter 4
Concept and design	100%	100%	100%
Data acquisition	80%	80%	100%
Data analysis and interpretation	80%	90%	80%
Preparation figures and tables	100%	75%	100%
Drafting of the manuscript	90%	90%	80%

Chapter 2

Enabling high temporal resolution sampling in biogenic carbonates $\delta^{13}C$ measurements of amino acids with methoxycarbonyl esterification

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To be resubmitted to Limnology and Oceanography: Methods

Abstract

High temporal sampling of biogenic carbonate structures with compound specific isotope analysis of $\delta^{13}C$ in essential amino acids can give insight into life-time resource use and movement by these organisms. Such insights are constrained by the current inability to extract minimal amounts of proteinogenic amino acids for $\delta^{13}C$ analysis from the small increments. To facilitate this for the first time, two derivatization methods, *N*-acetylmethyl esterification (NACME) and methoxycarbonyl esterification (MOC) were applied to different untreated biogenic carbonates, uncovering how to achieve low amount sampling. The observation of non-amino acid compounds in the NACME chromatography, absent with MOC derivatization, indicated that water retention after hydrolysis of calcium carbonate reacted with water-sensitive chemicals used in NACME. Such chromatographic performance is commonly improved with decalcification procedures, though can lead to amino acid losses and isotopic fractionation. MOC circumvents these losses by utilizing non-water sensitive derivatization chemicals and a lack of evaporation steps that enabled the analysis of smaller untreated sample amounts of 5 versus 10 mg previously used. Further adaptations to MOC procedure could decrease the sample amount to ~1 mg (0.7% protein content). Moreover, high accuracy and stability of $\delta^{13}C$ measurements over 56-63 days of MOC amino acid (AA) standards and in otoliths were achieved on a highly polar GC column, VF-WAXms, and not on a low polarity DB5 column due to insufficient baseline separation from low amounts of MOC derivatization byproducts. As a result, this study shows that MOC derivatization has considerable advantages over methods as NACME to achieve the minimal amount sampling of especially biogenic carbonates.

Keywords: biogenic carbonate, otolith chemistry, amino acids, carbon isotopes, derivatization

Introduction

Most biogenic carbonate skeletons, such as fish otoliths, coral skeletons and bivalve shells, are continuously formed during the life of the organism and retain the changing chemistry of the environment in the (in)organic matrix of the carbonate.

The carbonate matrix chemistry in terms of trace and minor elements, and their stable isotopes can be related to the chemistry of the organism's ambient environment (Elsdon and Gillanders 2004; Walther and Thorrold 2006). The combination of chemistry and continuous accretion, that often results in visible age bands, and metabolic inertness can provide us with a chronological record of the organism's environment and ecology (Campana 1999). Recently, a new chemical aspect of biogenic carbonates, the organic matrix, has been utilized for reconstructions of environmental resource utilization by these organisms (McMahon et al. 2011b; a). These deductions are based on the glycoproteins of biogenic carbonates that are involved in the biomineralization process, which are incorporated and preserved in the carbonate matrix (Nagasawa 2014). Their amino acid (AA) constituents enable us to make direct links between the organism and the primary producers (PPs) in its direct environment. As a result of the inability of animals to biosynthesize essential amino acids (EAAs), these EAAs need to be acquired from their diet (Howland et al. 2003). Biosynthesis of EAAs are limited to PPs, as well as fungi and bacteria, which determine the isotopic signature of the EAA molecules (Macko et al. 1987; Larsen et al. 2012; 2013). With the isotopic signature of EAAs remaining unaltered across trophic levels, animal resource use can be traced back to the PPs at the base of the food web (McMahon et al. 2010). Biogenic carbonates, as well as proteinaceous corals, could thus provide us with a longterm record of changes in ambient resources or use by organisms with biogenic carbonates (Ellis et al. 2014; McMahon et al. 2015a) and potentially the movement of fish between these resources or habitats (McMahon et al. 2011a; 2012).

The chemical information on the organism's ambient environment over time stored within the biogenic carbonate increments is the strength of its utilization. It allows us to potentially get annual or bi-annual information on environmental changes experienced by the organism building the calcium carbonate structure. The sample volume needed for compound specific isotope analysis (CSIA) of $\delta^{13}C$ in AAs would therefore ideally match or be lower than can be extracted from the increments. However, to date the sample volumes in previous studies have been too large to sample down to incremental level, which range from 10 mg to 2 g (Silfer et al. 1994; O'Donnell et al. 2007; McMahon et al. 2011b; Ellis et al. 2014; Schiff et al. 2014;

McMahon et al. 2015a). This is often necessary due to the typically low protein contents of biogenic carbonates with 0.3-1.5% for most fish otoliths (Degens et al. 1969; McMahon et al. 2011b), 0.3-3% for corals (Goffredo et al. 2011; Falini et al. 2015) and 2-4% for gastropods (Palmer 1983). However, previous methods used to measure AAs in calcareous structures can also complicate high-resolution sampling of the informative increments of biogenic carbonates. Derivation methods performed on biogenic carbonates are often preceded by a desalting procedure to remove the calcium carbonate matrix. These desalting procedures can lead to sample losses and alteration of isotopic composition of AAs due to partially hydrolysed protein leading to partial loss of AAs (Brault et al. 2014), loss of water soluble proteins (Delmas et al. 1984; Tuross et al. 1988) or hydrolysis breaking amide bonds within AAs (Bada et al. 1989; Silfer et al. 1992). Such desalting procedures are thus preferentially avoided, although necessary to remove non-AA compounds that interfere with isotopic measurements of AAs. Extra non-AA compounds that are incompletely resolved from AAs are undesirable as these can contribute their δ^{13}C signal to that of the AAs and consequently alter the δ^{13}C measurement of the target AA (Meier-Augenstein 1999; 2004; Elsner et al. 2012). This is detrimental to the use of EAAs in ecological studies that revolve around the unaltered isotopic exchange of EAAs between PPs and consumers throughout the food web by diet. Analytical error of individual δ^{13}C-EAA measurements due to coelutions can therefore contribute to incorrect identification of PPs in consumer diets.

Therefore, the aim of this study was to show the application advantages of methoxycarbonyl esterification (Walsh et al. 2014) over *N*-acetyl methyl esterification (NACME; (Corr et al. 2007) to untreated biogenic carbonates that avoid desalting procedures. The NACME method was chosen over the more commonly used trifluoroacetic anhydride derivatization (TFAA) due to the produced hydrogen fluoride poisoning that reduces combustion efficiency in the IRMS interface (Dunn et al. 2011). However, the NACME derivatization chemicals and protocols are highly similar and thus it is expected that the results are also applicable to TFAA and pivaloylchloride methods.

Although it is generally uncommon to exclude desalting procedures, in this study they are purposefully omitted to show the improved chromatographic performance of MOC over NACME derivatized AAs from biogenic carbonates. Consecutively, this is used to display why MOC avoids ecological application errors and how this method enables high-temporal sampling of biogenic carbonates for CSIA of $\delta^{13}C$ in EAAs.

It is further explored how to optimize and acquire accurate $\delta^{13}C$ measurements of MOC derivatized EAAs by gas chromatography-combustion-isotope mass spectrometry GC-c-IRMS analysis, with different GC column polarities and display their derivative stability over two months. The chromatography of MOC derivatized and untreated calcareous samples is displayed for fish otoliths of *Cynoscion acoupa* and *Pterois volitans*, shell of the bivalve *Mercenaria campechiensis* and hard coral *Stylophora pistillata*.

Materials and procedures

Standards and chemicals

A 20 µg/mL standard mixture of 14 L-AAs in 0.1 M HCl was prepared from individual AA stocks. Included were aspartic acid, glutamic acid, glycine, isoleucine, lysine, proline, serine, threonine, tyrosine and valine (all Sigma-Aldrich, Germany). Additionally, part of this standard were also alanine, leucine, methionine and phenylalanine of which the $\delta^{13}C$ values were precisely determined versus Vienna PeeDee Belemnite (VPDB) standards by an off-line dual inlet method at S.I. Science Co., Ltd, Japan. This served to increase precision to the correction factors of the AAs and decrease laboratory specific measurement deviations. As reference compounds in the isotopic analysis of MOC AA esters the internal standards 6-aminocaproic acid (6-ACA), $C_{12:0}$ and $C_{20:0}$ fatty acid methyl esters (FAME) were used. The $C_{12:0}$ FAME was an in house standard, whereas $C_{20:0}$ FAME was a standard certified by A. Schimmelmann, Indiana University, USA. All AA standards and 6-ACA were individually determined for their $\delta^{13}C$ values with repeated EA-IRMS (EA 1108, Finnigan MAT, coupled via a conflow to a MAT 252 interface, Finnigan MAT) measurements against CO_2 reference gas, calibrated against IAEA-CH-6 and NBS 22 (IAEA reference standards). All stable isotope ratios are reported in δ-notation to VPDB scale. To correct for kinetic fractionation effects due to derivatization, each AA was measured on an EA-IRMS and on a GC-c-IRMS with high polarity VF-WAXms column and calculated with the mass balance equation described by Docherty *et al.* (2001). It has to be noted that acid hydrolysis converts glutamine and aspartamine into glutamic acid and aspartic acid respectively, and thus analysis includes isotopic signatures of both AAs. All chemicals used in the hydrolysis and derivatization procedures were of high grade purity and purchased from Sigma-Aldrich, Germany.

Sample preparation

Biogenic carbonate material used for analysis in this study originates from whole adult *C. acoupa* and *P. volitans* otoliths, bivalve shell of *M. campechiensis* and the skeleton of the hard coral *S. pistillata*. Otoliths were scrubbed and rinsed with ultrapure water in an ultrasonic bath for 5 minutes. Subsequently, they were dried in an oven at 40°C for 2 hours. The organic outer layer of the bivalve shell and coral skeleton were removed with hydrogen peroxide. Mortar and pestle were used to homogenize the otoliths and bivalve shell and samples were stored in a muffled glass vial. The coral skeleton was cut and carbonate was removed from the inner parts with a handdrill. Protein content of all sample types was estimated from the total nitrogen content with a Euro EA 3000 elemental analyser and standardized with a Low Soil Standard OAS.

N-acetyl methyl esterification (NACME)

Homogenized material of *C. acoupa* (5 mg), *P. volitans* (5 mg), *M. campechiensis* (10 mg) and *S. pistillata* (15 mg) was weighed into 4 mL glass vials with PTFE faced cap. Per mg of carbonate material, 0.1 mL HCl together with 10 µL of 1 mg/mL of 6-ACA and norleucine was added. The headspace was filled with N_2 gas and placed into an oven at 110 °C for 20 hours. After hydrolysis, the samples were dried in the 4 mL vials in a heated aluminium block at 110 °C for approx. 30 minutes under a gentle stream of N_2.

Derivatization of the dried sample was performed according to the method described by Corr *et al.* (2007). Samples were methylated with a mixture of anhydrous methanol and acetylchloride (1.85 M) and acetylated with a mixture of acetic anhydride, triethylamine and acetone (1:2:5, v/v/v). Intermediate evaporation steps were performed in an aluminium heating block at 30 °C under a gentle stream of N_2. The dry and derivatized sample was dissolved in 1 mL ethylacetate and 2x1 mL saturated sodiumchloride solution was added, vortexed and the top organic phase extracted. The extracted phase was evaporated and redissolved in 100 µL ethylacetate. Derivatized samples were stored in a refrigerator at 4 °C.

Methoxycarbonyl esterification (MOC)

The same amounts of homogenized materials were weighed into a 2 mL heavy-walled borosilicate glass micro-reaction vessels with a PTFE-faced rubber septum (Sigma-Aldrich, Germany). Per 1 mg of carbonate material, 0.1 mL of 6 M HCl was added together with 20 µL of 0.1 mg/mL of 6-ACA. The headspace was filled with N_2 gas to prevent oxidation and put into an oven at 110 °C for 20 hours. After hydrolysis, the samples were dried in the micro-reaction vials in a heated aluminium block at 110 °C for ~30 minutes under a gentle stream of N_2. The dried otolith sample was redissolved in 100 µL of 0.1 M HCl before derivatization.

The derivatization was performed in the micro reaction vials according to the procedure described by Walsh *et al.* (2014). Methanol (30 µL) and pyridine (35 µL) were added with gentle shaking for mixing and followed by 15 µL of methyl chloroformate with 1 minute of reaction time. Finally, 100 µL of chloroform was added using a 250 µL gas tight Hamilton syringe, with gentle shaking and put aside for 5 minutes to allow the organic and aqueous layer to separate. The organic bottom layer was transferred to a GC vial with a 250 µL insert and a small amount of sodium sulphate to remove water that potentially came along with the extraction. To avoid suction of sodium sulphate into the GC injector needle, 40 µL was subsequently transferred to a new GC vial with 250 µL insert together with 4 µL of $C_{12:0}$/$C_{20:0}$ FAME. Derivatized samples were stored in the refrigerator at 4 °C. For the long term $\delta^{13}C$ stability measurements, 25 mg of *C. acoupa* otolith material was hydrolysed with 2.5 mL of HCl. Derivatization was performed by using five-fold the volumes of respective chemical volumes and the derivatized samples were stored in a 1.5 mL GC vial with screw cap. Aliquots of 40 µL were taken from this stock and 4 µL of $C_{12:0}$/$C_{20:0}$ FAME was added before analysis.

Separation performance of MOC vs. NACME

The NAMCE and MOC derivatization were applied to all untreated biogenic carbonates and compared for chromatographic performance of AA separation with focus on the presence of background signals. All samples were analysed on a gas chromatography-mass spectrometer (GC-MS) and injected via a cold injection system (PTV, ThermoFisher Scientific). The Trace GC-Ultra (ThermoFisher Scientific) equipped with a J&W DB-5HT (30 m, 0.1 µm film thickness and 0.25 mm i.d.) was coupled to a MS (TSQ Quantum, ThermoFisher Scientific). The GC temperature program was 60°C (2 min) increased with 3°C/min. to 350°C and held at 350°C for 12 minutes. Mass spectrometry was performed at 70 eV (EI) over a scan range of 50 to 650 n at 1 scan/0.5 seconds.

Separation performance of MOC on different GC column polarities

For comparison of AA separation and δ^{13}C measurement stability, MOC AA esters were injected via a cold injection system (KAS3, Gerstel) onto a nonpolar J&W DB-5 (30 m, 0.25 µm i.d., 0.25 µm film thickness) and a polar VF WAXms (30 m, 0.25 µm i.d., 0.25 µm film thickness). The DB-5 was installed in a HP5890 GC and interfaced to a MAT252 IRMS (Finnigan MAT) via a GC-C II combustion interface equipped with a NiO/CuO/Pt combustion reactor operated at 940°C. The GC temperature program utilised was 60°C (2 min.), first increased with 3°C/min. to 250°C and held at 305°C for 15 min. The VF-WAXms was installed in an Agilent 6890 GC and interfaced to a MAT253 IRMS (1.5 mA emission with 68 V) via a GC/C III (ThermoFisher Scientific) combustion interface that was equipped and operated equal to the MAT252 IRMS. The GC temperature program utilised was 60°C (2 min) and increased with 3°C/min to 245°C and held for 20.3 minutes. All samples were injected via a cold injection system (CIS4, Gerstel) in the splitless mode. Both GC-IRMS systems were run under similar conditions with strong checks of reproducibility by using CO_2 reference peaks and in house standards. Thus, a drift or precision change caused by the instruments could be excluded.

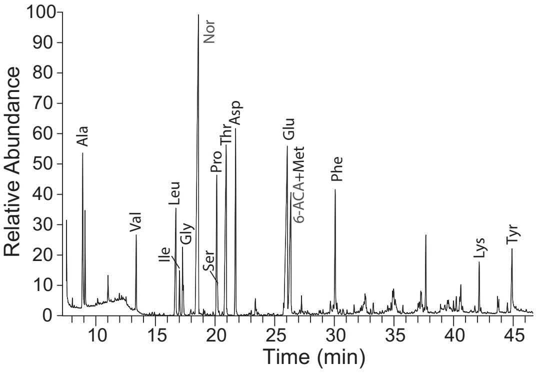
Repeated measurements of a single MOC derivatized otolith sample on the DB5 were performed on the day of derivatization (day 0) and repeated 7, 14, and 63 days later. The same MOC derivatized otolith and AA standard were repeatedly measured on the VF-WAXms on day 1, 42 and 56 and day 0, 30 and 63 respectively.

Assessment

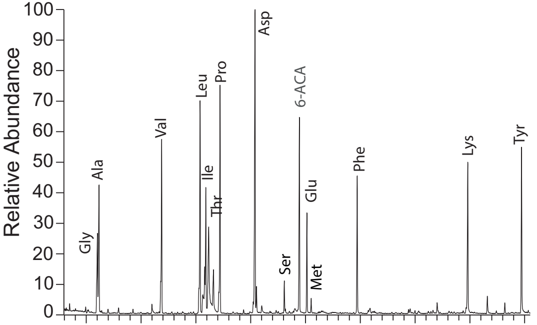
Chromatographic performance

GC-MS chromatography of NACME AA esters from the *C. acoupa* otolith shows additionally to the identified AAs many other signals that coelute or partially overlap with the AAs (Fig. 1A). The elution pattern of non-AA signals between 31-41 minutes is characteristic for the chromatography of NACME AA ester of all indicated calcareous samples and their intensity seemed to rely on the sample dryness during evaporation steps (data not shown). The origin of the extra compounds found in NACME chromatograms can be related to the interaction between the acid hydrolysis of $CaCO_3$ and the chemicals used in NACME derivatization procedure. Acid hydrolysing pure $CaCO_3$ will lead to the formation of a strongly hygroscopic $CaCl_2$, which can sometimes encase the water. Therefore, the evaporation of the HCl solution after hydrolysis is very difficult and incomplete dryness of the sample before derivatization is often the result. Within the derivatization procedure of NACME, there are a number of water reactive chemicals involved, i.e. acetylchloride and acetic anhydride. The reaction of these chemicals with the residual water in the sample can be observed during the derivatization procedure as ‘smoke’ development and a white residue in severe cases. In contrast, derivatization of the same *C. acoupa* otolith material with MOC shows chromatography with a very low amount of extra signals (Fig. 1B). The absence of water reactive chemicals in MOC procedures, hence, indicates that water related reactions are the cause for extra compounds in the untreated biogenic carbonate chromatography with NACME. Chromatography of MOC AA esters is consistent for carbonates of *M. campechiensis* bivalve (Fig 1C), *P. volitans* otolith, and *S. pistillata* coral carbonates (see Supplementary materials S1 and S2). The MOC AA esters signal intensities of several AAs are relatively increased compared to the NACME AA esters (e.g. lysine, valine), while others show a decreased signal (e.g. glutamic acid).

A) NACME - *C. acoupa* otolith



B) MOC - *C. acoupa* otolith



C) MOC - *M. campechiensis* bivalve

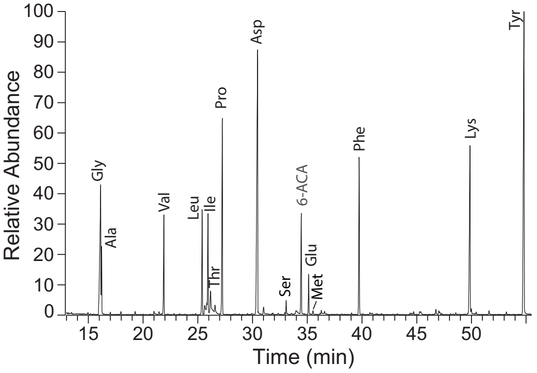


Figure 1. A comparison of GC-MS chromatograms of NACME AA esters (A) and MOC AA esters (B) from the same untreated *C. acoupa* otolith sample. Additionally, MOC AA esters in an untreated *M. campechiensis* bivalve shell sample (C) are also displayed. All samples were eluted on a J&W DB-5HT column (30 m, 0.1 µm, 0.25 µm i.d.).

Separation of MOC AA esters on two columns

The presence of derivatization chemical residues in MOC chromatography have often been reported (Hušek 1998; Zampolli et al. 2007; Chen et al. 2010; Walsh et al. 2014) and can be a source for $\delta^{13}C$ measurement inaccuracies. The nonpolar DB-5 was not able to completely separate these residues from the AAs, accomplish complete separation between AAs, and achieve stable retention of these residues on the column. Four coeluting AAs, glycine/alanine and threonine/isoleucine were observed and some additional non-AA peaks could be distinguished, in particular in front of valine and after isoleucine/threonine (Fig. 2A). Separation was much improved on the highly polar VF-WAXms coated capillary column and only partial coelutions were observed with glycine/leucine and serine/glutamic acid (Fig. 2B). Similar additional non-AA signals were observed on both the DB5 and the VF-WAXms, yet these were completely and constantly separated from the AAs on the VF-WAXms. Complete baseline resolution of glycine and leucine can be achieved with a 60 meter VF-WAXms column instead of 30 meters as in this study.

The voltages on the VF-WAXms column are twice as high compared to the DB5, mainly due to the higher sensitivity of the GC-c-IRMS with a MAT253. Eluting MOC AA esters on a DB5 column, however, required less time (70 min.) than on a VF-WAXms column (90 min.). Moreover, on the VF-WAXms lysine elutes comparatively late, resulting in an increased peak width, with tyrosine eluting later than lysine and could not be detected at the given concentration. This is a result of pronounced isothermal conditions at the end of the GC run and a disadvantage of polar GC columns, which allows comparable low temperature maxima (240°C VF-WAXms vs. 350°C DB5). As a result, the current use of a 30 meter VF-WAXms column for MOC AA esters displayed good baseline separation for the relevant EAAs, valine, isoleucine, threonine, phenylalanine (Fig. 2B), which is also observed for the *P. volitans* otolith, *M. campechiensis* bivalve shell and *S. pistillata* coral skeleton AA chromatography (see S3-5).

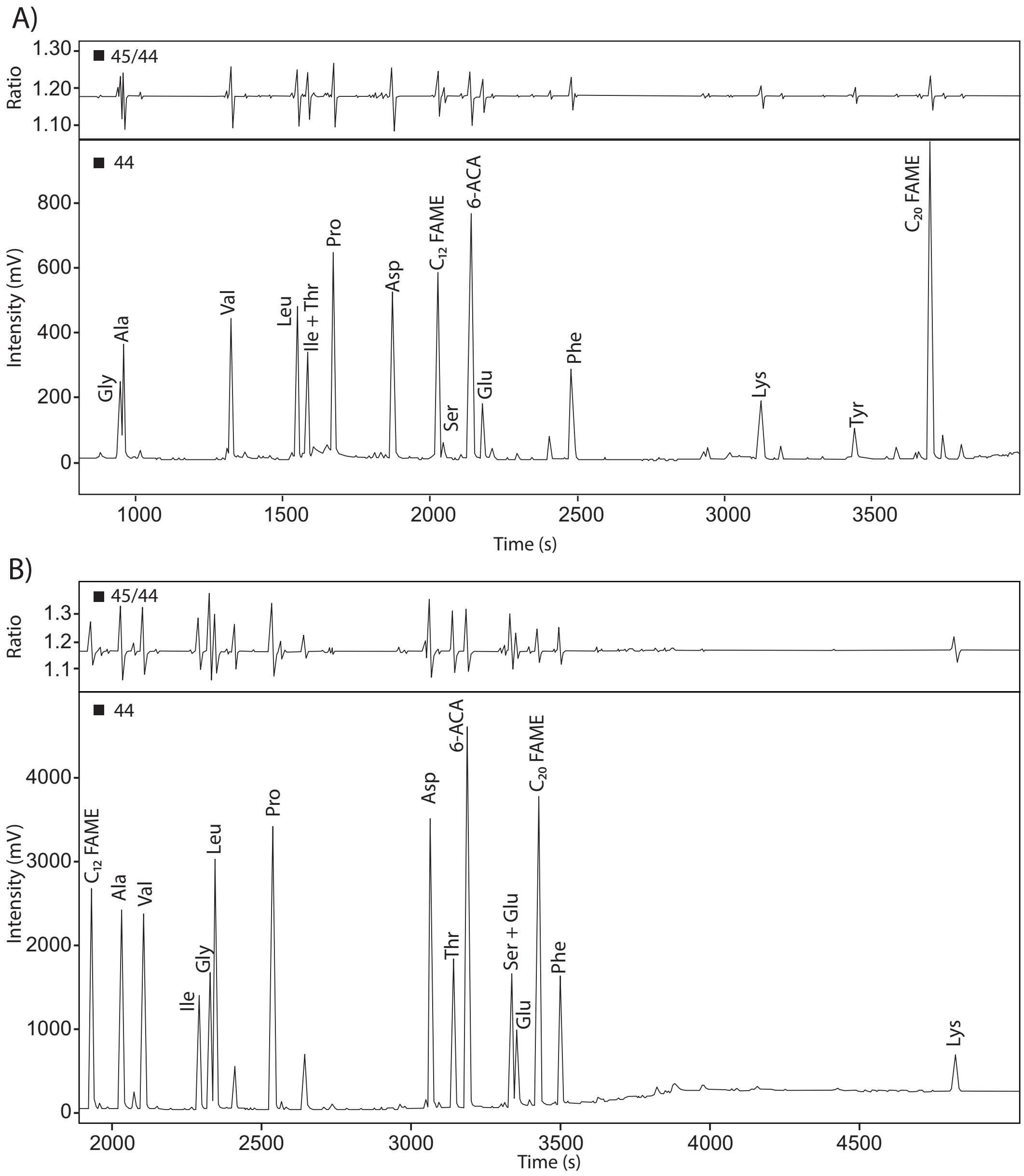


Figure 2. GC-c-IRMS chromatograms with 45/44 ratios of MOC AA esters from the same *C. acoupa* otolith sample. These were eluted on (A) a nonpolar J&W DB-5 column (30 m, 0.25 µm, 0.25 µm i.d.) and on (B) a highly polar VF-WAXms column (30 m, 0.25 µm, 0.25 µm i.d.).

Stability of MOC δ^{13}C-AA measurements

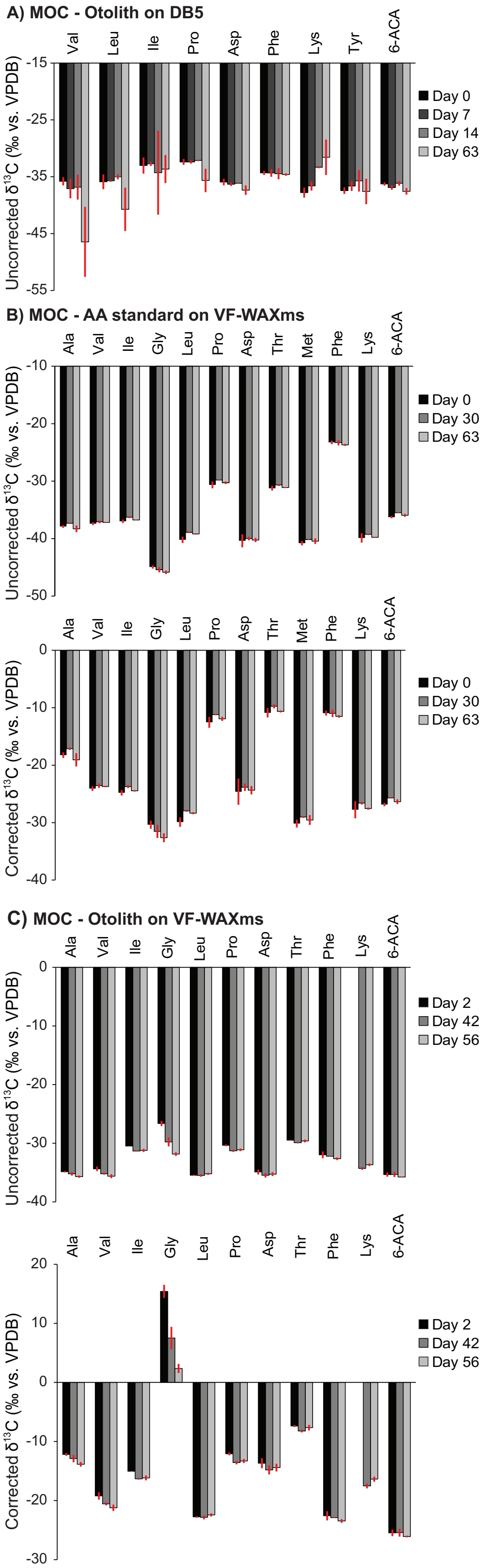
Accurate and long-term stable δ^{13}C values measurements of AAs can only be obtainable when there is a stable derivatization of the AAs and a good baseline separation by a GC column. Therefore, the observed incomplete baseline separation and instable retention behavior of the derivatization chemicals on the DB-5 column resulted in measurements with large $\delta^{13}C$ deviations within the three replicates injections and between measurement days (Fig. 3A). The $\delta^{13}C$ values shown in figure 3A represent raw data and are not corrected for the added carbon as that only enlarges the deviations. The uncorrected measurement errors were most apparent for valine (3.1 ± 2.2‰), leucine (1.4 ± 1.4‰), isoleucine (2.4 ± 2.9 ‰), lysine (1.2 ± 1.1‰) and tyrosine (3.1 ± 2.2‰). This can be linked to the partial coelutions of valine, leucine and isoleucine with non-AA compounds or other AAs as displayed in the chromatogram (Fig. 2A). Baseline separated AAs without coelutions, aspartic acid (0.40 ± 0.31‰), phenylalanine (0.52 ± 0.31‰), and the internal reference 6-aminocaproic acid (0.44 ± 0.11‰) had relatively stable $\delta^{13}C$ values over the entire period.

In contrast, the AA standard measured repeatedly on the VF-WAXms column over 63 days lead to more stable uncorrected $\delta^{13}C$ measurements with on average low standard deviations (0.30 ± 0.23‰) due to the increased baseline separation of the AAs. After correction for kinetic fractionation (Docherty et al. 2001) the average standard deviation increased (0.54 ± 0.45‰) due to error propagation (Fig. 3B).

Repeated measurement of the *C. acoupa* otolith sample also showed stable $\delta^{13}C$ measurements (raw SD 0.26 ± 0.17‰; corrected SD 0.48 ± 0.37‰), with the exception of glycine (corrected SD 1.26 ± 0.57‰) that was incompletely separated from leucine (Fig. 3C). Compared to the stable measurements in the AA standard, glycine $\delta^{13}C$ in the otolith sample shows a depletion in $\delta^{13}C$ values from day 2 to day 58. This could indicate an interference of glycine with sample matrix signals over time. Interestingly, correction of glycine entails a 40‰ increase, which is not displayed for glycine in the AA standard. Lysine was not measured on day 2 due to a technical issue. With the stability of MOC $\delta^{13}C$-AA measurements over a 2-month period, we show that MOC AA ester are more stable than described in previous studies which limited analysis time to measurement within 2 days (Chen et al. 2010) or 1 week (Hušek 1991; Walsh et al. 2014).

Complete separation was not achieved for all EAAs that are of interest for ecological studies. Glycine and leucine partially coelute, which implies that part of the leucine $\delta^{13}C$ signal is altered. This can be an issue when PPs show different intensities/concentrations of glycine and leucine compared to otoliths. When the increasing coelution alters the $\delta^{13}C$ measurement of leucine accordingly, the possibility of misidentifying the primary producers in consumer diets increases. Due to these implied inaccuracies we therefore suggest to exclude leucine for use in an ecological study until complete baseline resolution for leucine is reached, which is possible with a 60 meter VF-WAXms column.

It has to be mentioned with concern that some non-EAAs, i.e. glutamic acid and aspartic acid, are non-quantitatively derivatized with MOC. This is a trait of ethyl and methyl chloroformates that was previously observed for derivatization of amino and fatty acids (Meier-Augenstein 2004; Aoyagi et al. 2012). We also observed two signals for glutamic acid, from which one coeluted with serine. A side reaction of glutamic acid with methylchloroformate transforms it into 5-oxo-prolin methylester due to an internal condensation reaction. This might have consequences for isotopic fractionation of these particular AAs. However, redissolving AAs in 0.4M HCl instead of 0.1M HCl after acid hydrolysis can prevent this side reaction under a pH <1 (Yarnes and Herszage 2017). Nonetheless, based on sufficient concentration, complete baseline separation, and stability, our current MOC AA chromatography shows the utility of the EAAs valine, isoleucine, threonine, phenylalanine, and lysine for ecological studies concerning low protein containing calcareous structures.


A) MOC - Otolith on DB5

B) MOC - AA standard on VF-WAXms

C) MOC - Otolith on VF-WAXms

Figure 3. A) Repeated measurements of the *C. acoupa* otolith sample (uncorrected) on a DB5 column over 63 days. For comparison of stability of isotopic measurement

on a VF-WAXms column, B) an AA standard (uncorrected and corrected) over 63 days and C) *C. acoupa* otolith sample (uncorrected and corrected) over 56 days were analysed. Each day measurement is based on a triplicate injection of the sample.

Use and costs of MOC method

Due to the simplicity and low volumes of fewer chemicals, the MOC method is faster and less costly than conventional methods such as NACME. The use and reuse of micro reaction vessels lowers the costs for materials and facilitates easier extraction of the sample after organic separation with chloroform. However, high polarity columns tend to be slightly more expensive than nonpolar columns. Also the life expectancy of high polarity is often shorter than the nonpolar columns as the first tends to be more sensitive to oxygen and temperature exposure.

Discussion

Good AA chromatography for reliable isotope measurements often requires the removal of the non-AA matrix at a trade off with sample loss and unknown isotopic alterations. For biogenic carbonates specifically there have been different desalting procedures (Fig 4A) by filtration or acidification and aspiration (Ellis et al. 2014; Brault et al. 2014) or purified the sample of calcium after acid hydrolysis by cation exchange treatment (Serban et al. 1987; Silfer et al. 1994; Takano et al. 2010). In the current method comparison between NACME and MOC derivatization, it is shown for the first time that the minerals are not the direct cause for the extra compounds observed in the NACME chromatography. Such compounds were formed by the reaction of $CaCl_2$ retained water with the water reactive chemicals, i.e. acetyl chloride and acetic anhydride (Fig 4A). The NACME derivatization procedure and water reactive chemicals use are highly similar to TFAA derivatization acetylchloride and trifluoroacetic anhydride (McMahon et al. 2011b) and *N*-pivaloyl/isopropyl derivatization with thionyl and pivaloyl chloride (Takano et al. 2010). This is supported by GC-MS and GC-IRMS chromatography of derivatized AAs in untreated calcareous samples with *N*-pivaloyl/isopropyl and TFA derivatization methods also displaying extra signals in other studies (Takano et al. 2010; McMahon et al. 2011b).

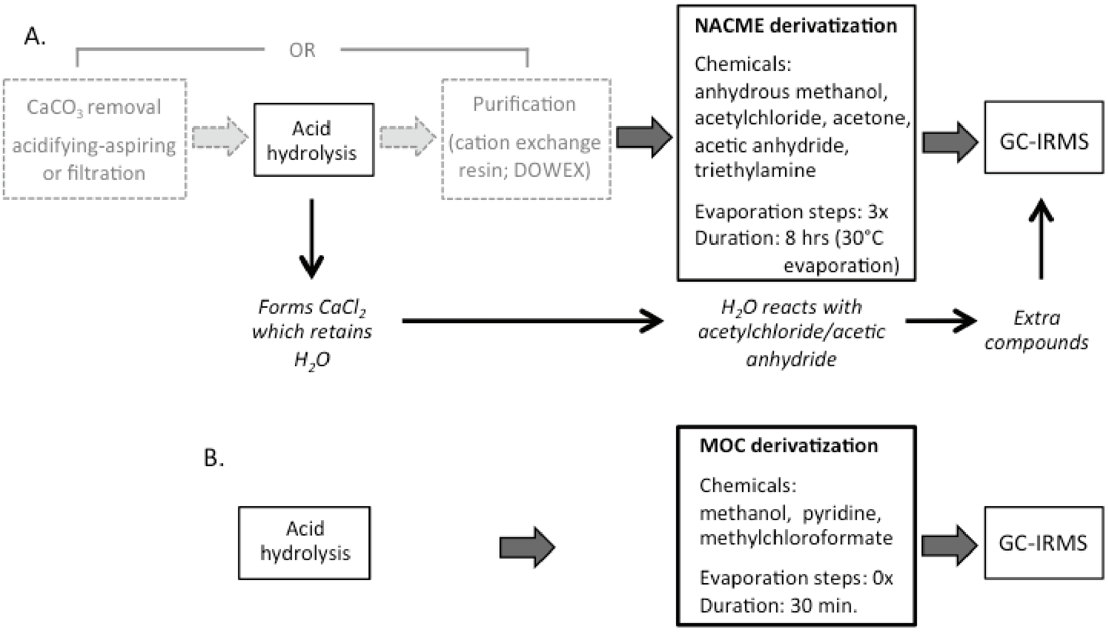


Figure 4. A) An overview of common preparation procedures and chemical requirements of the NACME method (similar to TFAA procedures) to biogenic carbonates. This includes the chemical consequences of applying NACME derivatization on untreated carbonates. B) In comparison, the MOC method applied to untreated $CaCO_3$, without additional procedures.

Thus, for derivatization methods as NACME, TFAA and *N*-pivaloyl/isopropyl desalting procedures are necessary to prevent water retention by $CaCl_2$ and obtain good chromatography. Such procedures have been observed to cause significant losses of certain AAs of up to 16% (Takano et al. 2010; Brault et al. 2014). Another type of AA loss that can occur with conventional methods as NACME and TFAA derivatizations is during evaporation steps of the derivatization procedures. Evaporation steps are sometimes performed at 60°C and can, especially with the hygroscopic $CaCl_2$, lead to overdrying volatilized AAs. To compensate for such AA loss during preparatory steps, sample volumes would have to be increased at the expense of high temporal sampling of small increments of biogenic carbonates. Without the need for purification/removal procedures for baseline resolved EAA chromatography, MOC derivatization thus avoids AA losses (Fig 4B).

The inherently good chromatography of untreated calcareous samples with MOC derivatization is also due to AA constituted compounds (i.e. proteins, proteoglycans and collagen) being the primary organic material in biogenic calcium carbonate matrices (Borelli et al. 2001; Falini et al. 2015). Other compounds identified in very low concentrations were lipids in corals and phosphorus containing compounds in crustaceans and polysaccharides in coccoliths (Farre et al. 2010; Nagasawa 2014). These compounds occur in such low concentrations that we do not expect them to have a significant effect on the $\delta^{13}C$ measurements of the target AAs. The MOC method without purification will not be as effective on for example bone material compared to biogenic carbonates due to the larger amounts of other compounds, including fatty acids. Particularly fatty acids (Aoyagi et al. 2012; Leggio et al. 2012) and di- and tricarboxylic acids (Villas-Bôas et al. 2003) are known to be simultaneously derivatized by methyl chloroformate. Consequently, the analysis of PP material, i.e. leaves, fungi, etc., with MOC derivatization should not be conducted without a purification method such as cation exchange resins. As the aforementioned compounds can occur in high concentrations in these PP materials, they can form another source for co-elution and analytical inaccuracy.

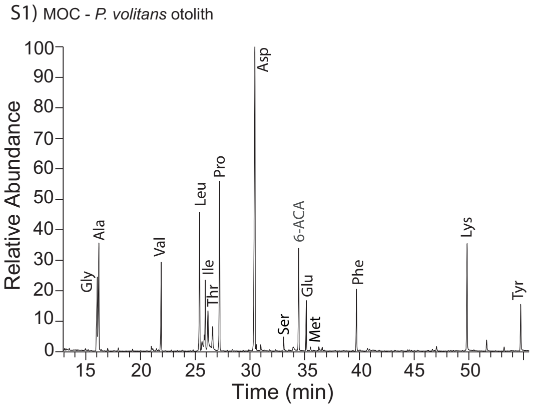
MOC derivatization therefore can decrease sample volumes to levels that are not possible with conventional derivatization methods by avoiding losses due to purification methods and procedural evaporations. Compared to untreated otolith carbonate of *Lutjanus ehrenbergii* with a similar protein content of 0.6% to 0.7% in *C. acoupa* otoliths, 10 mg was derivatized as TFA AA esters (McMahon et al. 2011b). In this study, that volume was reduced to 5 mg with MOC-AA esters without carbonate removal and relatively high injection volumes (2µL) allowed by the separation ability of the high polarity column.

Comments and recommendation

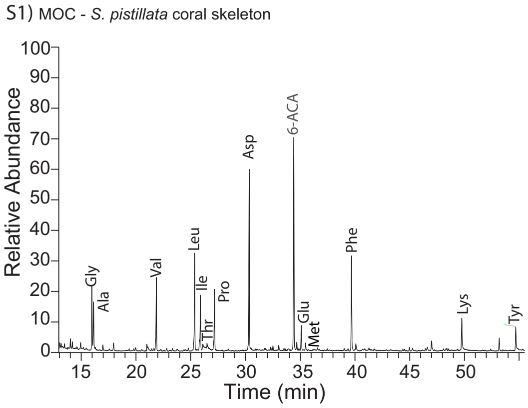
Low volume sampling of pure biogenic carbonate structures was achieved due to the MOC derivatization outperforming the more conventional method of NACME derivatization by providing chromatography without extra derivatization produced compounds. It avoids analyte loss due to the purification procedures and evaporation steps needed to get good chromatography with conventional methods. MOC derivatization, therefore, allows for stable δ^{13}C-EAA analysis over 2 months in small calcareous sample volumes with low AA content due to the high separation abilities of a high polarity column.

It might be feasible to further decrease the amounts needed to extract from biogenic carbonates and subsequently make this analysis available for smaller structures. The possibility remains to evaporate the chloroform extracted after the organic separation under a stream of N_2 at room temperature and redissolved in 15-30 µL of chloroform in low volume GC vials. As a result, we predict that the sample volume can be drastically reduced to ~1 mg with the 0.7% protein content of *C. acoupa* otoliths. Another option would be to slightly increase the section thickness that allows drilling deeper and more carbonate from the same microstructure. Yet, the increased section thickness should not lead to incorporation of other underlying growth increments, which would result in a loss of temporal resolution.

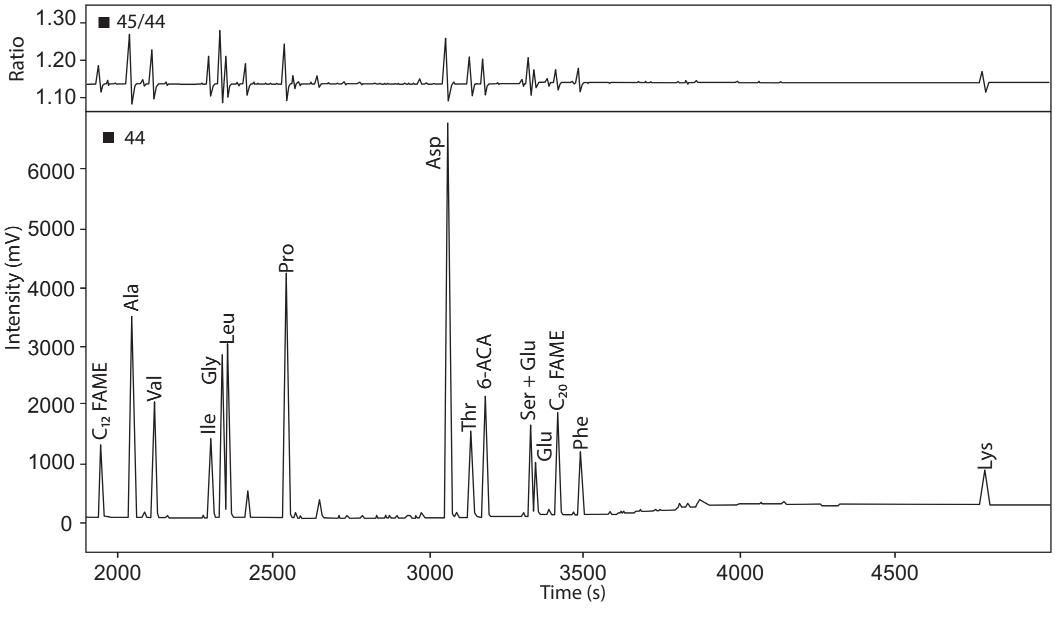
Supplementary Materials



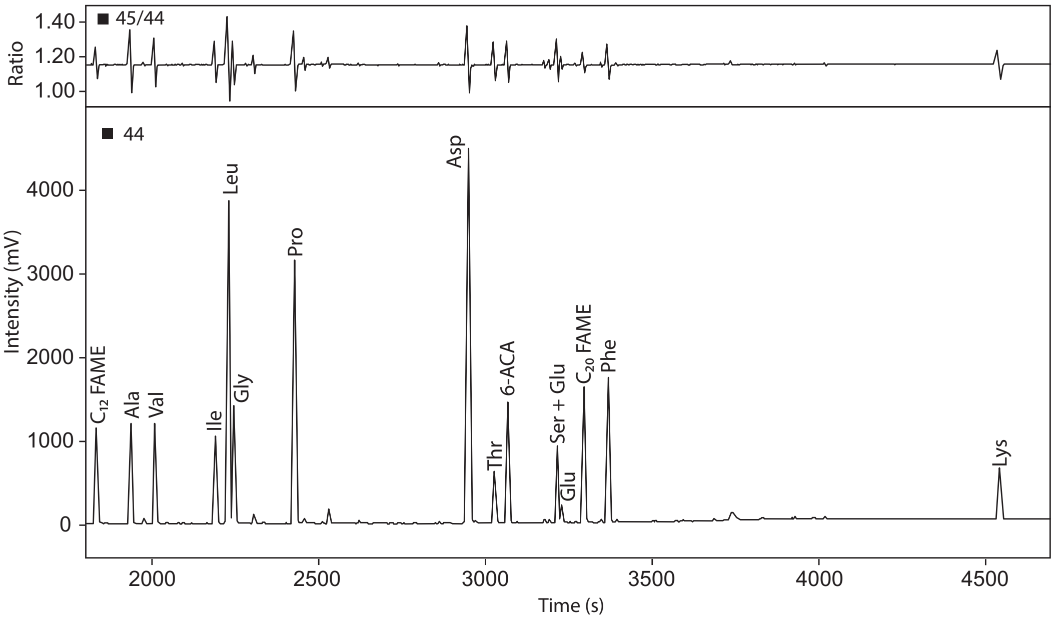
S1. GC-MS chromatography of MOC-AA ester in untreated *Pterois volitans* otolith on a J&W DB-5HT (30m, 0.1 µm, 0.25 µm i.d.).



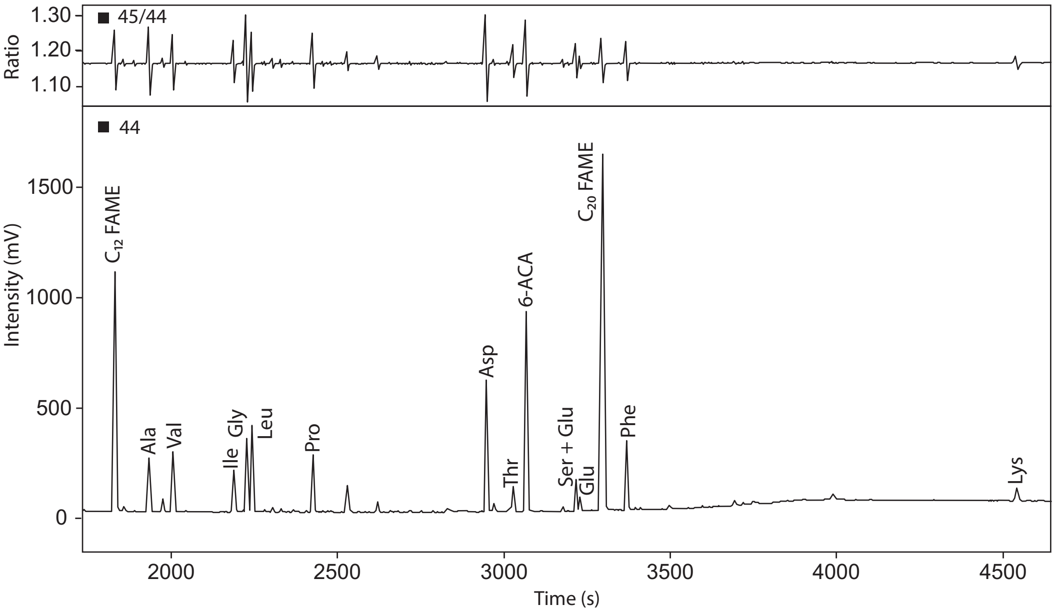
S2. GC-MS chromatography of MOC-AA ester in untreated *S. pistillata coral* skeleton on a J&W DB-5HT (30m, 0.1 µm, 0.25 µm i.d.).



S3. GC-IRMS MOC-AA chromatography of *P. volitans* otolith with a protein content of 1.13% (5 mg) on a VF-WAXms column.



S4. GC-IRMS MOC-AA chromatography of *M. campechiensis* bivalve shell with a protein content of 0.37% (10 mg) on a VF-WAXms column.



S5. GC-IRMS MOC-AA chromatography of hard coral *S. pistillata* with a protein content of 0.03% (12 mg) on a VF-WAXms column. Sample amount has to be at least doubled for $\delta^{13}C$ AA analysis.

Chapter 3

Ontogenetic resource utilization and migration reconstructions with $\delta^{13}C$ values of essential amino acids in the *Cynoscion acoupa* otolith

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Submitted to Ecology and Evolution

Summary

1. With the increasing anthropogenic impacts on fish habitats, it has become more important to understand which primary resources sustain fish populations. This resource utilization can differ between life stages of a fish species and individuals can migrate between habitats in search of resources. Such lifetime information is difficult to obtain due to the large spatial and temporal scales of fish behaviour. The otolith organic matrix has the potential to indicate this resource utilization and migration with δ^{13}C values of essential amino acids ($\delta^{13}C_{EAA}$), as they are a direct indication of the primary producers.

2. The *Cynoscion acoupa* is an example of a fish species with distinct ontogenetic migration patterns. While it inhabits the Brazilian mangrove estuaries during juvenile stages, it typically moves to the coastal shelf as an adult. In this study we investigated whether $\delta^{13}C_{EAA}$ values in otoliths can reconstruct *C. acoupa* resource utilization and migration.

3. By analysing the otolith edges across a size range of 11 to 119 cm, we found that baseline $\delta^{13}C_{EAA}$ values increased with size indicating estuarine to coastal shelf distribution. This trend is highly correlated with bulk inorganic δ^{13}C values. By utilizing source diagnostic $\delta^{13}C_{EAA}$ patterns, we found that estuarine algae rather than mangrove-derived resources supported the juvenile *C. acoupa* populations. Around the size of 40 cm, the resource utilization of juveniles and adults overlapped and mean $\delta^{13}C_{EAA}$ values increased, indicating a migration to coastal shelf habitats. This trend was confirmed by comparing otolith core and edges, although with some individuals potentially migrating over longer distances than others.

4. Hence, $\delta^{13}C_{EAA}$ values in otoliths have great potential to reconstruct ontogenetic shifts in resource use and habitats. The insight could aid in predictions on how environmental changes affect fish populations by identifying the controlling factors at the base of the food web.

Keywords: Amazon, carbon isotopes, life history, otolith chemistry, organic matrix

Introduction

Fish populations are increasingly influenced by human activities such as removal of estuarine vegetation for rural development, discharge of chemicals, climate change and ocean acidification (Jackson 2001b; Halpern et al. 2008; Cheung et al. 2015; Nagelkerken et al. 2015a). To understand the impact of these habitat changes to the stability of fish populations, it is imperative to know which resources sustain them. By providing the resources at the bottom of the food web, primary producers are a major factor in determining the productivity at lower trophic levels and thus also predatory fish populations at higher trophic levels (Pauly and Christensen 1995; Lynam et al. 2017). The use of resources can vary during fish life stages as is indicated by their utilization of distinct habitats or niches in an ecosystem (Huijbers et al. 2013; Kimirei et al. 2015). Moreover, fish actively undertake periodic migrations to synchronise their spawning time towards seasons with optimal resources for themselves and their offspring (Nakazawa and Yamamura 2006; Dingle and Drake 2007). Therefore, each life stage is sensitive to changes among primary producers, due to for example anthropogenic changes in the ecosystem, which affect the entire population. Shifts among primary producers affecting fish populations are not well characterized, as observational studies are often inconclusive in identifying key primary producers or habitat value for a fish species. Detecting a fish in a particular habitat does not directly identify the primary producers sustaining the food web it feeds on (Kruitwagen et al. 2010; Seitz et al. 2014). This lack of knowledge also impairs effective conservation strategies for maintaining fish populations (Sheaves et al. 2015).

Characterizing resource utilization by juvenile stages is key for understanding population dynamics as they determine the recruitment of the adult population. How habitats are used by juveniles has been difficult to resolve and can be attributed to the complications of tracking especially small juveniles between habitats, which can occur periodically and over large spatial scales (Beck et al. 2001; Gillanders et al. 2003; Dahlgren et al. 2006). For this reason, fish otolith inorganic chemistry has been often utilized to qualify the movements of fish between habitats as otoliths provide a complete record of the fish’s lifetime (Campana 1999; Elsdon et al. 2008). The otoliths calcium carbonate is accreted continuously from the early embryonic stage throughout the individual's life with often clear growth rings that are an indication of its age (Campana and Neilson 1985). Simultaneously, the otolith calcium carbonate matrix is metabolically inert and retains the environmental chemistry in terms of minor and trace elements and their isotopes (e.g. strontium, barium, bulk $\delta^{13}C$, bulk $\delta^{18}O$). By interpreting elemental concentrations or isotopic values in the otolith one could deduce whether the fish individual traversed distinct chemical environments such as freshwater and marine water habitats (Secor et al. 1995; Bath et al. 2000). However, there is growing evidence that the physiological and metabolic pathways of the fish have implications for the elemental concentrations and bulk $\delta^{13}C$ values in the otolith (Radtke et al. 1987; Campana 1999; Solomon et al. 2006; Geffen 2012; Sturrock et al. 2014). This causes uncertainty in the extent to which otolith inorganic chemistry can be interpreted for movement patterns and what is caused by physiological and metabolic changes (Chang and Geffen 2013; Grønkjær 2016).

The recent utilization of the otoliths organic part, consisting primarily of proteins, could provide a more direct and unperturbed link between fish and its ambient environment (McMahon et al. 2011b). Amino acids (AAs) are the building blocks of proteins and about half of the 20 protein AAs are essential for metazoans, because they cannot synthesize them *de novo* (Howland et al. 2003). Therefore, metazoans depend on the essential amino acids (EAA) through dietary sources. These EAAs can be traced to their biosynthetic origins such as algae, bacteria, fungi and vascular plants because each taxa produces characteristic $\delta^{13}C$ patterns among individual EAAs during biosynthesis (Macko et al. 1987; Larsen et al. 2009; 2012; 2013). Moreover, $\delta^{13}C$ baseline values of AAs among primary producers display geographical variations (i.e. isoscapes) and can therefore be utilized to reconstruct fish movement and habitat connectivity (McMahon et al. 2012; Vokhshoori et al. 2014). Provided that the carbon skeletons of EAAs remain intact during trophic transfers (McMahon et al. 2010), both baseline values and source diagnostic $\delta^{13}C$ patterns of EAAs ($\delta^{13}C_{EAA}$) can be used as a marker of primary producers (Larsen et al. 2013; Arthur et al. 2014). Thus, the otoliths temporal increments constitute an archive of lifetime resource utilization and migration through the fish's life stages.

However, only a limited amount of studies have looked into the potential of using the otolith organic matrix (McMahon et al. 2011b; a; 2012; Grønkjær et al. 2013).

The known distribution of the acoupa weakfish, *Cynoscion acoupa*, from mangrove estuaries in the early life stages to offshore coastal shelf areas as adults (Barletta et al. 1998; 2003) suggests an ontogenetic migration between the two habitats and changes in resource utilization. Although it is a commercially important species occurring along the entire coast of Brazil, little is known about its foraging ecology or migration. Thus, baseline $\delta^{13}C_{EAA}$ values and source diagnostic $\delta^{13}C_{EAA}$ patterns from *C. acoupa* otoliths could provide more insight into its ontogenetic resource utilization and migration. We collected fish otoliths of *C. acoupa* individuals across a size range to identify habitat change and primary production resources along the ontogenetic development of this species. The main aim of this study is therefore to investigate the potential of compound specific isotope analysis of $\delta^{13}C$ in EAAs of otoliths to extract lifetime records of movement and resource utilization of *C. acoupa*. This study explores the possibilities by answering the following questions: 1) Are baseline $\delta^{13}C_{EAA}$ values in otolith edges of *C. acoupa* individuals at different lengths consistent with the isotopic trend displayed by otolith inorganic $\delta^{13}C$ values? 2) Can we detect a pattern of lifetime resource utilization and distribution from estuary to coastal shelf by *C. acoupa* among different size classes? 3) Will the analysis of otolith core and edge reflect the expected estuarine to coastal shelf migration of *C. acoupa*?

Materials and methods

SAMPLING LOCATIONS

Sampling of *C. acoupa* otoliths was conducted in Bragança, one of the main landing ports in the state of Pará, northern Brazil. All 52 *C. acoupa* individuals with a standard length (SL) of 11-119 cm were collected from local fishermen at the Caeté river and the fish market in Bragança. Otoliths were removed, cleaned with demi-water and stored dry. Primary producers (PPs) were collected at the Caeté river mouth close to where the juvenile fish were collected by the fishermen. This area is characterized by macrotides of 4-5 meters and is dominated by large mangrove deltas. No seagrass or coral reefs are found in this region. Leaves of the *Rizophora mangle* mangrove tree, a rhodophyte growing on the mangrove roots *Bostrychia* sp., the freshwater algae *Spirogyra* sp., and degraded brown mangrove leaves, representing the bacterial community, were collected in the Caeté estuary. Coastal zooplankton, consisting predominantly of *Acartia tonsa*, was collected with a plankton net (mesh size 300 µm) to represent the coastal phytoplankton community. All PP samples were stored in a -20 degree freezer, rinsed with milliQ water and freeze dried.

OTOLITH PREPARATION

Juvenile otoliths of *C. acoupa* individuals between 11-25 cm SL were used whole, cleaned in an ultrasonic bath with ultrapure water and dried in the oven for 3 hours at 40 °C. Subsequently, otoliths were homogenized with mortar and pestle and stored in a combusted glass vial. Otoliths of post-juvenile and adults between 25-119 cm were embedded in Araldite 2020 epoxy resin. Embedded otoliths were cut with a diamond saw through the centre for a section of approximately 2 mm thickness. Sections were then glued with epoxy resin on a glass slide. Apart from an acetone wipe, the section surface was untreated to prevent contamination. The edges of these sectioned otoliths were micro-milled for a 5 mg sample. In a subsample of 7 otoliths also the cores were micro-milled according to the dimensions of the smallest otolith in the collection originating from an 11 cm SL *C. acoupa* individual.

OTOLITH BULK δ^{13}C ANALYSIS

The inorganic calcium carbonate δ^{13}C values from 42 homogenized *C. acoupa* otoliths and micro-milled otolith edges were measured on a Finnigan MAT 251 gas isotope ratio mass spectrometer. This was connected to a Kiel III automated carbonate preparation device where samples react with phosphoric acid at 74°C. Data are reported in delta-notation versus Vienna-Pee Dee Belemnite standard (V-PDB). The instrument was calibrated against the house standard (ground Solnhofen limestone), which in turn was calibrated against the NBS 19 standard reference material. Over the measurement period, the standard deviations of the house standard were 0.04‰ for δ^{13}C values.

OTOLITH δ^{13}C-AA ANALYSIS

The 5 mg of all homogenized and micro-milled otolith powder and 1 mg PP samples were hydrolysed with 100 µL of 6M HCl per mg of sample in a micro reaction vessel and 4 ml glass vial respectively. A reference of 20 µL 6-aminocaproic acid at the same time. After flushing the vials with nitrogen, the samples were heated at 110 °C for 20 hours. The acid was then evaporated in a heating block at 110°C for approximately 30 minutes. After acid hydrolysis only the PP samples were purified with a DOWEX 50WX8 (hydrogen form, 100-200 mesh) column and eluted with 2M ammonium hydroxide. This was then evaporated to dryness in a 110°C aluminium block for approximately 1 hour. The samples were then redissolved in 200 µL of 0.1M HCl and 100 µL was transferred to a micro reaction vessel. All otolith and PP samples were then derivatized with the methoxycarbonyl esterification protocol (Walsh et al. 2014). This included the addition of 35 µL methanol, 30 µL pyridine, and 15 µL methyl chloroformate. With a Hamilton syringe 100 µL of chloroform was added, mixed and rested for 5 minutes to separate into organic layers. The bottom chloroform layer was removed and transferred to a GC vial with 250 µL insert with a small amount of sodium sulphate. To prevent the sodium sulphate grains getting into the injector of the GC-c-IRMS, 40 µL was again transferred to a new GC vial with 250 µL insert and 4 µL of $C_{12:0}$/$C_{20:0}$ FAME standard was added.

A 2 µL aliquot of the sample was then injected in an Agilent 6890 GC with a VF-WAXms column (30 m, 0.25 µm i.d., 0.25 µm film thickness) and interfaced to a MAT253 IRMS (Finnigan MAT) via a GC-C II combustion interface. The GC temperature program utilised was 60°C (2 min), increased with 3 °C/sec. to 245 °C and held for 20.3 minutes. All samples were injected via a cold injection system (CIS4, Gerstel) in the splitless mode.

The δ^{13}C values of EAAs are shown as baseline δ^{13}C$_{EAA}$ values which are corrected for kinetic fractionation due to derivatization (Docherty et al. 2001). These are then converted to mean centred δ^{13}C$_{EAA}$ values and are calculated by subtracting each individual δ^{13}C$_{EAA}$ values from the mean δ^{13}C values of all EAAs. The δ^{13}C variability among EAAs for each measured sample is referred to as δ^{13}C$_{EAA}$ patterns (Larsen et al. 2009).

Results

OTOLITH BULK δ^{13}C VERSUS MEAN $\delta^{13}C_{EAA}$ VALUES

Otolith inorganic and organic baseline $\delta^{13}C_{EAA}$ values were compared among *C. acoupa* individuals ranging between 11-119 cm SL by analysing bulk inorganic δ^{13}C and mean $\delta^{13}C_{EAA}$ values of the same otoliths. These δ^{13}C values were tightly correlated (rho= 0.70, $p < 0.001$, Fig. 1A), but with the inorganic fraction being 10‰ more enriched than the organic fraction. While both the inorganic and organic δ^{13}C values become more positive with increasing SL, the range in mean $\delta^{13}C_{EAA}$ values was greater than for bulk inorganic δ^{13}C (2.5‰ vs. 4‰ respectively, Fig. 1B).

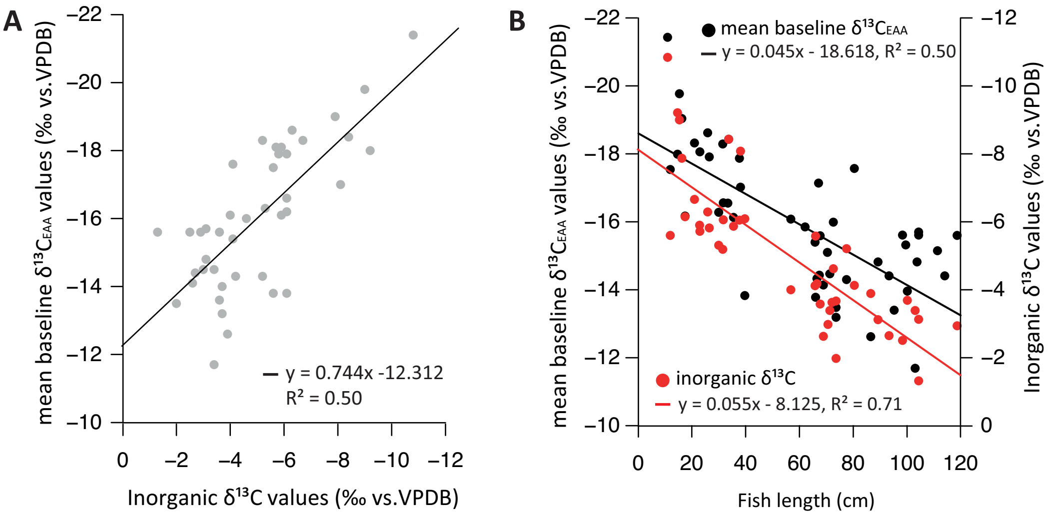


Fig. 1. A) Mean organic baseline $\delta^{13}C_{EAA}$ values versus the bulk inorganic δ^{13}C values measured in the same otolith material (N=42). B) Mean otolith organic baseline $\delta^{13}C_{EAA}$ values (N=52) and a subsample of the same otoliths measured as bulk inorganic δ^{13}C values (N=42) from individuals with a standard length from 12-119 cm.

INDIVIDUAL $\delta^{13}C_{EAA}$ VALUES IN OTOLITHS AND PRIMARY PRODUCERS

Coastal primary producers as mangrove root rhodophytes, degraded and fresh mangrove leaves had overall more negative baseline $\delta^{13}C_{EAA}$ values than the aquatic freshwater algae, and phytoplankton (Fig. 2A). Also, the baseline $\delta^{13}C_{EAA}$ values in otoliths from 12-119 cm SL individuals display an overall increasing trend from small to large individuals (Fig. 2B). When baseline $\delta^{13}C_{EAA}$ values of the PPs are converted to mean centred $\delta^{13}C_{EAA}$ values, distinctive $\delta^{13}C_{EAA}$ patterns are observed that are produced mainly by large variations in Thr and Phe (see fig. S1A). The same $\delta^{13}C_{EAA}$ pattern approach applied to the otolith measurements, resulted in a gradually changing trend across the 12-119 cm individuals. This trend consists of increasing Thr $\delta^{13}C$ values, while the $\delta^{13}C$ of Lys and Phe decreased from small to large sized individuals. In contrast, the mean centred EAAs, Val and Ile, did not show noticeable change in $\delta^{13}C$ values across different sizes. Individuals of 11-40 cm SL show similar $\delta^{13}C_{EAA}$ patterns and a transition to a different $\delta^{13}C_{EAA}$ pattern from 58-119 cm SL individuals (Fig. S1B).

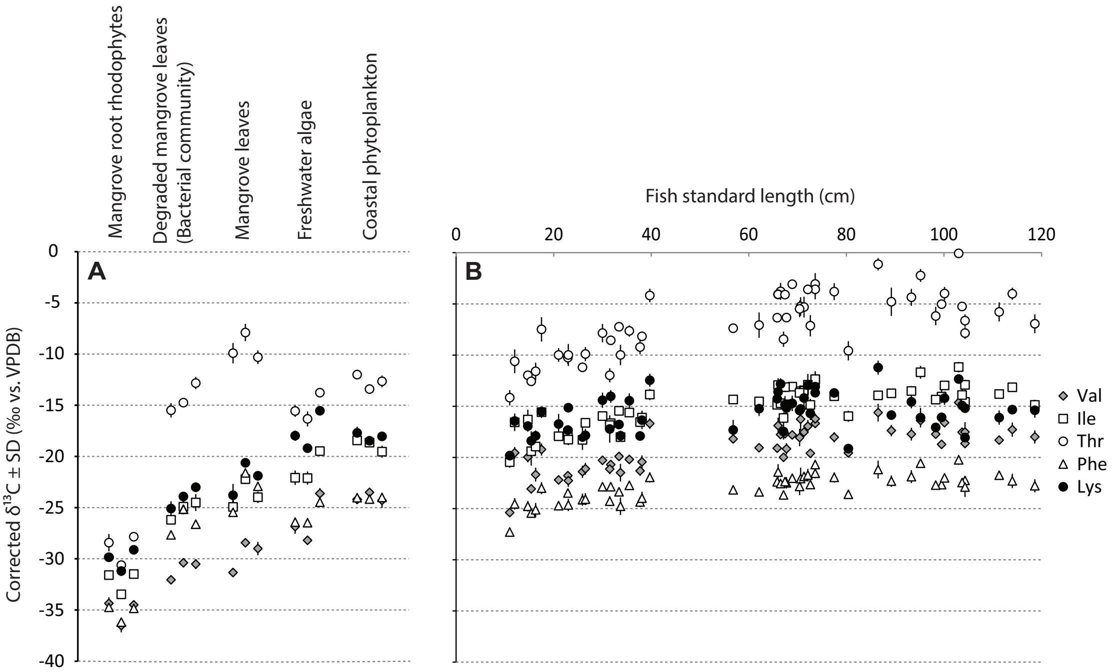


Fig. 2. Individual $\delta^{13}C$ values of Val, Ile, Thr, Phe and Lys with standard deviation from A) primary producers and B) whole fish otoliths from *C. acoupa* individuals with 11-25 cm SL and otolith edges from individuals with 25-119 cm SL. All measurements are based on triplicate injections.

To compare $\delta^{13}C_{EAA}$ patterns among otoliths and PP sources, a principal component analysis (PCA) was applied to investigate the relationship between individual AAs rather than variability in baseline $\delta^{13}C$ values among samples. This displayed that the first two PCA components account for 88% of the variation with the first component separating freshwater from marine resources and the second component separating algal from terrestrial resources. Three EAAs, Phe, Thr, and Val explain most of the variation of the first two components. The $\delta^{13}C_{EAA}$ patterns of fish otoliths resemble coastal phytoplankton and freshwater algae more than mangrove root rhodophytes and degraded/fresh mangrove leaves. This resemblance is stronger for juvenile otoliths than for adult otoliths (Fig. 3A). To assess the extent of *C. acoupa* specimens supported by marine resources, the PCA points were colour coded. Warm colours (red) represent more positive baseline $\delta^{13}C_{EAA}$ values (mean of all $\delta^{13}C_{EAA}$ values) and vice versa for cold colours (blue). The colour gradient shows that adults have more positive baseline $\delta^{13}C_{EAA}$ values than juveniles and coastal resources, and that about half of the juveniles share baseline $\delta^{13}C_{EAA}$ values with coastal resources (Fig. 3B).

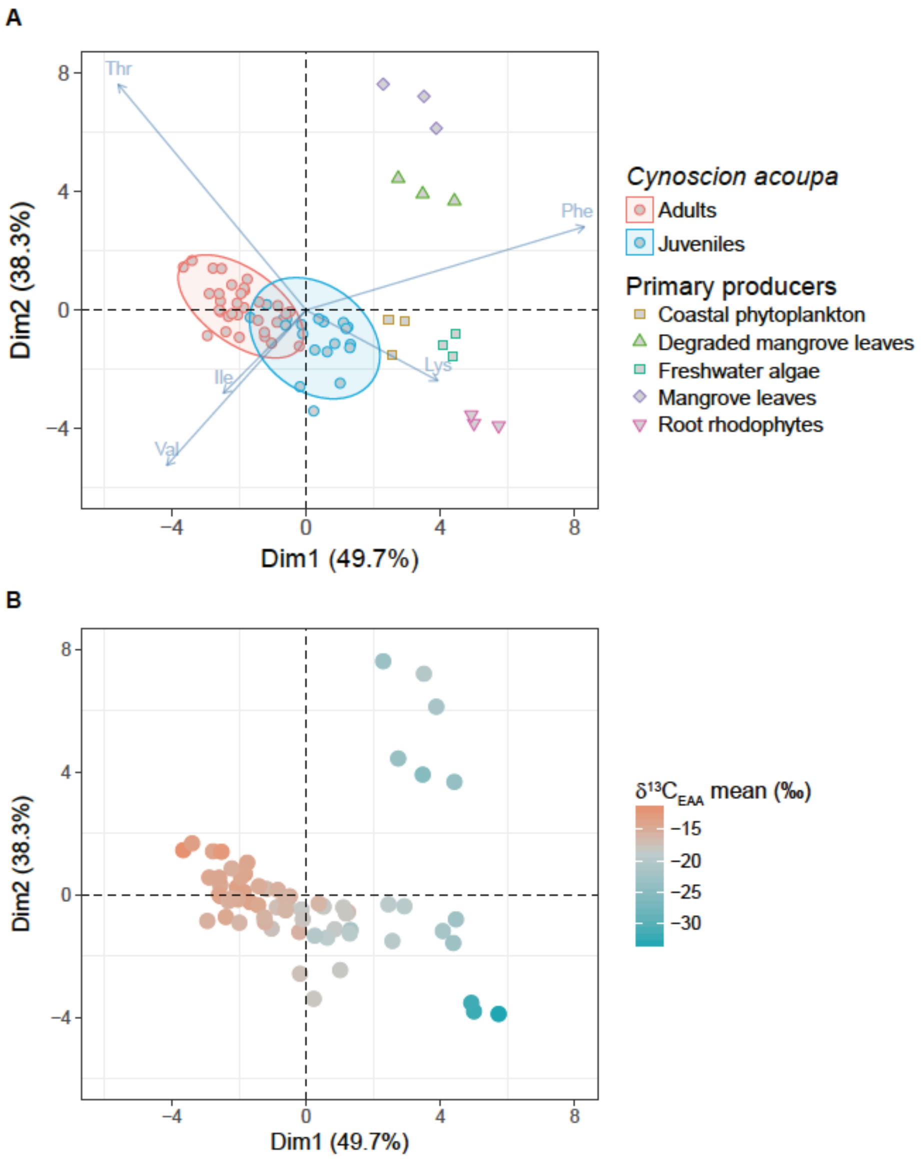


Fig. 3. A) Principal component analysis of $\delta^{13}C_{EAA}$ patterns in adult and juvenile otoliths and primary producers. B) Indication of the mean baseline $\delta^{13}C_{EAA}$ values overlaid on the PCA of the $\delta^{13}C_{EAA}$ patterns to display the change in isotopic values in PPs and otoliths from juveniles and adults.

$\delta^{13}C_{EAA}$ VALUES IN OTOLITH CORE AND EDGE

The seven *C. acoupa* otoliths of individuals with 38-110 cm SL, with cores micro-milled according to the otolith size dimensions of an 11 cm SL individual, show consistently lower baseline $\delta^{13}C_{EAA}$ values in the cores than in the edges. However, the baseline $\delta^{13}C_{EAA}$ values measured within individual otolith cores are very distinct from those measured in the otolith of the 11 cm individual and show considerable isotopic variation (Fig. S2A). More similarities between individual cores and edges are observable when converted to $\delta^{13}C_{EAA}$ patterns (Fig. S2B).

A linear discriminant function analysis was applied to investigate the commonality between $\delta^{13}C_{EAA}$ patterns from core and edge samples and their ability to distinguish between habitats and thus display ontogenetic habitat shifts of *C. acoupa*. Based on the four most informative EAAs in otoliths with the most pronounced estuarine and coastal shelf $\delta^{13}C_{EAA}$ patterns, the otolith core and edge measurements display potential habitat shifts. Practically all individuals, excluding SL38, have core $\delta^{13}C_{EAA}$ patterns that tend more towards estuarine $\delta^{13}C_{EAA}$ patterns than the edge measurements. However, some core or juvenile values, SL71 and SL87, overall incline more towards coastal shelf $\delta^{13}C_{EAA}$ patterns and approach edge or adult patterns (Fig. 4).

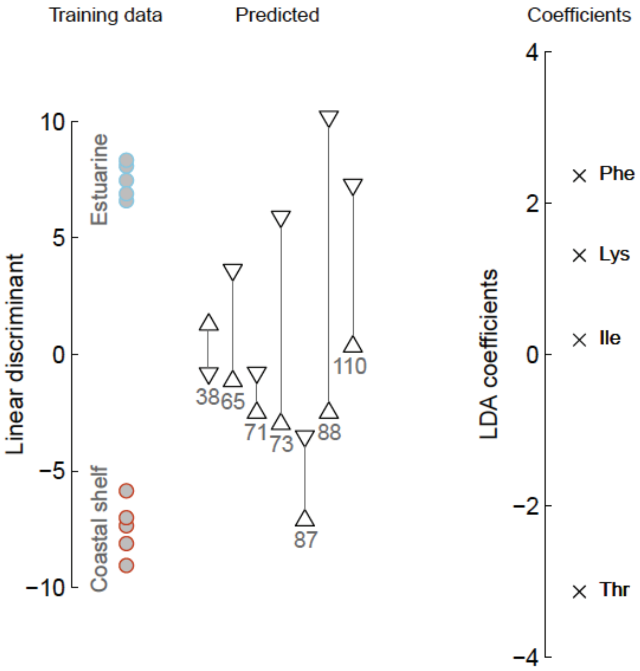


Fig. 4. Selected training data with the most pronounced estuarine and coastal shelf $\delta^{13}C_{EAA}$ patterns from 5 *C. acoupa* otolith samples each. These two habitats were differentiated by four of the most informative EAAs (Phe, Lys, Ile and Thr) and displayed on the right as coefficients for each independent variable (crosses). A linear discriminant function analysis was applied to predict group membership of $\delta^{13}C_{EAA}$ patterns derived from the otolith core and edge. Down-pointing triangles represent otolith cores and up-pointing triangles represent otolith edges. Numbers under the triangles represent standard length in cm for each individual from which the otolith was obtained.

Discussion

Since the dietary requirements of fish differ during different life stages, fish can move between habitats and rely on food webs supported by distinct primary producers. Reconstruction of such migrations and resource utilization can be complicated due to the temporal and spatial scales this fish behaviour occurs on. This study shows that the otolith organic matrix is informative of resource utilization and migration of *C. acoupa* individuals across an estuarine to coastal shelf gradient. The more positive mean baseline $\delta^{13}C_{EAA}$ values in otolith edges with the increasing size of *C. acoupa* individuals displays the known ontogenetic estuarine to coastal shelf distribution. The strong $\delta^{13}C$ correlation between organic and inorganic carbon of *C. acoupa* otoliths indicates that both fractions are sourced from ambient baseline dissolved inorganic carbon (DIC), where the otolith inorganic $\delta^{13}C$ becomes enriched in ^{13}C due to metabolic processing (Radtke et al. 1987; Thorrold et al. 1997). Since EAA carbon skeletons remain largely intact during trophic transfer, baseline $\delta^{13}C_{EAA}$ values reflect those at the base of the food chain (McMahon et al. 2010; 2015b). That a similar trend is present in both organic and inorganic $\delta^{13}C$ values implies that also inorganic $\delta^{13}C$ values are based on fractionation by PPs despite the metabolic enrichment. Disentangling metabolic change, diet and DIC contributions to otolith inorganic carbon has been complicated (Solomon et al. 2006; Elsdon et al. 2010; Nelson et al. 2011) and impede the deductions made from otolith records (Kalish 1991; Schwarcz et al. 1998; Jamieson et al. 2004).

Consequently, inorganic $\delta^{13}C$ trends are poor indicators for identifying $\delta^{13}C$ baseline or resource changes. The inability to characterize changes in ambient $\delta^{13}C$ baselines and resources across the entire migration range of the species and often unknown metabolic enrichment factor, limits ecological deductions from otolith inorganic $\delta^{13}C$ values. Also baseline $\delta^{13}C_{EAA}$ values from otolith edges of individual *C. acoupa* display large variations, even between similarly sized individuals. Variations in baseline $\delta^{13}C_{EAA}$ values are not unexpected in a species that lives in the highly variable Amazon region across a large distribution area with potential to move over considerable distances. In the wet season the Amazon River disperses large amounts of freshwater into the coastal areas and several kilometres offshore towards the north (Loick-Wilde et al. 2016; Weber et al. 2017). This can cause substantial spatial differences in baseline $\delta^{13}C$ values (Dittmar et al. 2006) across which *C. acoupa* individuals traverse. These geographical, seasonal and possibly long-term or sudden changes in baseline $\delta^{13}C$ values that can occur in the Amazon highly complicate the deduction of resources from baseline $\delta^{13}C_{EAA}$ values. This becomes very relevant when intending to infer resource utilization from several life years as represented in otoliths. Mean centred $\delta^{13}C_{EAA}$ values can overcome these $\delta^{13}C$ baseline variations because $\delta^{13}C_{EAA}$ patterns of PPs remain largely constant despite the $\delta^{13}C$ of the growth medium or spatial separation of PPs (Larsen et al. 2009; 2013).

The relative consistency of $\delta^{13}C_{EAA}$ patterns in otolith edges, despite the variations in baseline $\delta^{13}C_{EAA}$ values, shows that similar resources at the base of the food web support a cohort. Yet, the PCA results depict that the resource utilization of juvenile and adult *C. acoupa* gradually become distinct and overlap at intermediate life stages. This separation in resource utilization seems to start with individuals around 40 cm where $\delta^{13}C_{EAA}$ patterns seem to diverge and concurs with the known size of maturity of *C. acoupa* (Almeida et al. 2016). The gradual change in resource utilization also coincides with increasing mean baseline $\delta^{13}C_{EAA}$ values, which could indicate that at intermediate life stages there is a regular movement between estuarine and coastal shelf environments. An occurrence that is not unlikely in Amazon estuaries with their macro-tidal areas where water levels can change by 4-5 meters. Several fish species have been observed to move in and out of Amazon estuaries with the tides and resulting currents (2004; Brenner and Krumme 2007; Krumme et al. 2014). That such changes in resource utilization and migration are part of the life history of *C. acoupa* is demonstrated by the same trends observed in baseline $\delta^{13}C_{EAA}$ values and $\delta^{13}C_{EAA}$ patterns in some core and edge of individual otoliths. However, a few individuals seemed to have core and edge measurements that tend more towards coastal shelf resources. Such could imply that not all *C. acoupa* individuals spend their juvenile stage within the estuarine mangrove habitats. The possibility remains that some *C. acoupa* individuals undertake less extensive migrations during their lifetime.

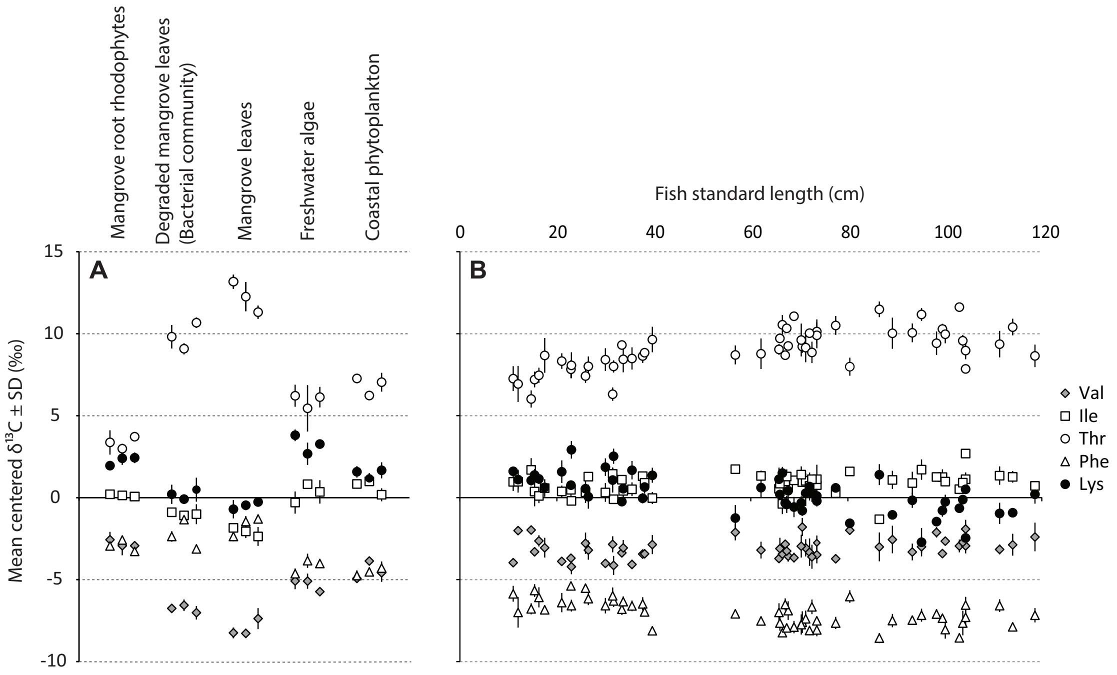
Our results also show that coastal phytoplankton and freshwater algae contribute the most as resources to the juvenile *C. acoupa* community and that direct contributions from mangrove derived resources are negligible. Algal resources supporting juvenile *C. acoupa* agrees with observations in the field and spawning behaviour. Spawning periods of *C. acoupa* in North Brazil are synchronised with the rainy seasons resulting in biannual spawning behaviour (Almeida et al. 2016). Large numbers of larval and juvenile *C. acoupa* coincide with large blooms in zooplankton and shrimp populations in the mangrove estuaries, which often rely on planktonic and algal diets (Barletta-Bergan et al. 2002a; b; 2004; Lima et al. 2015; Ferreira et al. 2016). However, mangrove carbon did not directly contribute to sustaining the juvenile population as has been indicated in other fish studies in mangrove estuaries (Melville and Connolly 2003; Kruitwagen et al. 2010; Igulu et al. 2013). Indirectly, mangroves can support fish populations by providing refuge to juvenile fish and supplying the coastal area with nutrients and mangrove carbon through the microbial and crustacean community (Sheridan and Hays 2003; Nagelkerken et al. 2008; Whitfield 2016). Amazon river macro-tides and wet season discharges increase phosphate and nitrate concentrations retained in estuarine and coastal areas, thereby leading to phytoplankton blooms (Smith and Demaster 1996; Dittmar and Lara 2001; Santos et al. 2008). Nevertheless, mangrove carbon can play an important role in the carbon cycling of aquatic environments offshore from the Amazon river. The mangrove crab *Ucides cordatus* and the bacterial community are responsible for decomposing the mangrove leaves (Schories et al. 2003; Nordhaus et al. 2006; Dittmar et al. 2006). Crab burials also enhance the mineralization of mangrove detritus into DIC, which leaches out with sediment pore water and becomes available for primary production (Bouillon et al. 2008). Mangrove forests are therefore an important factor in the productivity of coastal and offshore ecosystems supporting *C. acoupa* populations.

The diagnostic $\delta^{13}C_{EAA}$ patterns from otolith core and edge strongly suggest that primary producers produce a consistent $\delta^{13}C_{EAA}$ pattern across time scales of multiple years. Such temporal consistency is necessary for a reliable estimation of resource utilization over several years of a fish’s lifetime. It is known that such patterns are highly similar within taxonomic groups and distinct between the groups (Larsen et al. 2015; McMahon et al. 2015a). Nonetheless, all possible primary producers should be collected in the immediate environment and migration area of the species to correctly characterize the $\delta^{13}C_{EAA}$ patterns. More studies would need to confirm the temporal stability of diagnostic $\delta^{13}C_{EAA}$ patterns of specific primary producers.

The $\delta^{13}C_{EAA}$ application to otoliths can lead to even more detailed individual lifetime records on high temporal resolution. *C. acoupa* otoliths are relatively large and a 5 mg sample volume is sufficient for sampling the annual increments. For many smaller sized otoliths this sample volume can be too substantial for temporally resolved sampling. Yet, there are prospects for making $\delta^{13}C_{EAA}$ analysis accessible to smaller otoliths. The 5 mg sample mass could be decreased to 1-2 mg by adding an evaporation step at the end of the MOC procedure and increase concentration by dissolving in a smaller solvent volume. Moreover, small otoliths with higher protein content than *C. acoupa* (0.7%) will also enable lower sample volumes. Yet, we observed that overall AA concentrations differed among samples. This is not surprising as the protein content is known to be heterogeneously distributed within the otolith, with most organic content observed within the opaque zones (Watabe et al. 1982; Mugiya 1987; Murayama et al. 2004). These concentration variations can increase the standard deviation of $\delta^{13}C$ measurements for EAAs that have low peak signals, as they will go below the lower linearity range limit of the GC-IRMS and sometimes occurred for lysine in this study. One must therefore identify such protein concentration differences in otolith samples to avoid inaccuracies in $\delta^{13}C_{EAA}$ measurements and consequently in the correct identification of resources.

In summary, $\delta^{13}C_{EAA}$ measurements of the otoliths organic matrix display the resource utilization and migration of *C. acoupa* individuals. Baseline $\delta^{13}C_{EAA}$ values indicated its known coast to offshore distribution pattern and source diagnostic $\delta^{13}C_{EAA}$ patterns displayed a gradual change and overlap in resource utilization of *C. acoupa* juveniles and adults. The otolith organic matrix, hence, has great potential to be used as ontogenetic records with compound specific isotope analysis of $\delta^{13}C$ in EAAs. The insight could help with predictions on how environmental changes will affect fish populations by identifying the controlling factors at the base of the food web. In case of the *C. acoupa*, disappearance of the mangroves might not directly impact the juvenile food resources. However, the mangrove areas are likely a major factor in the productivity of the area, supplying nutrients for algal resources that support the food web of the juvenile *C. acoupa* population. Disappearance of the mangrove habitat might therefore severely impact the population in the long term.

Supplementary Materials



S1. The calculated mean centred $\delta^{13}C_{EAA}$ values or $\delta^{13}C_{EAA}$ patterns for A) primary producers and B) *C. acoupa* fish otoliths.

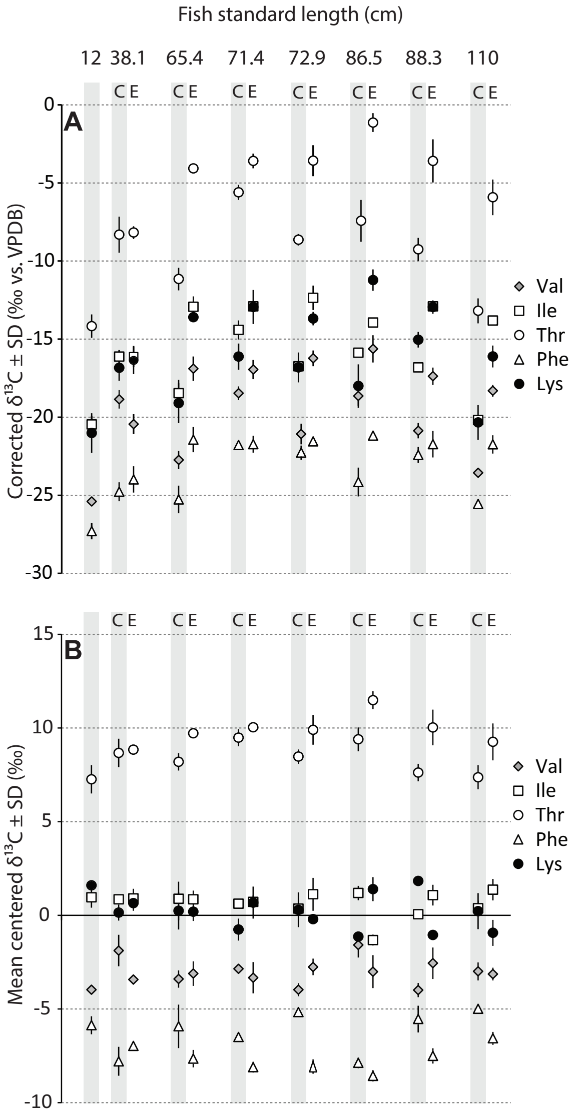


Fig. S2. A subsample of otoliths from differently sized individuals measured for $\delta^{13}C_{EAA}$ in core (C) and edge (E) of the otoliths. Otolith cores were micro drilled according to the dimensions of the 12 cm otolith and are shaded in grey. A) Individual $\delta^{13}C$ values of Val, Ile, Thr, Phe and Lys with standard deviation. B) Calculated mean centred $\delta^{13}C_{EAA}$ values or $\delta^{13}C_{EAA}$ patterns.

Chapter 4

Reconstructing lifetime nitrogen baselines and trophic position of *Cynoscion acoupa* from δ^{15}N values of amino acids in otoliths

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Abstract

Lifetime information about individual fish can be provided by their otoliths due to the structures continuous growth and metabolic inertness, incorporating environmental chemistry. Compound specific nitrogen isotope analysis of otolith proteinogenic amino acids could expand insight into individual lifetime migration, trophic position (TP) and environmental nitrogen dynamics. By comparing *Cynoscion acoupa* otoliths and muscle tissue from the Amazon area in Pará and otoliths from Rio Grande do Norte (RGN), this study demonstrates the ability to analyse juvenile and adult stages in individual fish otoliths across distinct Brazilian ecosystems.

Muscle tissue and otoliths gave comparable TP for both life stages, while weighted mean $\delta^{15}N$ values of all source amino acids differed between tissues. These differences reflected large seasonal and spatial changes in nitrogen cycles and anthropogenic nitrogen influences from the Amazon River onto the coastal shelf off Pará and the coast of RGN. While otoliths from Pará showed distinct TP and source amino acid $\delta^{15}N$ values between life stages, RGN otoliths showed overlap on both indicators. This could indicate an earlier migration and habitat overlap of juvenile and adult *C. acoupa* in the offshore RGN than in the Pará ecosystem, possibly due to the level of productivity in their environments. However, for both areas adult TP ranged across two TPs, whereas juvenile TP was relatively constant. Otoliths could therefore be an insightful mean to show changing $\delta^{15}N$ baselines, migration and TP over the lifetime of individual fish across distinct ecosystems and perhaps geological timescales.

Keywords: otolith chemistry, organic matrix, Brazil, migration, Amazon, trophic level

Introduction

Fish live in dynamic ecosystems where primary productivity can change suddenly, seasonally or over large time scales with increasing alteration in recent decades due to anthropogenic influences (e.g. climate change and chemical inputs) (Cloern et al. 2014; Levin et al. 2015). Such changes in the level of ecosystem primary productivity can have profound effects on the growth rate and maturation time of fish. Especially when changes are enduring or permanent, the growth and persistence of the fish population may eventually be affected (Audzijonyte et al. 2016). Nitrogen supply in marine environments can often limit the level of ecosystem primary productivity (Glibert 1988; Beardall et al. 2001). However, an excess supply of anthropogenic nitrogen from untreated sewage and wastewater discharge or from agricultural runoff, currently impacts many coastal ecosystems. This often leads to eutrophication of coastal waters that can induce severe algal and bacterial blooms that culminate in hypoxia and loss of ecosystem diversity (Vitousek et al. 1997). While elevated nitrogen levels can enhance primary productivity and therefore fish growth, it also can lead to habitat degradation with the resulting hypoxia increasing fish mortality and reproductive dysfunction (Nixon and Buckley 2002; Thomas et al. 2006). Overall, a monitor of nitrogen biogeochemical processes can indicate the living conditions and fitness of fish.

Nitrogen isotope ratios ($^{15}N/^{14}N$ or $\delta^{15}N$ values) of primary producers often reflect these biogeochemical processes in aquatic ecosystems and are commonly referred to as baseline isotope values (Deutsch et al. 2004; Hannides et al. 2009; Loick-Wilde et al. 2012). An individual fish can traverse environments with multiple baseline isotope values due to ontogenetic habitat shifts, which can be distinct from individuals in subpopulations experiencing other environmental conditions (Nagelkerken et al. 2015b). Moreover, over time, individuals of the same species can encounter different environmental conditions when baseline isotope values change significantly (Lindegren et al. 2012).

While traversing through these distinct ecosystems, the fish individual’s tissue proteins record the baseline nitrogen isotope values through its diet. Proteinogenic amino acids (AAs) appear to have consistent differences in $\delta^{15}N$ values which lead to their classification as source and trophic AAs (Popp et al. 2007). Source AAs directly indicate the nitrogen baseline as they do not change from primary producers to consumers by remaining largely unaffected by metabolic processes (McClelland and Montoya 2002; Chikaraishi et al. 2007). Source AAs therefore reflect nitrogen biogeochemical processes of the ecosystems in which the fish resided (Choy et al. 2012; Lorrain et al. 2015; Hetherington et al. 2016). Since nitrogen biogeochemical processes can vary across geographical landscapes, the $\delta^{15}N$ values of source AAs can be used to reconstruct the movements or migrations of fish (Madigan et al.

2014; 2016). Trophic AAs are enriched in ^{15}N relative to source AAs due to complex nitrogen cycling in organisms (Chikaraishi et al. 2007; O'Connell 2017). This ^{15}N enrichment with each trophic transfer is thought to occur when trophic AAs undergo transamination, transfer of an amino group to a ketoacid, or deamination by removing amine functional groups (McClelland and Montoya 2002; Chikaraishi et al. 2007; Braun et al. 2014). Importantly, the difference in $\delta^{15}N$ values between trophic and source AAs provide an estimate of the trophic position (TP) of the fish that is normalized to baseline isotopic compositions (McClelland and Montoya 2002; Chikaraishi et al. 2009; Nielsen et al. 2015; Bradley et al. 2015).

Nitrogen isotopic composition of muscle tissue has commonly been used to study fish ecology (Choy et al. 2012; Lorrain et al. 2015; Madigan et al. 2016). These tissues provide relatively short term insights into the individual life history, the length of which is dictated by the tissue turnover time (Tieszen et al. 1983; Madigan et al. 2012; Bradley et al. 2014). However, fish otoliths provide archival information about different life stages of an individual. The calcium carbonate structures often display visible age increments and incorporate a low amount of proteins during its daily accretion without metabolic turnover (Campana and Neilson 1985; Nagasawa 2014). Otoliths elemental and bulk isotope chemistry are considered lifelong archives of the individual fish's movement and environmental conditions such as salinity and temperature (Thorrold et al. 1997; Elsdon and Gillanders 2002; Elsdon et al. 2008). Recently, also the $\delta^{13}C$ values of essential AAs in the otoliths organic matrix were used to identify fish residency and habitat connectivity (McMahon et al. 2011b; a; 2012). Yet, any inferences made from $\delta^{13}C$ values of essential AAs have to be linked to known isotopic values of primary producers synthesizing the essential AAs. With $\delta^{15}N$ values of source and trophic AAs, ecosystem biogeochemistry, TP and migration for the life history of the fish can be inferred directly from the otolith.

Limitations exist due to analysis sensitivity for AA nitrogen isotope analysis of otoliths. This is primarily due to the extra volume requirement for current $\delta^{15}N$-AA analysis compared to $\delta^{13}C$-AA analysis (~9x). Together with the typically small otolith sizes and low protein contents of 1-4% (Degens et al. 1969), most otoliths do not contain enough material for either total or temporally sampled analysis. Yet, some fish species possess relatively large otoliths and can provide sufficient material for

δ^{15}N-AA analysis. Such large fish otoliths can be found in the *Cynoscion acoupa*, a large commercial fish with adults possessing 8 gram otoliths up to 5 cm in length. The species occurs along the entire coast of Brazil, which has a diverse array of climatic and hydrodynamic conditions. Early life stages of the *C. acoupa* are generally associated with mangrove areas while adults are exclusively caught in offshore coastal shelf areas (Barletta et al. 2005). This study therefore aims to identify whether δ^{15}N values of AAs from *C. acoupa* otoliths can be used to infer biogeochemical processes, migration and TP at different life stages. Otoliths of *C. acoupa* and muscle tissue were collected at the same time in the dry season of the Amazon state Pará, Brazil, to indicate whether baseline isotope values, in terms of source AA δ^{15}N values, and TP of two life stages extracted from both tissues are similar. To compare the nitrogen dynamics at juvenile and adult life stages of the *C. acoupa* in two distinct Brazilian ecosystems, adult otoliths were collected in the wet Amazon area of Pará and the semi-arid area of Rio Grande do Norte (RGN).

Methods

Sample collection

Four otoliths of *C. acoupa* with a body length of 92-114 cm standard length (SL) and muscle tissue of two respectively sized individuals (91 and 100 cm SL) were collected at the fish market in Bragança, Pará, Brazil. Simultaneously, two 34-36 cm SL and two ~26 cm SL individuals were sampled in mangrove areas near Bragança in November and December 2014 (dry season). An additional four *C. acoupa* otoliths were collected at the fish market of Natal, Rio Grande do Norte, Brazil and original body sizes were unknown. However, with our own collection (n=66) of *C. acoupa* otoliths of which otolith weight (mg) and fish standard length (mm) was known, the standard length could be estimated with the exponential relationship 24.649 x otolith weight$^{0.4379}$.

All otoliths were embedded in resin and sectioned to 2 mm thickness and mounted on a glass slide. Otolith surfaces were not polished after sectioning and were only wiped with acetone. Material of the outer edge and inner part of all otoliths were subsampled with a mounted hand-microdrill with a 0.5 mm drillbit to a calcium carbonate sample mass of 45 mg (average protein content 0.7%).

Stable isotope analysis

Otolith powder of ~45 mg and 5 mg of homogenized muscle tissue, respectively, were hydrolyzed with 0.1 ml 6N HCl per 1 mg of material (4.5 mL for otolith material and 0.5 mL for muscle tissue) at 150°C in a heating block for 70 minutes. The resulting 5.8N HCl was evaporated at 110°C in a heating block under a gentle stream of N_2 for approximately ~3-4 hours. Amino acids were isolated using cation exchange resin (DOWEX 50WX8, 100-200 mesh, hydrogen form; (Metges et al. 1996; Takano et al. 2010). The sample was again dried under a stream of N_2 at 80°C in a heating block and 0.2N HCl added which was heated to 110°C for 5 minutes and dried at 55°C under N_2. Amino acids were acetylated with an acetyl chloride and isopropanol mixture (4:1, v/v), heated to 110°C for 60 minutes and dried at 60°C under a N_2 stream. Subsequently, AAs were methylated with 600µL methylene chloride and 200µL trifluoroacetic anhydride by heating at 100°C for 15 minutes and dried under a N_2 stream at room temperature. The derivatized sample was further purified with a liquid/liquid extraction of 2mL 1M P-buffer and 1 mL chloroform as described by Hannides et al. (2009). The AAs in chloroform were evaporated under N_2 at room temperature, methylated again and stored at -20°C. Before analysis the derivatization agents were evaporated under N_2 at room temperature and dissolved in 15µL (otolith sample) and 125µL (muscle tissue sample) ethyl acetate in a GC vial.

The $\delta^{15}N$ values of AAs in the otolith and muscle tissue samples were measured using a Delta V Plus mass spectrometer interfaced to Trace GC gas chromatograph through a GC-C III combustion furnace (980°C), reduction furnace (650°C), and liquid nitrogen cold trap via a GC-C III interface. Otolith samples were hand injected (between 2.0-4.5 µL according to sample concentration) onto a *forte* BPx5 capillary column (60 m x 0.32 mm x 1.0 µm film thickness) with a split/splitless injector in splitless mode. The injector temperature was 180°C and had a constant helium flow rate of 1.4 mL min^{-1}. The column was initially held at 50°C for 2 minutes and increased to 120°C at 15°C min^{-1}. Subsequently, temperatures were raised to 195 °C at 4°C min^{-1} and 255 °C at 5 °C min^{-1} and to 300°C at 15°C min^{-1} where it was held for

8 min. All samples were analysed in triplicate and measured $\delta^{15}N$ values were normalized to the known nitrogen composition of internal reference compounds (L-2-Aminodipic acid and L-(+)-Norleucine) co-injected with each sample. When the co-injected reference compounds were not useable due to co-elution effects, a linear correction was applied to measured isotope values. The linear correction was derived from a suite of 14 AAs of known isotopic composition that was analysed between each triplicate sample analysis.

Trophic position estimations

The nitrogen isotopic composition of six trophic AAs; alanine (Ala), leucine (Leu), isoleucine (Iso), proline (Pro), aspartic acid (Asp), glutamic acid (Glu), and four source AAs; glycine (Gly), serine (Ser), phenylalanine (Phe), lysine (Lys) could be measured consistently. Weighted mean values for groups of trophic and source AAs were calculated with

$$\delta^{15}\mathrm{N}_{\bar{x}_w} = \frac{\sum \frac{\delta^{15} N_x}{\sqrt{\sigma_x^2}}}{\sum \frac{1}{\sqrt{\sigma_x^2}}}$$

where $\delta^{15}N_x$ is the nitrogen isotopic composition of a specified AA within the grouping and σ_x is the standard deviation of the specific AA based on triplicate analysis of each sample.

TP was calculated with the equation:

$$\mathrm{TP_{CSIA}} = [(\delta^{15}\mathrm{N_{Trp}} - \delta^{15}\mathrm{N_{Src}} - ß)/\mathrm{TDF_{AA}}] + 1$$

where $\delta^{15}N_{Trp}$ and $\delta^{15}N_{Src}$ are the nitrogen isotopic composition of selected trophic and source AAs, respectively. In this study, the combination of source AAs (Gly, Lys, Phe) and trophic AAs (Ala, Glu, Leu) was used, which has been shown to provide reliable TP calculations for teleosts (Bradley et al. 2015). The symbol ß is the difference between the $\delta^{15}N$ values of trophic and source AAs in primary producers and was calculated to be 3.4 ± 0.9‰ for the aforementioned combination of AAs (see Bradley et al. 2015). The trophic discrimination fractionation factor (TDF_{AA}) is the weighted mean average ^{15}N enrichment in trophic AAs (Ala, Glu, Leu) relative to source AAs (Gly, Lys, Phe) per trophic level and was found by Bradley *et al.* (2015) to be 5.7 ± 0.3‰ for this combination of trophic and source AAs.

Uncertainty in TP was calculated by propagation of errors:

$$\sigma_{TP} = \sqrt{\left(\frac{\partial \mathrm{TP}}{\partial \delta^{15}\mathrm{N_{Trp}}}\right)^2 \sigma^2_{\delta^{15}\mathrm{N_{Trp}}} + \left(\frac{\partial \mathrm{TP}}{\partial \delta^{15}\mathrm{N_{Src}}}\right)^2 \sigma^2_{\delta^{15}\mathrm{N_{Src}}} + \left(\frac{\partial \mathrm{TP}}{\partial \beta}\right)^2 \sigma^2_{\beta} + \left(\frac{\partial \mathrm{TP}}{\partial \mathrm{TEF}}\right)^2 \sigma^2_{\mathrm{TEF}}}$$

where σ is the standard deviation of TP, $\delta^{15}N$ values for trophic and source AAs, ß and TDF (see Bradley *et al.* 2015, Jarman et al. 2017). Indicated uncertainties are reported here as two standard deviations.

Results

Reconstructed individual *C. acoupa* body lengths from the RGN otoliths (92-107 cm SL) were in the same size range as those collected in Pará (92-114). Samples obtained from the outer edge of Pará and RGN otoliths were estimated to cover a life period of ~2 years based on the drill holes covering two opaque and translucent areas (Fig. 1). A wider surface was micro-drilled in the inner part of the otoliths compared to the edges (Fig. 1). However, otolith growth is known to be generally higher in the early life stages of the fish and therefore the wider surface of the inner part was also estimated to cover ~2 year life span in the juvenile stage of the individual.

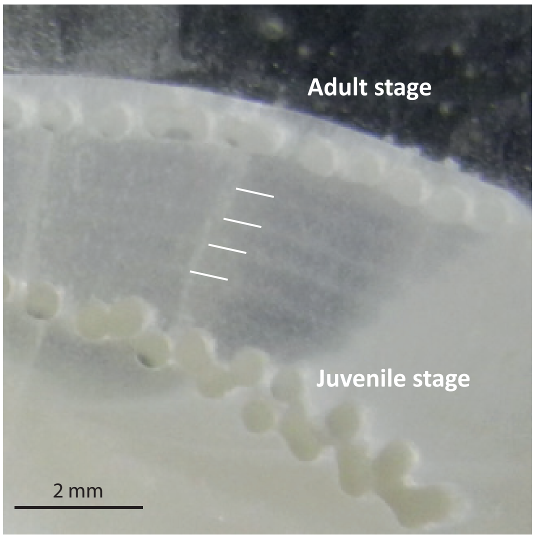

Fig. 1. Example of drill holes (0.5 mm diameter) in the inner part (juvenile stage) and outer edge (adult stage) of a Rio Grande do Norte *C. acoupa* otolith of 2 mm section

thickness. Displayed are also the incremental rings indicated with white lines, which are only detected in the otoliths collected in Rio Grande do Norte.

Comparable TPs were derived from adult muscle tissue and otolith adult stages from Pará with TP averages of 3.8 and 3.4±0.3 respectively. In addition, TPs obtained from the adult stage in RGN otoliths (3.6±0.4) were similar to that in adult stage Pará otoliths (3.5±0.3) (Wilcoxon rank-sum, W=9, p=0.885, Fig. 2A, Table 1). TPs acquired from juvenile muscle tissue from Pará were similar to the juvenile stage of Pará otoliths (averages 3.0±0.2 and 2.8±0.3 respectively, Wilcoxon rank-sum, W=15, p=0.057). Muscle tissue from RGN juveniles was not available for analysis. TPs from the juvenile and adult stage in Pará otoliths were different (Wilcoxon rank-sum, W=0, p=0.028) in contrast to the similar juvenile and adult stage TPs from RGN otoliths (Wilcoxon rank-sum, W=2, p=0.114, Fig. 2A, Table 1). Furthermore, a comparison between otoliths from Pará and RGN indicated similar TPs in juvenile (Wilcoxon rank-sum, W=15, p=0.057) and adult stages (Wilcoxon rank-sum, W=9, p=0.885, Fig. 2B, Table 1).

In Pará, significant differences were observed between otolith juvenile and adult stages for both source (Wilcoxon rank-sum, W=16, p=0.028) and trophic AA $\delta^{15}N$ values (Wilcoxon rank-sum, W=0, p=0.028). Source AA weighted mean $\delta^{15}N$ values of juvenile and adult *C. acoupa* muscle tissue collected during the dry season of Pará were similar (mean values 5.9±0.2‰ and 5.7‰ respectively), although different in trophic AA $\delta^{15}N$ values (19.5±1.8‰ and 24.5‰ respectively). However, source and trophic AA $\delta^{15}N$ values of adult muscle tissue were lower than those in otolith edges (Table 1). Juvenile stage otolith and muscle tissue from Pará also displayed distinct source AA $\delta^{15}N$ values (Wilcoxon rank-sum, W=0, p=0.028), although comparable trophic AA $\delta^{15}N$ values (Wilcoxon rank-sum, W=0, p=0.114, Fig. 3, Table 1). In RGN, no differences were found between otolith juvenile and adult stages for either source (Wilcoxon rank-sum, W=3, p=0.2) or trophic AA $\delta^{15}N$ values (Wilcoxon rank-sum, W=2, p=0.114). Nor were source or trophic AA $\delta^{15}N$ values in RGN otoliths different from Pará otolith juvenile (Wilcoxon rank-sum, W=8, p=1 and W=0, p=0.114) or adult stages (Wilcoxon rank-sum, W=2, p=0.114 and W=1, p=0.057, Fig. 3, Table 1).

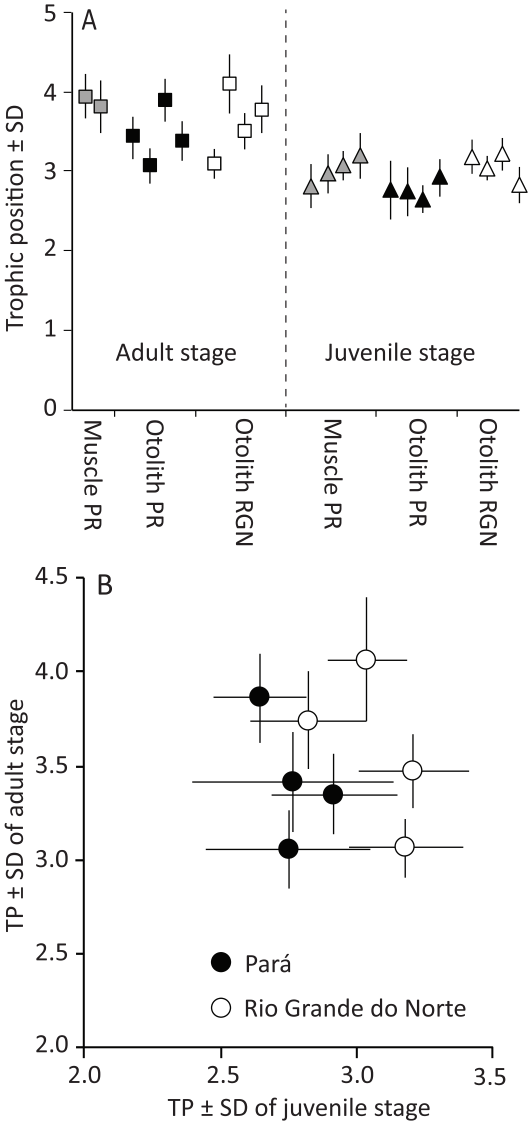

Fig. 2. A) Overview of the trophic position (TP) and its uncertainty calculated from adult and juvenile muscle tissue from Pará (PR) and adult and juvenile stages within the adult otoliths from Pará and Rio Grande do Norte (RGN). B) Trophic position of juvenile and adult stage measured in each individual otolith from Pará and Rio Grande do Norte.

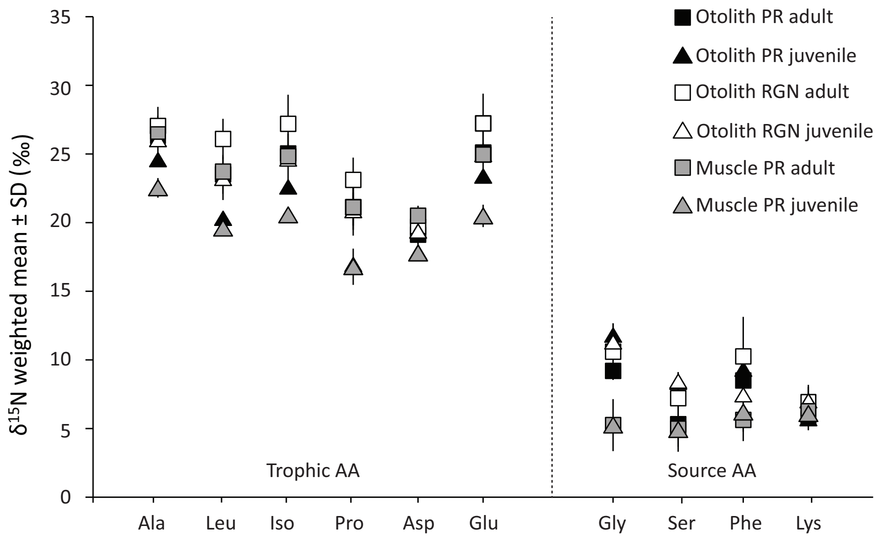

Fig. 3. Individual AA $\delta^{15}N$ values of muscle tissue of juvenile and adult *C. acoupa* from Pará (PR) and otoliths from Pará and Rio Grande do Norte (RGN). Error bars are ± standard deviation (SD) of all *C. acoupa* individuals collected within each category.

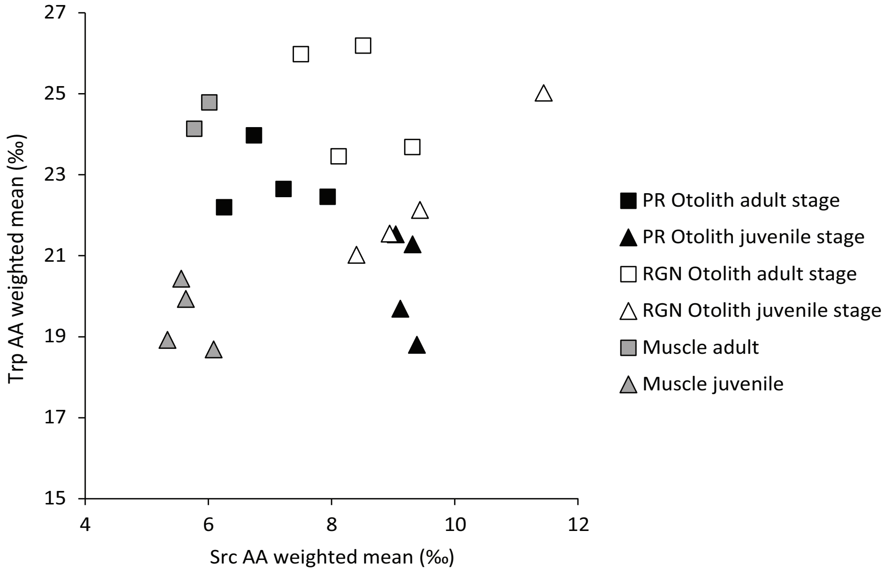

Fig. 4. Weighted mean of all source AAs versus weighted mean of all trophic AAs in otolith and muscle tissue of *C. acoupa* from Pará (PR) and Rio Grande do Norte (RGN).

Table 1. An overview of individual AA measurements with standard deviations and calculated weighted means of all source and trophic AAs. TP estimations and its uncertainty are based on Src AAs (Gly, Phe, Lys) and Trp AAs (Ala, Leu, Glu). This is displayed for muscle tissue from different sized *C. acoupa* individuals from Pará and the edge (adult stage) and inner (juvenile stage) of otoliths from both Pará and Rio Grande do Norte.

			Src AAs				Trp AAs							
	SL (cm)	Stage	Gly	Ser	Phe	Lys	Ala	Leu	Pro	Asp	Glu	Weighted mean Src AAs	Weighted mean Trp AAs	TP
Muscle	91	Adult	5.4 ± 0.1	3.1 ± 0.4	7.4 ± 0.9	6.2 ± 0.0	26.5 ± 0.0	23.8 ± 0.2	20.1 ± 0.2	20.4 ± 0.1	24.8 ± 0.2	6.0	24.8	3.9 ± 0.2
Pará	100	Adult	4.9 ± 0.2	5.5 ± 0.1	5.3 ± 0.2	7.2 ± 0.2	26.9 ± 0.3	23.6 ± 0.2	22.4 ± 0.3	20.9 ± 0.4	25.3 ± 0.4	5.8	24.1	3.8 ± 0.3
	36.5	Juvenile	8.1 ± 0.3	6.3 ± 0.3	5.6 ± 0.1	6.1 ± 0.3	22.6 ± 0.5	19.4 ± 0.2	15.2 ± 0.2	17.5 ± 0.1	19.2 ± 0.2	6.1	18.7	2.8 ± 0.3
	34.5	Juvenile	7.3 ± 0.2	4.4 ± 0.1	6.6 ± 0.5	5.7 ± 0.2	23.4 ± 0.2	20.0 ± 0.0	17.6 ± 0.1	16.8 ± 0.4	21.1 ± 0.1	5.6	19.9	3.0 ± 0.3
	25.5	Juvenile	4.7 ± 0.2	4.9 ± 0.3	6.2 ± 0.4	5.7 ± 0.1	21.7 ± 0.2	19.3 ± 0.4	17.7 ± 0.2	17.8 ± 0.1	20.6 ± 0.2	5.3	18.9	3.1 ± 0.2
	26.5	Juvenile	4.2 ± 0.1	4.9 ± 0.7	7.1 ± 0.1	7.2 ± 0.2	22.5 ± 0.1	19.0 ± 0.2	15.7 ± 0.3	18.1 ± 0.1	20.7 ± 0.1	5.6	20.4	3.2 ± 0.3
Otolith	92	PR-1 Adult	10.0 ± 0.8	6.9 ± 0.9	7.9 ± 0.1	7.1 ± 0.5	26.8 ± 0.4	23.2 ± 0.8	20.6 ± 0.2	19.9 ± 0.1	24.8 ± 0.2	7.9	22.5	3.4 ± 0.3
Pará	103	PR-2 Adult	8.7 ± 0.3	3.6 ± 0.2	11.3 ± 0.6	7.0 ± 0.8	26.2 ± 0.3	23.4 ± 0.1	21.0 ± 0.1	19.3 ± 0.1	25.3 ± 0.3	6.3	22.2	3.1 ± 0.2
	114	PR-3 Adult	8.7 ± 0.9	7.1 ± 0.3	8.8 ± 0.4	5.5 ± 0.1	26.7 ± 0.5	26.2 ± 0.4	22.2 ± 0.1	19.7 ± 0.3	26.3 ± 0.2	6.7	24.0	3.9 ± 0.2
	100	PR-4 Adult	9.4 ± 0.3	6.8 ± 0.6	9.3 ± 0.7	5.4 ± 0.2	25.1 ± 0.5	23.3 ± 0.0	19.2 ± 0.3	18.4 ± 0.1	24.8 ± 0.0	7.2	22.6	3.4 ± 0.2
	92	PR-1 Juvenile	11.6 ± 0.3	7.3 ± 0.5	9.3 ± 0.0	5.6 ± 0.6	24.9 ± 0.2	19.2 ± 0.3	16.1 ± 0.5	18.8 ± 0.3	23.5 ± 0.3	9.3	21.3	2.8 ± 0.4
	103	PR-2 Juvenile	11.8 ± 0.4	7.2 ± 1.0	11.0 ± 0.8	5.8 ± 0.4	24.9 ± 0.2	20.2 ± 0.2	16.0 ± 0.1	18.5 ± 0.2	23.2 ± 0.1	9.1	19.7	2.8 ± 0.3
	114	PR-3 Juvenile	11.9 ± 0.3	9.4 ± 0.7	7.3 ± 1.0	5.7 ± 0.6	23.4 ± 0.8	21.0 ± 0.5	17.5 ± 0.1	17.2 ± 0.1	22.7 ± 0.7	9.4	18.8	2.7 ± 0.2
	100	PR-4 Juvenile	11.9 ± 0.4	9.1 ± 0.5	8.9 ± 0.3	5.8 ± 0.4	24.2 ± 0.2	21.2 ± 0.3	19.0 ± 0.2	17.6 ± 0.1	23.7 ± 0.1	9.0	21.5	2.9 ± 0.2
Otolith	100	RGN-1 Adult	11.3 ± 0.1	7.7 ± 0.4	6.1 ± 0.9	6.0 ± 0.5	25.8 ± 0.2	24.5 ± 0.1	21.6 ± 0.4	19.0 ± 0.4	24.4 ± 0.3	9.3	23.7	3.1 ± 0.2
Rio Grande	92	RGN-2 Adult	6.9 ± 0.7	3.8 ± 0.9	11.7 ± 0.8	7.5 ± 0.1	28.5 ± 0.4	27.9 ± 0.4	25.5 ± 0.3	20.3 ± 0.3	29.2 ± 0.3	7.5	26.0	4.1 ± 0.3
do Norte	96	RGN-3 Adult	10.2 ± 0.9	7.5 ± 0.3	9.4 ± 0.3	5.2 ± 0.8	26.1 ± 1.1	26.5 ± 0.5	23.0 ± 0.3	18.8 ± 0.3	25.8 ± 0.3	8.1	23.5	3.5 ± 0.2
	107	RGN-4 Adult	11.1 ± 0.4	7.7 ± 0.3	12.5 ± 0.5	6.8 ± 0.1	28.1 ± 0.4	27.1 ± 0.2	22.8 ± 0.1	20.4 ± 0.3	28.2 ± 0.1	8.5	26.2	3.7 ± 0.3
	100	RGN-1 Juvenile	10.9 ± 0.4	8.6 ± 0.1	5.3 ± 0.8	6.0 ± 0.6	25.3 ± 0.5	23.4 ± 0.2	19.9 ± 0.1	17.9 ± 0.2	23.8 ± 0.5	8.4	21.0	3.2 ± 0.2
	92	RGN-2 Juvenile	10.8 ± 0.0	8.7 ± 0.7	7.2 ± 0.1	6.4 ± 0.5	25.4 ± 0.3	23.6 ± 0.4	19.6 ± 0.2	18.1 ± 0.2	24.2 ± 0.4	9.4	22.1	3.0 ± 0.2
	96	RGN-3 Juvenile	13.7 ± 0.2	9.3 ± 1.3	8.9 ± 0.5	8.5 ± 0.4	28.7 ± 0.4	25.8 ± 0.7	23.6 ± 0.3	21.9 ± 0.2	27.6 ± 0.4	11.4	25.0	3.2 ± 0.2
	107	RGN-4 Juvenile	11.2 ± 0.4	7.8 ± 0.3	11.6 ± 1.3	6.2 ± 0.8	25.0 ± 0.4	22.0 ± 0.2	21.2 ± 0.2	19.4 ± 0.1	24.5 ± 0.2	8.9	21.5	2.8 ± 0.2

Discussion

Fish otoliths can provide individual lifetime information about migration, TP and environmental nitrogen dynamics based on $\delta^{15}N$ AA values. This study showed that the average TP calculated from AA $\delta^{15}N$ values for *C. acoupa* muscle tissue and otoliths agreed well for each life stage in fish collected from the Pará region. On the contrary, these tissues often recorded different source $\delta^{15}N$ AA values. These differences could be due to dissimilar rates of AA incorporation into muscle tissue compared to the otolith matrix. The turnover time of specific AAs in muscle tissue can range from months to a year (Madigan et al. 2012, Bradley et al. 2014) and thus some AAs can record (inter-)seasonal variations of $\delta^{15}N$ values in the environment. Due to the low protein content in otoliths, samples here average isotopic values over approximately two years. Otolith proteins are produced *de novo* daily by the saccular epithelium (Payan et al. 1999, Takagi et al. 2005) of which only ~1% is deposited within the calcium carbonate structure without turnover (Edeyer et al. 2000, Borelli et al. 2001). A strong relationship between essential AA $\delta^{13}C$ values of muscle and otolith edge of *Lutjanus* spp. indicated that both tissues derive AAs from the bloodstream with minor fractionation (McMahon, Fogel, et al. 2011, McMahon, Berumen, et al. 2011). Subtle $\delta^{15}N$ value differences of particular AAs might be expected between tissues, depending also on an individual's metabolism, although this 'biological noise' might be overcome by measuring the isotopic composition of multiple AAs (O'Connell 2017). The similarities in AA abundances between muscle tissues and otoliths of this study also suggested a comparable AA incorporation into both tissues. Although muscle tissue and otolith AA $\delta^{15}N$ measurements from the same individual would confirm the comparability of AA incorporation, the large temporal differences represented by these tissues (months vs. ~2 years) and individual fish behaviour might account for the observed differences in source AA $\delta^{15}N$ values found in this study. Thus, we attribute the differences in source AA $\delta^{15}N$ values found between the tissues as a likely result of the temporal differences represented by the otolith sampling and muscle turnover time.

Seasonal nitrogen isotopic variations in the Amazon

The estuarine and coastal shelf habitats in the Amazon region are influenced by strong seasonal hydrodynamics that can create distinct baseline $\delta^{15}N$ values. During the wet season most of the dissolved inorganic nitrogen is discharged by the Amazon River into coastal areas and dispersed several kilometres offshore (Demaster & Pope 1996, Subramaniam et al. 2008). Much of this nitrogen originates from organic nitrogen fertilization in the increasing agricultural use of the Amazon rainforests and untreated sewage from adjacent cities (Martinelli et al. 2012, Bustamante et al. 2015). Anthropogenic organic nitrogen input often leads to high $\delta^{15}N$ values (>10‰) of dissolved inorganic nitrogen as well as in the tissues of local organisms (Montoya 2007, van de Merwe et al. 2016). This is reflected in bulk $\delta^{15}N$ values of surface zooplankton collected in the wet season at an Amazon estuary with values of 8-11‰ (Giarrizzo et al. 2011), which decreases to 3-5‰ at oceanic areas (Loick-Wilde et al. 2012). No bulk $\delta^{15}N$ values are published for the dry season in the Amazon. However, diminished river discharge and lower offshore dissolved nitrate concentrations during this season (23 µmol kg^{-1} with rising river discharge and 12 µmol kg^{-1} with falling river discharge; Demaster & Pope 1996) might imply that anthropogenic nitrogen is contained in the estuaries and leads to lower offshore $\delta^{15}N$ values.

Source $\delta^{15}N$ AA values in adult and juvenile muscle tissue samples collected in the Pará dry season were similar, however they were lower than those measured in otoliths. Due to the turnover time of muscle tissue AAs of on average several months, we posit that muscle tissue likely recorded the dry season baseline $\delta^{15}N$ values. On the contrary, otolith AA measurements, which represent two life years, probably average the changing baseline $\delta^{15}N$ values of the wet and dry season and result in overall elevated source $\delta^{15}N$ AA values. Higher source $\delta^{15}N$ AA values in otolith juvenile stages compared to otolith adult stages could indicate habitat separation. Generally, the mangrove estuaries are important feeding areas for *C. acoupa* juveniles and these fishes can be found there before and after major rainfall events, following the growth cycles of zooplankton and shrimp populations (Barletta-Bergan et al. 2002, Krumme et al. 2004, Nóbrega et al. 2013, Lima et al. 2015). Adults remain in offshore coastal shelf areas during the entire year and, thus, the similar juvenile and adult muscle tissue source $\delta^{15}N$ AA values might suggest seasonal movement of juveniles towards the coastal shelf during the dry season. Yet, it cannot be excluded that the $\delta^{15}N$ baseline differences between estuaries and coastal shelf areas are negligent during the dry season.

C. acoupa in distinct Brazilian ecosystems

In contrast to Pará otoliths, the source $\delta^{15}N$ AA values and average TP estimations of juvenile and adult stage from RGN otoliths are highly similar. This may indicate a habitat overlap during both life stages in RGN or that the fish lived in different habitats with indistinguishable baseline $\delta^{15}N$ values. Nonetheless, TP remained constant at the two life stages of the fish in RGN. Rio Grande do Norte is a semi-arid state, where mangrove coverage is significantly lower than the wet Amazon area in Pará (Schaeffer-Novelli et al. 1990). As in Pará, anthropogenic influences are also present in RGN mangrove estuaries due to intensive shrimp farming, agriculture and bovine husbandry (de Lacerda et al. 2006; Bustamante et al. 2015). The comparable source $\delta^{15}N$ AA values of otolith juvenile stages from Pará and RGN suggest that all *C. acoupa* juveniles reside in estuaries with anthropogenic nitrogen input. Nevertheless, coastal nitrogen will not be deposited in large amounts onto the coastal shelf due to low river run off at the RGN coast (Schaeffer-Novelli et al. 1990) and thus will have negligible effect on offshore baseline $\delta^{15}N$ values. Yet, *C. acoupa* adults that are exclusively caught offshore from RGN have similar otolith source AA $\delta^{15}N$ values in the adult and juvenile stage. The high source $\delta^{15}N$ AA values in otolith adult stages from RGN are therefore likely not of anthropogenic origin. A more likely explanation is a different and unknown nitrogen cycling process at the RGN coast compared to the Pará coast. The coast of RGN is an oligotrophic environment (Medeiros et al. 1999), which is very distinct from the eutrophic Pará coast due to input of nutrients from the Amazon River (Nittrouer and Demaster 1996; Smith and Demaster 1996). It is thus possible that estuarine and offshore coastal habitats at RGN have similar baseline $\delta^{15}N$ values where estuarine anthropogenic nitrogen and offshore nitrogen cycling lead to similar source $\delta^{15}N$ AA values. This seems to be supported by similar bulk $\delta^{15}N$ values of zooplankton of 6-8‰ during the dry season in RGN estuaries and the coastal shelf 10 km offshore (Schwamborn et al. 1999).

Hence, a habitat overlap of juvenile and adult *C. acoupa* at offshore RGN habitats at an earlier stage than Pará *C. acoupa* individuals is conceivable. Through microbial transformations of nutrients in mangrove ecosystems, the estuarine mangrove habitats play a large role in the productivity of aquatic food webs (Holguin et al. 2001). With smaller mangrove coverage, the ecosystem productivity of RGN estuaries and offshore habitats is lower and of shorter time period than at the Pará coast (Medeiros et al. 1999; Neumann-Leitao et al. 1999). As a result of such low productivity, RGN juveniles could be utilizing the mangrove estuaries for a short time and proceed in an early life stage to feed at a similar TP as the adults. We speculate that Pará juveniles might benefit from larger estuarine productivity on lower trophic levels for a longer time period than in RGN.

Variations in trophic position

While juvenile stage TP of both Pará and RGN otoliths are highly consistent between individuals with 0.2-0.4 TP variation, individual TP from otolith adult stages in both areas varied by 0.8-1 TP. Whether such TP ranges in adult *C. acoupa* are an indication of individual diet variation in the adult stage or a remnant of TP calculation issues with AA δ^{15}N values has to be evaluated. The TP estimate on Fishbase (2017) of 4.1 ± 0.7 for *C. acoupa* agrees with the highest adult TP calculations of both Pará and RGN. Nonetheless, TP estimations of higher trophic level organisms have been complicated. This often lies in the uncertainties surrounding TDF values due to different turnover times of AAs (Nielsen et al. 2015; Chikaraishi et al. 2015; McMahon and McCarthy 2016). Calculating TP with AAs that have very different turnover times can lead up to 1 TP variation, which can be more accurate with multiple source and trophic AAs (Bradley et al. 2014; 2015). In this study, a large discrepancy in δ^{15}N values of glycine and lysine between muscle tissue and otolith was observed. While otolith glycine displays the expected changes in baseline δ^{15}N values, lysine remains homogenous across tissues, life stages, and areas. Lysine is considered a representative source AA with a short half life (131 days in *Thunnus orientalis*) and barely changes across TPs (Bradley et al. 2014). However, it is suggested that lysine is not homogenized with other transaminating AAs due to the metabolic pathways of two N groups (McMahon and McCarthy 2016). The consistent lysine δ^{15}N values in this study implies that this AA does not capture the baseline changes in the environment perhaps due to a metabolic pathway that buffers variations in δ^{15}N values or has a longer turnover time in *C. acoupa* tissues (>2 years). Contrary to lysine, the source AA designation of glycine is more controversial due to substantial TDF variations across trophic transfers (Nielsen et al. 2015; Chikaraishi et al. 2015; McMahon et al. 2015a). However, glycine was most influential in differentiating the baselines between *C. acoupa* life stages and areas, changes that were expected based on known spatial differences in nitrogen cycles, which indicates in this fish it has the isotopic characteristics of a typical source AA. Using a source AA with relatively constant δ^{15}N values as phenylalanine for TP calculations (as Glu/Phe combination, see (Chikaraishi et al. 2009) in this study results in TP estimations that did not distinguish between juvenile and adult *C. acoupa*. As a TP distinction was expected for *C. acoupa* life stages in Pará due to the overall distinct habitat use and diet, the use of glycine might have been necessary for the TP estimations to capture a more accurate baseline δ^{15}N value change across life stages.

It has been proposed that the TDF of trophic AAs can be influenced by diet quality, whereas diets with a low protein content and distribution similar to the consumer results in decreased TDF values (McMahon et al. 2015d; Chikaraishi et al. 2015) and thus creates variation in TP. Diets low in protein or starvation can therefore underestimate TP when standard TDF values are used, although are improbable in *C. acoupa* with a high protein diet of shrimp and fish. Moreover, such situations are unlikely to occur often in natural settings as such undernourished fish might be more susceptible to predation. A variation in diet among individual fish seems a more plausible explanation than issues with TDF values. Individual TP variation has also been reported in giant trevally, *Caranx ignoblis*, by CSIA studies (Papastamatiou et al. 2015). Bulk isotope analysis in freshwater fish also displayed dietary variations in concordance with lower TP at littoral areas than at pelagic areas (Beaudoin et al. 1999; Svanbäck et al. 2015). Striking is that the otolith adult stages of *C. acoupa* displaying the highest TP also often had the lowest source δ^{15}N AA values, indicating less productive oceanic environments based on the bulk isotope trends at both RGN and Pará. It could indicate that individual adult *C. acoupa* diet can change according to the coastal distance, which represents a larger productivity at lower trophic levels than at offshore or oceanic environments.

Future perspectives

This study shows that δ^{15}N values of AAs in otoliths indicate significant differences between life stages and various ecosystems that can be explained relatively well with known nitrogen cycle processes in the Amazon area. However, changes in nitrogen cycling and indicative δ^{15}N values of AAs over various connected habitats and time scales are not yet fully understood as was illustrated with RGN otoliths. With a better understanding of such nitrogen cycling changes and variations, δ^{15}N values of AAs in otoliths could also be used to derive migration patterns from archaeological otoliths. Isotopic measurements of AAs in shells and bones of ~100,000 years old indicate stable preservation in biogenic carbonate structures (Serban et al. 1987, Edgar Hare et al. 1991) with low probability of contamination. Thus, also archaeological fish otoliths could potentially provide a unique insight into how trophic ecology and habitat connectivity of fish populations differed during periods before anthropogenic influences, and large environmental and climate change

Chapter 5. Discussion

This thesis is among the first to show that the otolith proteinogenic matrix can be utilized for extracting information on the lifetime connectivity and habitat use of the *Cynoscion acoupa* in two distinct Brazilian ecosystems. It is shown that choosing the simple derivatization method methoxycarbonyl esterification supports the reconstructions by enabling low internal increment sampling. This method is applied to measure δ^{13}C values of essential amino acids ($\delta^{13}C_{EAA}$) in Amazonian *C. acoupa* otoliths. The results demonstrate a variable estuarine to coastal shelf resource utilization and migration with increasing fish length. Also, δ^{15}N-AA analysis of juvenile and adult otolith sections of large adult (~100 cm) otoliths indicated that *C. acoupa* have a distinct life history in the monsoonal Amazon compared to the semi-arid Rio Grande do Norte. Possibly as a function of their local ecosystem productivity, lifetime habitat use and feeding on trophic positions (TP) were observed to be significantly different.

The findings presented in this thesis show that compound specific isotope analysis of amino acids (CSIA-AA) in otoliths opens new possibilities to assess fish movement as well as life history parameters such as resource utilization and TP. The CSIA-AA application to otoliths and other biogenic carbonates, however, is in its very early stages of development and there are still many areas that can be further improved and advanced.

Understanding habitat connectivity and use of *C. acoupa*

The analysis of *C. acoupa* otoliths in this thesis has elucidated some life history strategies of this little studied fish species of commercial importance in Brazil. Multiple studies have described the use of mangrove rivers and creeks by *C. acoupa* larvae and juveniles across its distribution range (de Morais and de Morais 1994; Barletta-Bergan et al. 2002a; b; Barletta et al. 2003; 2005; Barletta and Barletta-Bergan 2009; Lima et al. 2015; 2016). However, there is little knowledge on the successive life stages of *C. acoupa* and its migration to the coastal shelf.

Migration patterns of C. acoupa

The gradual habitat separation of juvenile and adult *C. acoupa* life stages in the Amazon region is displayed by both baseline $\delta^{13}C_{EAA}$ and source δ^{15}N-AA values in their otoliths. Significantly different nitrogen dynamics in the habitats of the two life stages was indicated by distinct source δ^{15}N-AA values. However, a more variable and coinciding lifetime use of estuarine and coastal shelf habitats and resources by *C. acoupa* juveniles and adults are exhibited by baseline $\delta^{13}C_{EAA}$ values and $\delta^{13}C_{EAA}$ patterns in otolith core and edges. These two observations do not necessarily exclude each other. The same habitats can be differently represented by baseline δ^{15}N and δ^{13}C values due to distinct mechanisms driving their spatial and temporal variability. Nitrogen is generally a limited nutrient in the aquatic environment and algal NO_3^- assimilation can drive very large spatial gradients of δ^{15}N values in organic matter (Somes et al. 2010). However, δ^{13}C values of dissolved inorganic carbon in the Amazon ecosystem can be highly variable on small local estuarine scales likely due to differences in freshwater outflow (Dittmar et al. 2006). Thus, to resolve whether the perceived variability in habitat use and resource utilization by *C. acoupa* life stages are due to these small spatial scale differences in baseline δ^{13}C values of estuarine habitats, the use of a second tracer indicating an estuarine habitat would be desirable. As such lifetime analysis requires minimal sampling of otolith increments, this tracer needs to be measured in small sample volumes. One could consider using δ^{18}O values alongside the AA analysis, which can be analysed in calcareous volumes of 10-30 µg, (Kimirei et al. 2013; Huijbers et al. 2013; Trueman et al. 2013) as an indicator of estuarine freshwater input represented by enriched δ^{18}O values.

In contrary, a habitat overlap by *C. acoupa* juvenile and adult stages from Rio Grande do Norte was shown by their otolith source δ^{15}N-AA values. This might indicate that migrations towards the coastal shelf, where the adults exclusively reside, are undertaken at an earlier stage in this semi-arid environment than in the Amazon. However, there is still a poor understanding of nitrogen dynamics in connected areas and how they are indicated by δ^{15}N-AA values. The similar otolith source δ^{15}N-AA values in both life stages could thus also indicate two habitats with distinct nitrogen cycling that result in comparable source δ^{15}N-AA values.

Feeding strategy and resource utilization by C. acoupa

Apart from elucidating migration patterns, CSIA-AA in *C. acoupa* otoliths also enables the exploration of lifetime feeding strategies and resource utilization of *C. acoupa* individuals. These parameters can give some insight into the drivers and perceived variations of lifetime *C. acoupa* migrations from mangrove estuaries to coastal shelf in different ecosystems.

Early life stage (<25 cm SL): Otolith $\delta^{13}C_{EAA}$ patterns of early life stage *C. acoupa* from the Caeté river in the Amazon indicate that their food web is largely supported by algal resources such as phytoplankton and freshwater algae. This agrees with the observed presence of larvae and juveniles in the mangrove estuaries right after the rainy season when phyto/zooplankton abundances increase and spawning is induced (Barletta-Bergan et al. 2002a; Almeida et al. 2016; Lima et al. 2016). However, otolith core sampling displays variable resource utilization in the same early life stages of individual *C. acoupa* in the Amazon. These individual $\delta^{13}C_{EAA}$ patterns resemble estuarine as well as coastal shelf resources, implying that early life stage habitats can be distinct for individual *C. acoupa* and does not only include estuarine mangrove habitats. This is also observed for multiple coral reef species, which are also known for their ontogenetic coast to offshore coral reefs migration (Kimirei et al. 2013, McMahon et al. 2012). Variable habitat use can occur in euryhaline marine species and is thought to be a mechanism that increases survival chances as a trade off between for example predation risk and food availability (Alofs and Polivka 2004; Able 2005; Kimirei et al. 2011; McMahon et al. 2012).

Juvenile stage (between 25 to 40 cm SL): Periodic movements by Amazonian *C. acoupa* juveniles between estuaries and coastal shelf can precede their definitive residence in coastal shelf habitats as adults. This is represented by a gradual shift in estuarine to coastal shelf resources by mean baseline $\delta^{13}C_{EAA}$ values and $\delta^{13}C_{EAA}$ patterns in individual juvenile otolith edges. However, source δ^{15}N-AA values of late dry season muscle tissue indicated a juvenile habitat overlap with adults, while ~2 life year otolith source δ^{15}N-AA values display a distinct habitat use by juveniles and adults. The causation for such juvenile offshore movement during the dry season is unknown, as most seasonal studies in the Amazon involve *C. acoupa* up to 29 cm length (Barletta et al. 2003, 2005). Yet, it can't be excluded that baseline $\delta^{15}N$ differences between estuaries and coastal shelf are negligent during the dry season. Despite the perceived resource and habitat variations, all juveniles feed on prey of low trophic levels, as similar TPs inferred from juvenile stage muscle tissue and juvenile otolith sections indicated.

In contrast, a diet and habitat overlap by juvenile and adult *C. acoupa* in Rio Grande do Norte is displayed by similar TPs and source $\delta^{15}N$-AA values in adult otolith sections. Confirming such a perceived co-occurrence of both stages is impeded by a lack of studies on *C. acoupa* in this area. Yet, Rio Grande do Norte fishermen also observed a simultaneous offshore catch of juvenile and adult *C. acoupa* (Vane pers. comm.). The overlap might thus imply an earlier juvenile offshore migration towards the adult habitat than in the Amazon. Such can be hypothesised to be a function of lower productivity in semi arid Rio Grande do Norte estuaries than in Amazon estuaries (Medeiros et al. 1999; Neumann-Leitao et al. 2008).

Adult stage (between 40 to 120 cm SL): While some *C. acoupa* adults show a distinct use of estuarine and coastal shelf resources in their early life and adult otolith stages, others display use of estuarine-like resources during adult stages. This variability is also reflected in individual adult TPs from both the Amazon and Rio Grande do Norte ranging from 3 to 4. Moreover, high source $\delta^{15}N$-AA values (suggesting more coastal habitats) are measured with the lowest adult TPs, and vice versa. This also suggests that adults utilize different food webs and resources according to coastal distance. A low TP and estuarine-like resource utilization could indicate that some *C. acoupa* adults prey on estuarine feeding fish species, which by the fishermen have been suggested to make large-scale migrations over several kilometres per tide (Krumme et al. 2004). Such individual specialisation of large marine predators is little understood, although could be a consequence of resource availability, inter- and intra-specific competition, food-predation risk trade-offs or spatial overlap of food webs (Bolnick et al. 2003; Laidre and Jameson 2006; Matich et al. 2011; Papastamatiou et al. 2015). However, a complete sampling of all source endmembers across the distribution range of *C. acoupa* populations to identify

$\delta^{13}C_{EAA}$ patterns will be necessary for a better understanding of such variations in individual lifetime resource utilization and migrations.

C. acoupa populations in an environment under stress

Understanding the life history of a fish species in terms of habitat utilization and connectivity is prominent part in fisheries management. With CSIA-AA in otoliths, the primary resources utilized at the food web base define lifetime habitat utilization by different life stages. It cannot identify which exact habitats are utilized, however, such identification is complicated even by visual census as observing a fish in a particular habitat does not directly identify the habitat value for a species. Thus, identification of resource utilization by different life stages provides a bottom up insight in the regulation of the food web. In the case of *C. acoupa*, the results in this thesis indicated that phytoplankton is an important resource for the early life stages. The phytoplankton blooms in the Amazon occur after heavy rainfall, during which nutrients are released from the mangrove sediments (Schories et al. 2003; Nordhaus et al. 2006; Dittmar et al. 2006). With the forecast of climate change decreasing local precipitation and the moist tropical forests in the Amazon (Levine et al. 2016; Zanin et al. 2017), this nutrient discharge and thus phytoplankton biomass can be speculated to decrease. Yet, at the same time their coastal nursery habitats receive high anthropogenic nitrogen inputs from sewage and aqua- or agricultural origin (Bustamante et al. 2015). This can lead to harmful algal blooms but also to a shift in phytoplankton composition and changes in food web structure with decreases in zooplankton or composition which can be unfavourable for early life stages of fish (Vitousek et al. 1997; Capriulo et al. 2002). Fisheries management of *C. acoupa* thus should also include a more holistic approach by protecting the mangrove estuaries to ensure optimal early life stage growth by reducing anthropogenic nitrogen by untreated sewage and agricultural fertilizers.

Although the ontogenetic estuarine to coastal shelf migration has already been hypothesized, both baseline $\delta^{13}C$ values and source $\delta^{15}N$ AA values of otolith life stages in this thesis confirm this lifetime movement of *C. acoupa* in the Amazon. Yet, the results also showed potential flexibility in individual life history with non-estuarine resource and habitat use in early life stages and differing adult TP with close to shore and offshore coastal shelf habitat use. These indications of intrapopulation flexibility in dietary life history can suggest that the population has some resilience and adaptability to environmental changes. This can lead to a selection of those individuals that possess the best traits to deal with such changes (Bolnick et al. 2003; 2011).

Development of δ^{13}C-AA analysis and methodological progress for otoliths

To utilize the full extent of the ecological CSIA-AA application to otoliths and other biogenic carbonates, some aspects of the δ^{13}C analysis need to be further studied and developed. The reconstruction of resource utilization from *C. acoupa* otoliths made use of source diagnostic $\delta^{13}C_{EAA}$ patterns to overcome the spatial and temporal differences in δ^{13}C values of EAAs. The consistency of these $\delta^{13}C_{EAA}$ patterns across temporal sampling of otolith core and edges does indicate that $\delta^{13}C_{EAA}$ patterns persist over time. Moreover, $\delta^{13}C_{EAA}$ patterns of a diatom with high phenotypic plasticity, *Thallassiosira weissflogii*, remained constant under different salinity, pH, temperature and irradiance conditions (Larsen et al. 2015) The concept of $\delta^{13}C_{EAA}$ patterns has also been applied to long term records from proteinaceous corals spanning 80-1000 years to identify changes in plankton regimes (Schiff et al. 2014; McMahon et al. 2015a) due to main taxa of source endmembers displaying distinct and consistent $\delta^{13}C_{EAA}$ patterns (Larsen et al. 2009; 2013). Yet, it remains unconfirmed whether $\delta^{13}C_{EAA}$ patterns of a source endmembers remains constant over timescales up to thousands of years in the same habitat or geographically separated habitats. A long-term study sampling the same source endmembers across seasons and years in different locations might confirm the temporal persistence of their $\delta^{13}C_{EAA}$ patterns. More confirmation of the temporal and spatial consistency of $\delta^{13}C_{EAA}$ patterns of various source endmembers might be necessary to reinforce its application to lifetime resource reconstructions by sampling increments of recent and archaeological biogenic carbonates.

Another issue in the ecological application of $\delta^{13}C_{EAA}$ analysis is the often incomplete representation of all source endmembers in the determination of resource utilization by organisms. Food web reconstructions with isotopic tracers require the characterisation of all possibly utilised sources to accurately determine their contributions to consumer diets (Phillips et al. 2014). Especially the roles of phytoplankton species or size fractions, microbes and fungi in providing resources at the food web base have been poorly characterised. Microbes and fungi can play an important role in the diet of first trophic level consumers (Yi et al. 2017; Steffan et al. 2017; Pascal et al. 2017), but are difficult to sample in the field. Culturing microbes might be an option, although the prerequisite is that the culturing conditions do not influence the microbial AA fractionation processes. Moreover, first trophic level organisms such as copepods are often used to represent phytoplankton endmembers and thus their $\delta^{13}C_{EAA}$ patterns can be distorted by those of microbes in their diet. In environments with high aquatic detrital loads such as Amazonian rivers, copepod sampling is however an accessible approximation of phytoplankton $\delta^{13}C_{EAA}$ patterns. Filtering the generally cleaner marine water column for phytoplankton can still misrepresent their $\delta^{13}C_{EAA}$ patterns as some detritus will be included. Size fractionation is one way to roughly distinguish between phytoplankton species as a factor of size and this has been observed to display differences when analysed for δ^{15}N-AA values (Loick et al. 2007; Mompeán et al. 2016). Currently, there is not yet an approach to acquire pure phytoplankton samples to species level for δ^{13}C-AA analysis. Yet, the limitations of the aforementioned sampling approaches for phytoplankton and microbes have to be taken into account when interpreting $\delta^{13}C_{EAA}$ patterns for resource utilization.

The use of δ^{13}C-AA analysis in biogenic carbonates can only be widely applied if the sample volume can be kept low, in order to analyse internal increments and small structures. In chapter 2 of this thesis it was described how avoidance of decalcification procedures in methoxycarbonyl esterification can decrease calcareous sample volumes for δ^{13}C-AA analysis. It was also mentioned that possibilities still remain to reduce the sample volume of 5 mg extracted from *C. acoupa* otolith (0.7%) to potentially ~1 mg for MOC δ^{13}C-AA measurements. This could be achieved by a combination of an evaporation step after organic separation and redissolving in 20 µL solvent, and increasing GC injection volumes according to the injector volume (liner) limits. Nonetheless, ~1 mg is no set limit as with higher protein content in biogenic carbonate structures and maximised section thickness, sample volumes can be even lower.

Lowering the calcareous sample volume for δ^{15}N-AA measurements is more complicated. The analysis in Chapter 4 included increasing concentrations with evaporation and hand-injecting up to 4.5 µL into the GC-IRMS. Moreover, rigorous decalcification or purification procedures for all sample types are necessary to acquire clean samples for δ^{15}N-AA analysis. Due to the splitless injection onto the gas chromatograph (GC) column and generally large sample volumes (~9x larger relative to δ^{13}C-AA analysis) for δ^{15}N-AA analysis; there is more potential for contamination and effects on the GC and combustion and reduction furnace functioning. This interferes with accurate δ^{15}N-AA measurements due to the non-AA matrix causing background noise in the chromatography and system malfunction (Takano et al. 2010). Consequently, with the current sensitivity for AA nitrogen isotope analysis, it is expected that it allows only for sampling of large otoliths.

Future perspectives

The application of CSIA-AA in biogenic carbonates opens new avenues to explore the life history of fish and changes in resources in the aquatic environment of sessile organisms. With the ability to sample small biogenic carbonate increments and temporal stability of source diagnostic $\delta^{13}C_{EAA}$ patterns, complete lifetime resource utilization records can be reconstructed. This can gain insight into life history and long-term environmental changes with a relatively simple approach and aid in management and conservation approaches. These life history insights might be extended to past ecosystems with large paleontological, archaeological and historical fish otoliths. The isotopic composition of AAs in biogenic carbonates and skeletal structures also remain uncompromised over long timescales (Serban et al. 1987; Edgar Hare et al. 1991). The δ^{15}N-AA analysis is well suited for studying past ecological interactions. AAs provide the δ^{15}N baseline values as well as the trophic enrichment without the need to quantify the δ^{15}N baseline through primary production samples (Naito et al. 2013; 2016). The perceived earlier migration onset of *C. acoupa* individuals from a semi-arid and monsoonal ecosystem, illustrate how a fish species can adapt their life history to distinct environments. The ecosystems inhabited by fish are by no means static and have changed due to physical processes occurring over thousands of years but also on shorter timescales as result of well-known human impacts. This can fundamentally change the ecosystem by for example removing freshwater input by climate change or increasing nitrogen concentrations due to agriculture or wastewater discharge (Vitousek et al. 1997; Halpern et al. 2008). Whether and how fish species could adapt to such changes is little understood as current ecological study approaches are too short term to investigate processes over long time scales (MacCall 1996; Jackson 2001a). Thus, δ^{15}N-AA analysis of archaeological otoliths can be used to increase our understanding on nitrogen dynamics of past ecosystems and how fish species adapt to changing habitats. Additional insights into past resource utilization of fish can also be provided by δ^{13}C-AA analysis, when $\delta^{13}C_{EAA}$ patterns of source endmember groups prove to be stable across time.

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Versicherung an Eides Statt

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Ich, _________________________________ (Vorname, Name, Anschrift, Matr.-Nr.)

versichere an Eides Statt durch meine Unterschrift, dass ich die vorstehende Arbeit selbständig und ohne fremde Hilfe angefertigt und alle Stellen, die ich wörtlich dem Sinne nach aus Veröffentlichungen entnommen habe, als solche kenntlich gemacht habe, mich auch keiner anderen als der angegebenen Literatur oder sonstiger Hilfsmittel bedient habe.
Ich versichere an Eides Statt, dass ich die vorgenannten Angaben nach bestem Wissen und Gewissen gemacht habe und dass die Angaben der Wahrheit entsprechen und ich nichts verschwiegen habe.
Die Strafbarkeit einer falschen eidesstattlichen Versicherung ist mir bekannt, namentlich die Strafandrohung gemäß § 156 StGB bis zu drei Jahren Freiheitsstrafe oder Geldstrafe bei vorsätzlicher Begehung der Tat bzw. gemäß § 161 Abs. 1 StGB bis zu einem Jahr Freiheitsstrafe oder Geldstrafe bei fahrlässiger Begehung.

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Ort, Datum Unterschrift