INSTITUTO NACIONAL DE PESQUISAS DA AMAZÔNIA - INPA
PROGRAMA DE PÓS-GRADUAÇÃO EM ECOLOGIA

FLUXO DE ENERGIA EM TEIAS ALIMENTARES DE ECOSSISTEMAS AQUÁTICOS TROPICAIS:

DAS FONTES AUTOTRÓFICAS ATÉ OS GRANDES CONSUMIDORES ECTOTÉRMICOS

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Manaus, Amazonas

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Sinopse:

Estudou-se o fluxo de energia em teias alimentares de ecossistemas aquáticos tropicais do Território Norte da Austrália e da Amazônia central. Aspectos como alocação de energia para reprodução em um peixe herbívoro-detritívoro e origens da energia e posição trófica de crocodilianos amazônicos foram avaliados.

Palavras chave: isótopos estáveis, RNA:DNA, energia, consumidores ectotérmicos

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Mis abuelas Cecilia y Aída me enseñaron con cariño la importancia de la simplicidad y perseverancia en la vida. A ellas, dedico

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Resumo

A estrutura das teias alimentares é considerada um dos atributos mais fundamentais dos ecossistemas. Além disso, as conexões entre hábitats e o fluxo de matéria através dos limites dos ecossistemas têm importantes implicações na produção de biomassa de animais e plantas, que é facilitada pelas relações tróficas entre consumidores e recursos.

Migrações de peixes podem transferir nutrientes e produção de origem aquática, mas informações sobre como essa produção contribui para a alocação individual da energia e o investimento reprodutivo de animais é escasso. A posição trófica dos predadores de topo de cadeia influencia fortemente a estrutura das teias alimentares, pois reflete o número de passos que a energia atravessou a partir do produtores primários até os consumidores terciários. Os crocodilianos exercem uma forte influência em diversas teias alimentares ao longo das suas vidas, desde água doce, salobre, marinha e hábitats terrestres adjacentes. Os crocodilianos amazônicos apresentam diferenças interespecíficas na dieta. Porém, desconhece-se em que medida diferem nas fontes primárias dos recursos que consomem e até que ponto essas diferenças são refletidas em mudanças ontogenéticas na posição trófica. É também desconhecido em que medida as diferenças interespecíficas na dieta podem ser um reflexo de divergências no comportamento de forrageio, além de ser uma função do uso de hábitat.

No presente estudo, utilizamos um contexto espaço-temporal para traçar as origens e o fluxo da energia utilizando ferramentas químicas, como a razão de isótopos estáveis de carbono e nitrogênio e RNA:DNA. Em relação ao funcionamento das teias alimentares dos ecossistemas aquáticos tropicais, buscamos entender três aspectos: A sazonalidade e espacialidade no investimento reprodutivo da tainha-diamante (*Liza alata*) do território Norte da Austrália (Capítulo 1); as diferenças interespecíficas nas origens da energia que sustenta as quatro espécies de crocodilianos amazônicos dentro de um contexto espacial (Capítulo 2); e as mudanças ontogenéticas na posição trófica desses predadores dentro do contexto das teias alimentares em que estão inseridos (Capítulo 3).

Os resultados indicam que o investimento reprodutivo da tainha-diamante acontece durante a época seca, quando os recursos são limitados e os peixes mostram uma pobre condição corporal. Existe um forte compromisso entre o investimento somático e reprodutivo. Isso pode ser explicado porque a tainha-diamante mostra um desacoplamento temporal entre a ingestão de recursos nas planícies de inundação durante a época cheia, o estoque de energia por alguns meses em corpos lipídicos mesentéricos e a posterior alocação para reprodução durante a época seca. Devido às migrações entre hábitats desses peixes para desovar, o desacoplamento temporal e espacial entre a aquisição de energia e sua alocação para reprodução tem importantes implicações para a preservação dos regimes hidrológicos naturais das planícies de inundação. A manutenção desses regimes naturais é importante para garantir a capacidade dos peixes dessa região de manter populações viáveis.

No contexto dos ecossistemas lóticos da Amazônia central, os resultados mostram evidências de que existem diferenças nos recursos basais que sustentam as quatro espécies de crocodilianos amazônicos. Essas diferenças resultam de divergências comportamentais e estratégias de forrageio além da seleção de macrohábitat. Encontramos também uma relação positiva entre o tamanho dos crocodilianos e a posição trófica que ocupam, mas existem diferenças interespecíficas na forma dessa relação. Os crocodilianos ocupam níveis tróficos mais altos do que peixes piscívoros. Essa estrutura pode gerar divergências significativas nas

estimativas do comprimento das cadeias alimentares, pois tradicionalmente, apenas os peixes têm sido considerados como predadores de topo em estudos de comprimento das cadeias tróficas.

As informações apresentadas nesse estudo trazem aspectos inovadores sobre a biologia e ecologia dos consumidores estudados, retratando o funcionamento trófico dos ecossistemas e as origens dos recursos que os sustentam.

Abstract

Energy fluxes in tropical aquatic ecosystem foodwebs: From autotrophic sources to large ectotherm consumers

Food webs constitute one of the main research frameworks in ecology, and food web structure is considered one of the most fundamental attributes of ecosystems. Linkages among habitats and the flux of matter across ecosystem boundaries have important implications for biomass production of animals and plants, which is facilitated by trophic relations between consumers and their resources.

It has been well documented that fish movements may transfer nutrients and aquatic production, but information on how this production contributes to individual energy allocation and reproductive investment is scarce. Furthermore, the trophic position of top predators in a food chain strongly influences food-web structure because it reflects the number of steps that energy takes from primary producers to tertiary consumers. Crocodilians are likely to influence the structure of food webs because of their pronounced ontogenetic shifts in diet. Some species of large crocodiles may be trophic links in diverse food webs throughout their lives, from freshwater, brackish, marine and adjacent terrestrial food webs. Amazonian crocodilians show interspecific differences in diet. However, it is unknown to what extent they differ in the primary sources of their diet and to what extent those differences are reflected in ontogenetic shifts in trophic position. It is also not well understood to what extent interspecific differences in diet are a reflection of foraging behavior or are a function of macrohabitat selection.

We used a spatiotemporal context to trace the origins and the flux of energy by applying chemical tools, such as the natural ratio of carbon and nitrogen stable-isotopes and RNA:DNA. Within the tropical aquatic ecosystems we studied, we aimed to understand three aspects of food-web functioning: the seasonality and spatiality in the reproductive investment of diamond mullet (*Liza alata*) from the Northern Territory of Australia (Chapter 1); interspecific differences in the origins of energy sustaining the four species of Amazonian crocodilians within a spatial context (Chapter 2) and ontogenetic shifts in trophic position of these predators within the context of the food web (Chapter 3).

The results show that reproductive investment of diamond mullet takes place during the dry season, when resources are limited and fish are in poor body condition. There is a strong trade-off between somatic and reproductive investment. This may be explained because diamond-mullet show a temporal uncoupling between resource acquisition from floodplains during the wet season, energy storage within lipid mesenteric bodies for some months and a subsequent reproductive allocation during the dry season. Because this fish undertakes spawning migrations across habitats, temporal and spatial uncoupling between energy acquisition and reproductive allocation have important implications for the preservation of natural hydrological regimes of water bodies. The maintenance of these hydrological regimes is important to enhance the capacity for fishes of this region to maintain viable populations.

In the context of lotic ecosystems from central Amazonia, the results show evidence of differences in the basal resources sustaining the four species of Amazonian crocodilians. These differences result from behavioral divergences and foraging strategies in addition to macrohabitat selection. Furthermore, we found a positive relationship between crocodilian

size and trophic position. However, there are interspecific differences in the shape of these relationships. Crocodilians occupy higher trophic levels than piscivorous fishes. This structure may create significant divergences in food-chain-length estimates because, traditionally, only fish have been considered as top predators in most food-chain-length studies.

The information presented in this study brings novel insights about the biology and ecology of the studied consumers, depicting the trophic functioning of ecosystems where they occur in relation to the resources that sustain them.

Sumário

O presente trabalho foi escrito utilizando a estrutura de capítulos em forma de artigos. A seguir, apresenta-se de maneira suscinta o conteúdo do trabalho na ordem em que o assunto aparece no texto.

As listas de tabelas e figuras são apresentadas nas páginas xiv e xv, respetivamente. Tabelas e figuras foram inseridas o mais próximo possível do texto a que se referem dentro de cada capítulo.

Na parte inicial do texto, apresenta-se uma introdução geral do trabalho (Págs. 1 - 5), fazendo uma breve síntese sobre os fundamentos teóricos e metodológicos envolvidos no estudo das teias alimentares dos ecossistemas aquáticos. Posteriormente, apresenta-se de maneira introdutória o assunto de cada capítulo. A introdução do Capítulo 1 (6 - 8) é apresentada de maneira separada dos outros dois capítulos (9 - 11).

Os objetivos do presente estudo são sintetizados de maneira geral e depois apresentados como objetivos específicos para cada capítulo (12).

O capítulo 1 é apresentado a partir da página 13. Nesse ponto existe uma mudança no tipo de numeração das páginas. O capítulo 1 já foi publicado, portanto a numeração da revista é usada para se referir ao conteúdo desse capítulo. Nesse capítulo analiza-se a alocação de energia para reprodução em uma espécie de peixe australiano.

O capítulo 2 é apresentado entre as páginas 14 - 50. Questões sobre as origens da energia que sustenta os crocodilianos amazônicos são abordadas nesse capítulo. O capítulo 3 (51 - 72) trata de entender as mudanças ontogenéticas no nível trófico dessas espécies de crocodilianos.

Uma síntese geral é apresentada (73 - 75) como uma discussão integrada dos resultados obtidos nos capítulos e as conclusões resultantes no contexto das teias alimentares dos ecossistemas estudados.

Apresenta-se de maneira unificada as referências bibliográficas do trabalho completo nas páginas 76 - 88.

Finalmente, na página 89 apresenta-se como apêndice as informações prévias sobre as contribuições relativas de cada tipo de presa que compõe a dieta dos crocodilianos utilizadas para o agrupamento dos *endmembers* terrestres e aquáticos.

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INTRODUÇÃO GERAL

O estudo das teias alimentares

A presente síntese está baseada na revisão de Layman et al. (2015) sobre a evolução histórica no estudo das teias alimentares. As teias alimentares podem ser definidas como as redes de interações entre consumidores e seus recursos dentre grupos de organismos, populações ou unidades tróficas e constituem um dos principais marcos de estudo na área da ecologia (Layman et al., 2015). O estudo da ecologia das teias alimentares teve seu início com o clássico trabalho de Elton (1927), que acreditava que as interações tróficas constituem uma parte estrutural fundamental para entender o funcionamento dos ecossistemas. Elton introduziu o conceito da pirâmide dos números, no qual a base está composta por organismos produtores primários e herbívoros, que são mais abundantes, enquanto animais que ocupam lugares mais altos na pirâmide tendem a ser maiores e mais raros. Essas ideias inspiraram o trabalho de Lindeman (1942) sobre o fluxo de energia através dos ecossistemas. Usando presunções sobre a eficiência na transferência de energia, estimativas de produção primária e relações tróficas, estimou a quantidade de biomassa que poderia ser sustentada em níveis tróficos superiores (Layman et al., 2015).

As décadas subsequentes estiveram marcadas por estudos em que a tendência general foi o uso de modelos matemáticos para explicar o funcionamento, estabilidade e estrutura das teias alimentares (May, 1972; Pimm, 1979; 1982; Pimm e Lawton, 1977; Dunne et al., 2002; 2013). No entanto, uma das principais críticas ao uso de modelos matemáticos para representar as teias alimentares enfatiza que os dados empíricos utilizados para parametrizar e testar os modelos não retratam de maneira satisfatória a complexidade das teias alimentares do mundo real (Cohen et al., 1993; Winemiller e Layman, 2005). Em decorrência disso, foi adotada uma abordagem diferente no estudo empírico das teias alimentares, na qual utilizam-se estimativas mais diretas, detalhadas e realistas das teias alimentares (Polis, 1991). Polis foi pioneiro ao integrar o estudo das teias alimentares com a ecologia da paisagem, particularmente na forma como o fluxo de energía é acoplado no ecossistema entre ambientes/habitats através de limites da paisagem (Layman et al., 2015). Polis (1991) definiu como "subsídios espaciais" os recursos controlados pelo doador de um hábitat (presas animais, detritos ou nutrientes) e transportados para outro hábitat diferente. Dessa forma, a produtividade aumenta no hábitat beneficiário do recurso alterando as dinâmicas consumidor-recurso (Polis et al., 1997). Trabalhos posteriores mostraram que o fluxo de recursos que atravessam limites na paisagem é esperado na maioria de ecossistemas (Polis et al., 2004).

A necessidade de usar dados empíricos sobre a dieta dos consumidores para entender o fluxo de energia através das teias alimentares promoveu o aprimoramento de ferramentas químicas como os isótopos estáveis. A razão natural de isótopos estáveis, principalmente de carbono ($\delta^{13}C$) e nitrogênio ($\delta^{15}N$), proporciona informações integradas sobre as relações tróficas entre os organismos e o fluxo de energia através das teias alimentares de maneira espacial e temporal (revisão em Layman et al., 2012). A partir dos trabalhos de Peterson e Fry (1987) que mostraram a ampla utilidade dos métodos isotópicos no ramo da ecologia trófica, seu uso tem se tornado a principal ferramenta no estudo das teias alimentares nos últimos 30 anos.

Fundamentos do uso da razão de isótopos estáveis de carbono ($\delta^{13}C$) e nitrogênio ($\delta^{15}N$) em estudos de fluxo de energia em teias alimentares

O uso da razão natural de isótopos estáveis de carbono ($\delta^{13}C = {}^{13}C/{}^{12}C$) e nitrogênio ($\delta^{15}N = {}^{15}N/{}^{14}N$) tem se tornado a ferramenta mais eficaz para rastrear as fontes de produtividade que abastecem as teias alimentares (i.e. hábitats, tipos de recursos ou em alguns casos, taxa específicos) e a posição trófica dos consumidores (Finlay e Kendall, 2007). Os isótopos estáveis são considerados marcadores químicos presentes nos tecidos biológicos que carregam informações provenientes dos recursos adquiridos pelos consumidores. Dessa forma, os isótopos estáveis são análogos a "impressões químicas digitais" e permitem rastrear as fontes primárias da energia que sustentam os diferentes organismos.

Quimicamente, os isótopos estáveis são átomos do mesmo elemento com o mesmo número de prótons e elétrons, mas diferente número de nêutrons, resultando em diferentes números de massa. Esses elementos são energeticamente estáveis por não apresentarem diminuição radioativa (revisão em Sulzman, 2007). Em geral, as plantas apresentam depleção de ${}^{13}C$ com relação ao CO_2 atmosférico do qual dependem para fazer a fotossíntese. Essa depleção é causada por processos enzimáticos e físicos que discriminam contra o ^{13}C e em favor do ^{12}C. A discriminação isotópica varia entre plantas que utilizam diferentes rotas fotossintéticas (C3, C4 e CAM) (revisão em Marshall et al., 2007). A razão dos isótopos de carbono das plantas C3 depende da atividade conjunta da difusão atmosférica do CO_2 e da enzima Ribulose bisfosfato carboxylase/oxygenase (Rubisco). Os valores de $\delta^{13}C$ de plantas C3 apresentam portanto, uma média de -27‰ (Marshall et al., 2007). Por outro lado, a composição isotópica das plantas C4 difere substancialmente daquela das plantas C3, pois sua rota fotossintética é catalisada pela enzima Fosfoenolpiruvato carboxylase (PEP), que apresenta um fator de discriminação diferente. Portanto, os valores médios de $\delta^{13}C$ para essas plantas são próximos a -14‰ (Marshall et al., 2007). Já os valores de $\delta^{13}C$ das algas é amplamente variável porque dependem em grande parte da quantidade de carbono inorgânico dissolvido (DIC) na água. Os processos que afetam o $\delta^{13}C$ do DIC em corpos hídricos lóticos são a degaseificação e o intercâmbio de CO_2 com a atmosfera; a dissolução / precipitação de minerais de carbonato no riacho; a discriminação durante a fotossíntese que deixa o residual de DIC enriquecido em ^{13}C e a respiração (Finlay e Kendall, 2007).

Devido a todos os processos descritos, a discriminação isotópica do carbono é diferenciada entre diferentes rotas fotossintéticas e condições físico-químicas nos ecossistemas aquáticos. No entanto, é conservada ao longo das teias alimentares, mostrando mudanças muito baixas em cada transferência trófica (0.5-1‰). Dessa forma, é possível traçar a origem dos nutrientes em níveis tróficos superiores (DeNiro e Epstein, 1978; Layman et al., 2012; Peterson e Fry, 1987). Assim, o uso de isótopos estáveis de carbono facilita o entendimento das origens da energia que sustenta os consumidores.

Por outro lado, independente da fonte autotrófica, a razão dos isótopos estáveis de nitrogênio ($\delta^{15}N = {}^{15}N/{}^{14}N$) exibe um enriquecimento contínuo a cada transferência trófica. Dessa forma, os isótopos estáveis de nitrogênio constituem uma ferramenta útil para estimar a posição trófica dos diferentes organismos dentro das teias alimentares (Cabana e Rasmussen, 1996; DeNiro e Epstein, 1981; Layman et al., 2012; Minagawa e Wada, 1984; Peterson e Fry, 1987; Post, 2002a). O enriquecimento de 3.4‰ na razão dos isótopos estáveis de nitrogênio ($\delta^{15}N$) relacionado com cada transferência trófica tem sido amplamente aplicado (Cabana e Rasmussen, 1996; Minagawa e Wada, 1984; Peterson e Fry, 1987; mas ver Rosenblatt e Heithaus, 2013; Marques et al., 2014). No entanto, Vander Zanden e Rasmussen (1999) encontraram uma marcada variação na assinatura isotópica dos organismos de linha base (consumidores primários) relacionada com mudanças no hábitat. Os autores sugeriram que os estudos isotópicos de teias alimentares deveriam incluir medidas do mais amplo espectro possível de consumidores primários de uma forma sítio- específica. Valores referentes à posição trófica devem ser interpretados com base em valores de linha base do δ^{15}N. Essa linha base pode ser estabelecida utilizando a assinatura isotópica dos consumidores primários (e não dos produtores primários) devido ao maior tamanho e longevidade dos herbívoros resultarem em menor sazonalidade nas assinaturas do δ^{15}N (Cabana e Rasmussen, 1996).

Post (2002b) sugeriu que a posição trófica máxima ou comprimento das cadeias alimentares é uma característica importante das comunidades ecológicas e influencia na sua estrutura. Entre outras coisas, o comprimento da cadeia alimentar pode modificar a organização das interações tróficas, funções ecológicas como ciclagem de nutrientes, produtividade primária e intercâmbio de carbono atmosférico (Pace et al., 1999; Persson, 1999; Post, 2002b). Adicionalmente, o uso de representações das teias alimentares baseadas na posição trófica dos organismos pode melhorar a capacidade de modelar e entender importantes processos ecossistêmicos (Vander Zanden e Rasmussen, 1999).

Fundamentos do uso da razão de RNA:DNA como indicadores bioquímicos de síntese proteica

A busca de um método confiável e preciso para estimar a condição nutricional e o crescimento instantâneo de organismos aquáticos tem sido o foco de um grande número de estudos (ver Chícharo e Chícharo, 2008). Os avanços mais recentes estão baseados no uso de ferramentas bioquímicas como índices com base em ácidos nucleicos. O uso da razão de RNA:DNA como um indicador bioquímico do estado fisiológico e nutricional dos organismos aquáticos em condições naturais foi proposto pela primeira vez quase cinco décadas atrás (Holm-Hansen et al., 1968). Desde então, o número de estudos em que a técnica é aplicada tem aumentado consideravelmente (Bulow, 1970; 1987; Clemmesen, 1994; Chícharo et al., 1998; Berdalet et al., 2005), especialmente a partir da década de 1990 (Chícharo e Chícharo, 2008).

Os ácidos nucleicos desempenham um papel importante no crescimento e desenvolvimento celular. O índice da razão RNA:DNA é uma medida da capacidade de síntese celular e geralmente está correlacionada com o estado nutricional do organismo (Buckley et al., 1999). Essa correlação está baseada no princípio de que a quantidade de DNA, que é o principal portador da informação genética, é estável dentro da célula e suas concentrações nos tecidos refletem o número de células presentes (Regnault e Luquet, 1974; Dortch et al., 1983). Por outro lado, a quantidade de RNA na célula é diretamente proporcional à capacidade de síntese proteica. Portanto, organismos com um bom estado nutricional tendem a apresentar valores mais altos na razão RNA:DNA do que aqueles com uma pobre condição corporal (Bulow, 1987; Robinson e Ware, 1988). A relação entre o RNA e o DNA é um índice da intensidade metabólica da célula e tem sido aplicado para medir crescimento recente em peixes adultos (Bulow, 1987). Também, tem se mostrado muito útil como indicador da condição nutricional em estudos de larvas de peixes (Clemmesen, 1993). Por ser muito sensível, a razão RNA:DNA pode proporcionar estimativas da taxa de crescimento em períodos curtos na ordem de um dia até uma semana (Bulow, 1987).

Os primeiros trabalhos que analisaram a quantidade de ácidos nucleicos em tecidos de peixes para estimar a sua relação com a alimentação e crescimento utilizaram uma variedade de técnicas colorimétricas baseadas em raios UV. Essas técnicas geralmente demandam um grande volume de amostras e, portanto, em muitos casos as amostras de vários indivíduos têm sido agrupadas, perdendo resolução (Buckley, 1979). No final da década de 1980 uma quantidade crescente de pesquisadores começou a usar procedimentos fluorimétricos mais sensíveis para analizar os níveis de RNA e DNA em larvas de peixes de maneira individualizada, ao invés de utilizar amostras agrupadas (Clemmesen, 1988; Robinson e Ware, 1988; Westerman e Holt, 1988). Além de mais sensíveis, os métodos fluorimétricos são rápidos e igualmente precisos do que o método de UV. Prestando a devida atenção aos procedimentos metodológicos (Buckley et al., 1999; Chícharo e Chícharo, 2008), a razão RNA:DNA é uma ferramenta que oferece respostas refinadas sobre a condição corporal dos organismos, o crescimento instantâneo dos diferentes tecidos e, portanto, pode potencialmente proporcionar informações sobre as interações tróficas dentro das teias alimentares.

O fluxo de energia em teias alimentares de ecossistemas lóticos no Território Norte da Austrália e na Amazônia central

Capítulo 1:

Desacoplamento temporal entre a aquisição de energia e a alocação para reprodução em uma espécie de peixe herbívoro-detritivoro no Território Norte da Austrália

As conexões entre hábitats e o fluxo de matéria através dos limites dos ecossistemas têm importantes implicações na produção de biomassa de animais e plantas (Vannote et al., 1980; Polis et al., 1997; Vanni, 2002; Jardine et al., 2012). Muitas espécies de peixes atravessam limites ecossistêmicos durante migrações para desova. Por exemplo, nos trópicos úmido-secos do Território Norte da Austrália, os peixes que tipicamente habitam canais de rio, lagoas e estuários durante a época seca se movimentam até as planícies de inundação durante a época cheia, onde aproveitam a abundância de recursos. Subsequentemente, quando as águas de inundação retrocedem, muitos peixes retornam às lagoas, canais do rio e estuários. Tem sido bem documentado que as migrações dos peixes podem transferir efetivamente nutrientes e produção de origem aquática (Vanni, 2002; Moore et al., 2007), mas informações sobre como essa produção contribui para a alocação individual da energia e o investimento reprodutivo desses animais é escasso (mas, ver Jardine et al., 2012). Grande parte dessa energia pode ser obtida da produtividade das planícies de inundação.

As planícies de inundação proporcionam grandes quantidades de recursos de alta qualidade para os consumidores (Junk et al., 1989; Jardine et al., 2012). No entanto, não é bem entendido como os subsídios temporalmente abundantes das planícies de inundação são alocados nos tecidos dos consumidores. Os organismos diferem na forma como alocam os recursos para reprodução. Espécies de peixes diádromos empreendem migrações energeticamente dispendiosas para desovar e usam os excedentes de energia estocados durante periodos anteriores para abastecer a produção reprodutiva (Boulcott e Wright, 2008; Palstra e Van den Thillart, 2010; McBride et al., 2015), uma estratégia chamada de *‘capital breeding’*. Por outro lado, o acoplamento do ciclo reprodutivo com a produção de recursos temporalmente abundantes durante a época de reprodução é denominada *´income breeding´*.

Uma espécie muito comum nos trópicos úmido-secos da Austália, a tainha-diamante (*Liza alata*), é um peixe catádromo amplamente distribuído no Pacífico Indo-ocidental-central. Essa espécie de peixe herbívoro-detritívoro se reproduz nos estuários onde desova grandes quantidades de ovos pelágicos (Bishop et al., 1980). Os juvenis são recrutados nos estuários e migram rio acima até as lagoas das planícies de inundação. É desconhecido se essa espécie é um *´capital breeder´* ou um *´income breeder´*. Se os recursos das planícies de inundação são alocados instantaneamente para reprodução, então maior crescimento de tecidos reprodutivos é esperado durante a época cheia. Por outro lado, se as tainha-diamantes empreendem migrações para desovar no mar durante a época cheia, eles devem possuir suficientes recursos estocados para sintetizar as gônadas antes do início da enchente e esperar-se-ia maior investimento reprodutivo durante a época seca. Nesse caso, o investimento reprodutivo estaria desacoplado temporalmente da disponibilidade de recursos das planícies de inundação.

A maioria de estudos de condição de peixes em estado natural foram baseados em estimativas morfológicas de investimento reprodutivo como a massa total da ninhada, o número de juvenis na ninhada e a frequência de ninhadas (Glazier, 1999). No entanto, além das limitações para quantificar a fecundidade ou o esforço reprodutivo baseado na contagem de ovos (McBride et al., 2015), essas estimativas não expressam o crescimento instantâneo dos tecidos somáticos ou reprodutivos. Um método alternativo, a razão de RNA:DNA de uma célula é um indicador bioquímico de crescimento recente nos tecidos dos organismos (Holm-Hansen et al., 1968; Bulow, 1970; Clemmesen, 1994; Berdalet et al., 2005), porque a quantidade de DNA presente numa célula se mantém relativamente constante, enquanto que as concentrações de RNA variam em proporção à síntese protéica (Bergeron, 1997; Bulow, 1987; Buckley et al., 1999). Dessa forma, através da quantificação da razão desses ácidos nucleicos nas células dos peixes, é possível estimar o investimento instantâneo no crescimento dos diferentes tecidos, o que facilita determinar se o peixe estava investindo em crescimento somático ou reprodutivo no momento da captura.

Outro método, a razão na abundância natural de isótopos estáveis de carbono e nitrogênio tem se convertido em uma ferramenta comum para entender as interações tróficas e as rotas da energia nas teias alimentares (DeNiro e Epstein, 1978; 1981; Peterson e Fry, 1987; Fry, 2006; Layman et al., 2012). Esse método pode ser utilizado para distinguir a origem dos recursos dos tecidos reprodutivos (Jardine et al., 2012). Os valores isotópicos dos tecidos dos vertebrados dependem em parte das taxas de substituição, sendo que o fígado tem um tempo de substituição menor do que o músculo (Tieszen et al., 1983; Hobson e Clark, 1992; Suzuki et al., 2005; Buchheister e Latour, 2010). A meia-vida do carbono e nitrogênio em tecidos metabolicamente ativos dos peixes, como o fígado, varia na ordem de dias a semanas, enquanto que em tecidos estruturais como o músculo, o tempo varia entre semanas a meses (Buchheister e Latour, 2010; Ankjærø et al., 2012; Heady e Moore, 2013). Por tanto, em teoria, o fígado tem o potencial de proporcionar informação de dieta mais recente. Ao comparar os valores isotópicos desses tecidos de substituição rápido e lento com os das gônadas é possível inferir se as gônadas são formadas utilizando recursos disponíveis no momento da captura ou com antecedência de alguns meses (Jardine et al., 2012).

No primeiro capítulo do presente estudo, combinamos informações sobre a razão de RNA:DNA e análises de isótopos estáveis de carbono (δ^{13}C) e nitrogênio (δ^{15}N) de múltiplos tecidos para entender as estratégias espaço-temporais na alocação reprodutiva de *L. alata*. O uso dessas ferramentas permitiu estimar a importância temporal de hábitats aquáticos diferentes como subsídio para a síntese de tecidos somáticos e reprodutivos.

Capítulos 2 e 3

Isótopos estáveis e análises espaciais revelam partição de recursos e mudanças ontogenéticas na posição trófica dos crocodilianos amazônicos

A estrutura das teias alimentares é considerada um dos atributos mais fundamentais dos ecossistemas (Elton, 1927; Lindeman, 1942; Hutchinson, 1959; Pimm, 1982). Um dos principais componentes dentro da estrutura das teias alimentares é a posição trófica dos predadores de topo de cadeia, pois reflete o número de passos que a energia atravessou dos produtores primários até os consumidores terciários.

A coexistência de espécies dentro de comunidades ecológicas é determinada parcialmente pelas formas em que elas partilham os recursos disponíveis (Finke e Snyder, 2008). Espécies em coexistência devem diferir nos requerimentos ecológicos de um recurso compartido por pelo menos uma quantidade mínima para evitar exclusão competitiva (Pianka, 1974). A bacia amazônica é o único ecossistema que sustenta quatro espécies de crocodilianos vivendo em simpatria. Essa diversidade de predadores aquáticos em conjunto com as enormes abundâncias reportadas em algumas regiões da Amazônia central (Da Silveira, 2002), requer a partição das presas de base para que a sua coexistência seja garantida.

Como predadores de topo de cadeia, os crocodilianos potencialmente exercem uma forte influencia na estrutura das teias alimentares. A maioria de espécies de crocodilianos apresenta notáveis mudanças ontogenéticas de dieta e, portanto, podem representar importantes elos tróficos em diversas teias alimentares ao longo das suas vidas, desde água doce, salobre, marinha e hábitats terrestres adjacentes (Radloff et al., 2012; Hanson et al., 2015). Dependendo da espécie, os crocodilianos podem aumentar seu comprimento de 6 a mais de 20 vezes ao longo da vida. Como resultado, eles experimentam mudanças progressivas na dieta, consumindo desde invertebrados terrestres e aquáticos durante as fases juvenis até dietas mais ricas em proteína compostas principalmente por peixes e vertebrados terrestres durante a fase adulta (Ross, 1998). Apesar de a maioria dos crocodilianos ser considerada predadora generalista e oportunista, as espécies amazônicas mostram amplas diferenças nas proporções de presas aquáticas e terrestres que consomem (Magnusson et al., 1987).

As trajetórias ontogenéticas de dieta nos crocodilianos amazônicos leva a diferenças interespecíficas visíveis quando são adultos. Análises tradicionais de conteúdos estomacais têm demonstrado que indivíduos jovens de *Paleosuchus palpebrosus, Caiman crocodilus* e *Melanosuchus niger* apresentam dietas compostas principalmente por invertebrados terrestres e aquáticos, mudando progressivamente para um maior consumo de peixes quando são adultos (Magnusson et al., 1987; Da Silveira e Magnusson, 1999). *Paleosuchus trigonatus*, por outro lado, muda de uma dieta composta por invertebrados terrestres para vertebrados terrestres ao longo do desenvolvimento (Magnusson et al., 1987). Acredita-se que muitas das diferenças interespecíficas na dieta são um reflexo de divergências no uso dos macrohábitats. No entanto, é desconhecido em que medida as diferenças na dieta podem representar divergências no comportamento de forrageio e mudanças ontogenéticas, além de ser uma função do uso de hábitat. Desconhece-se também em que medida os crocodilianos amazônicos diferem nas fontes primárias dos recursos que consomem e como eles evitam a exclusão competitiva em corpos hídricos onde ocorrem de maneira sintópica. Baseado em estudos de outras espécies, espera-se que a posição trófica dessas espécies aumente ao longo do desenvolvimento (Radloff et al., 2012; Hanson et al. 2015; Bontemps et al. 2016) e que hipoteticamente os indivíduos maiores apresentem uma posição alta refletindo a sua condição de predadores de topo. No entanto, os crocodilianos são comumente excluídos de estudos em que o nível trófico dos predadores de topo é analisado (Vander Zanden e Fetzer, 2007) e desconhece-se até que ponto o uso de peixes piscívoros pode produzir divergências significativas nas estimativas do comprimento das cadeias tróficas nos diferentes estudos.

Nesse estudo, analisamos a razão de isótopos estáveis de carbono (δ^{13}C) e nitrogênio (δ^{15}N) das quatro espécies de crocodilianos amazônicos (*Melanosuchus niger, Caiman crocodilus, Paleosuchus palpebrosus* e *Paleosuchus trigonatus*) e suas potenciais presas para avaliar as diferenças interespecíficas na dependência de recursos aquáticos e terrestres e na mudança ontogenética na sua posição trófica. Essas informações foram colocadas em um contexto espacial utilizando mapas classificados que refletem as classes de macrohábitat (riachos de cabeceira, riachos de médio porte de florestas alagadas e planícies de inundação de várzea) para esclarecer se as diferenças na dieta são explicadas pela seleção de hábitat ou refletem o comportamento de forrageio associado com mudanças ontogenéticas. Além disso, estimamos o nível trófico dos crocodilianos e o comparamos com os diferentes componentes da teia alimentar para entender qual nível trófico que esses predadores ocupam.

De maneira geral, no presente estudo utilizamos um contexto espaço-temporal para traçar as origens e o fluxo da energia utilizando ferramentas químicas para entender três aspectos interessantes no funcionamento das teias alimentares dos ecossistemas aquáticos tropicais estudados: A sazonalidade e espacialidade no investimento reprodutivo do peixe *Liza alata* do território Norte da Austrália (Capítulo 1); as diferenças interespecíficas nas origens da energia que sustenta as quatro espécies de crocodilianos amazônicos dentro de um contexto espacial (Capítulo 2); e as mudanças ontogenéticas na posição trófica desses predadores dentro do contexto das teias alimentares em que estão inseridos (Capítulo 3).

OBJETIVOS

O presente estudo busca aportar com conhecimentos sobre a sazonalidade e as origens da energia sustentando os grandes consumidores ectotérmicos, como peixes e crocodilianos. Especificamente, os objetivos propostos para cada capítulo são:

Capítulo 1

1) Descobrir quando os recursos de energia são alocados para reprodução e crescimento somático em *Liza alata*.

2) Comprovar se existe um compromisso entre o investimento reprodutivo e somático nessa espécie.

3) Quantificar as semelhanças dos tecidos com substituição de curto e longo prazo em termos de isótopos estáveis de carbono e nitrogênio com relação às gônadas.

Capítulo 2

1) Quantificar a proporção em que os crocodilianos amazônicos dependem dos recursos de origem aquática ou terrestre.

2) Estimar em que medida as diferenças interespecíficas na dependência de recursos aquáticos ou terrestres são uma função da seleção de macrohábitat.

Capítulo 3

1) Descobrir em que medida as trajetórias ontogenéticas da dieta refletem as mudanças na posição trófica dos crocodilianos amazônicos.

2) Após estimar a posição trófica de todos os consumidores da teia alimentar, avaliar em que medida o uso dos valores de posição trófica de peixes piscívoros subestima os crocodilianos como predadores de topo de cadeia.

Capítulo 1

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RESEARCH ARTICLE

Temporal Uncoupling between Energy Acquisition and Allocation to Reproduction in a Herbivorous-Detritivorous Fish

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Abstract

Although considerable knowledge has been gathered regarding the role of fish in cycling and translocation of nutrients across ecosystem boundaries, little information is available on how the energy obtained from different ecosystems is temporally allocated in fish bodies. Although in theory, limitations on energy budgets promote the existence of a trade-off between energy allocated to reproduction and somatic growth, this trade-off has rarely been found under natural conditions. Combining information on RNA:DNA ratios and carbon and nitrogen stable-isotope analyses we were able to achieve novel insights into the reproductive allocation of diamond mullet (*Liza alata*), a catadromous, widely distributed herbivorous-detritivorous fish. Although diamond mullet were in better condition during the wet season, most reproductive allocation occurred during the dry season when resources are limited and fish have poorer body condition. We found a strong trade-off between reproductive and somatic investment. Values of $\delta^{13}C$ from reproductive and somatic tissues were correlated, probably because $\delta^{13}C$ in food resources between dry and wet seasons do not differ markedly. On the other hand, data for $\delta^{15}N$ showed that gonads are more correlated to muscle, a slow turnover tissue, suggesting long term synthesis of reproductive tissues. In combination, these lines of evidence suggest that *L. alata* is a capital breeder which shows temporal uncoupling of resource ingestion, energy storage and later allocation to reproduction.

Introduction

Linkages among habitats and the flux of matter across ecosystem boundaries have important implications for biomass production of animals and plants [1–4]. As part of spawning movements and migrations, many species of fish cross ecosystem boundaries. For example, in the wet-dry tropics, fish that typically live in river channels, waterholes and estuaries during the dry season move onto floodplains during the wet season, where they take advantage of

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abundant resources. Subsequently, as flood waters recede, many fish disperse back to waterholes, river channels and estuaries. It has been well documented that fish movements may transfer nutrients and aquatic production [3,5], but information on how this production contributes to individual energy allocation and reproductive investment is scarce (but see [4]). Much of this energy might be obtained from floodplain productivity.

Floodplains provide large amounts of high quality resources to consumers [4,6], with higher production of macrophytes, phytoplankton and attached algae during periods of inundation [7]. In general, abundant food resources support earlier maturation and higher fecundity in fish (see [8]). However, it is not well understood how the temporally abundant subsidies from floodplains are allocated to consumer tissues. According to life-history theory, allocation to reproduction results in a trade-off with somatic growth or survival because of limitations of the resource budget [9–14]. However, trade-offs between somatic growth and reproduction have rarely been observed under natural conditions [14,15].

Organisms differ in their allocation of resources to reproduction. Diadromous species engage in energetically expensive migrations for spawning and use surplus energy stored from previous periods to fuel reproductive output [8,16–18], a strategy known as 'capital breeding' [19–21]. Conversely, the coupling of the reproductive cycle with temporally abundant resources during the breeding period is characterized as ´income breeding´. On floodplains with short inundation periods (~2 months) in the Australian wet-dry tropics, the development of reproductive tissues was fueled by resources available at the time of spawning in the herbivorous fish, *Nematalosa come* [4]. Therefore, the reproductive cycle of this 'income-breeding' fish, not known to undertake upstream spawning migrations [22], is coupled with the temporally abundant resources from floodplains, despite the short inundation duration.

Another common species in the Australian wet-dry tropics, the diamond mullet (*Liza alata*), is a catadromous fish broadly distributed in the Indo-west-central Pacific. This herbivorous-detritivorous fish breeds in estuaries and spawns large numbers of pelagic non-adhesive eggs [23], as do most of the other members of the Mugilidae [24]. Juveniles recruit in estuarine areas from where they move up river. Although information on reproduction of this species is scarce, Bishop [23] found higher values of gonadosomatic index (GSI) during the early-wet season in the Alligator Rivers region and suggested that spawning migrations must occur during the wet season, which is the only time when seasonally isolated water bodies are connected to the sea for more than 4 months. It is unknown whether this species is a capital or an income breeder. If resources from floodplains are instantly allocated to reproduction, then greater growth of reproductive tissues is expected during the wet season. On the other hand, if mullet carry out spawning migrations to the sea during the wet season, they must have enough stored resources to synthesize gonads before flooding begins and we would expect higher reproductive investment during the dry season. In this case, reproductive investment would be temporally uncoupled from resource availability from floodplains.

Most studies under natural conditions have relied on morphological estimates of reproductive investment, such as total clutch mass, the number of young in a clutch and frequency of clutches (see [14]). However, besides the limitations on quantifying fecundity or reproductive effort based on counting fish eggs [8], those estimates do not express instantaneous growth of reproductive or somatic tissues. An alternative method, RNA:DNA ratio of a cell, is a biochemical indicator of recent growth in aquatic organisms [25–28]. This is because the amount of DNA present in a cell remains relatively constant, whereas RNA concentrations vary in proportion to protein synthesis [29–31]. Thus, by quantifying the ratio of these nucleic acids in the cells of fish it is possible to estimate the instantaneous investment in growth of different tissues. This can allow inferences as to whether the fish was investing in somatic or reproductive growth at the time of capture. Another method, stable-isotope analysis (SIA) has become a common tool for investigating trophic interactions and energy pathways in food webs [32–35] and can be used to determine the origin of resources in reproductive tissues [4]. Isotope values of vertebrate tissues depend in part on the isotopic turnover rates, with liver having a shorter half-life than muscle [36–39]. Half-lives of carbon and nitrogen in metabolically active fish tissues, such as liver, range in the order of days to weeks, while structural tissues, such as muscle, show half-lives in the range of weeks to months [39–41]. Therefore, theoretically, liver has the potential to provide more recent dietary information. Comparing isotope ratios of these fast and slow turnover tissues with those of gonads may be useful to indicate whether gonads were formed using resources available at the time of capture or a few months in advance [4].

Here we combine information on RNA:DNA ratios and stable-isotope analyses to achieve novel insights into the reproductive allocation strategies of diamond mullet. We analysed RNA:DNA and ratios of stable isotopes of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) in multiple tissues of this fish to estimate the temporal importance of different aquatic habitats as a subsidy for the synthesis of somatic and reproductive tissues. Specifically, we asked the following questions: 1) When do mullet allocate energy resources for reproductive and somatic growth? 2) Is there a trade-off between reproductive and somatic investment? 3) How different are short- and long-term turnover tissues in terms of carbon and nitrogen stable isotopes?

Materials and Methods

Study area

This study was undertaken within the limits of Kakadu National Park, a protected area located in the Alligator Rivers region in the Northern Territory, Australia (Fig 1). This region is situated about 150 km east of the city of Darwin and is part of a bio-geographical region known as the Australian wet-dry tropics. The most conspicuous climatic characteristic of this region is the presence of a warm dry season and a warm-humid wet season [42,43]. The dynamic hydrology drives most ecosystem processes and structure, including primary productivity and subsidies to food webs through fish movements [4,44].

One fourth of the area of the Australian wet-dry tropics is comprised of floodplain wetlands [44]. These ecosystems experience extensive seasonal inundations and high river flows during the wet season between November and April. As rainfall decreases during the dry season, flows are reduced to zero in most rivers and waterbodies are contracted and isolated [45,46]. During this period, creeks and floodplain areas dry out except for a few permanent swamps and lagoons, known locally as billabongs or waterholes [47]. The Alligator Rivers region area is covered by water during March and April, which recedes to approximately 25%–30% of its maximum extent by August and September. It takes about 5 months to reduce to 50% of the maximum recorded flooded area in a given wet season [48]. Tidal influence extends for 70–90 km along the major rivers [47].

Permission to access biological resources in a commonwealth area for non-commercial purposes was provided by the Australian Government. Permit number: AU-COM2012-171.

Mean monthly water-level data at the South Alligator River Data Warehouse (12°39´42´´S, 132°30´26´´E) from 1979 to 2011 was obtained from the Department of Land Resource Management Water Data Portal < http://www.lrm.nt.gov.au/water/water-data-portal>. These data were used to broadly characterize water-level variation at the study area.

Fish and primary resources sampling

We sampled 13 water bodies, including waterholes and floodplains, but none of those waterbodies were visited during both dry and wet seasons because of lack of water or access. Nevertheless, most waterbodies sampled during the dry season were represented by their surrounding

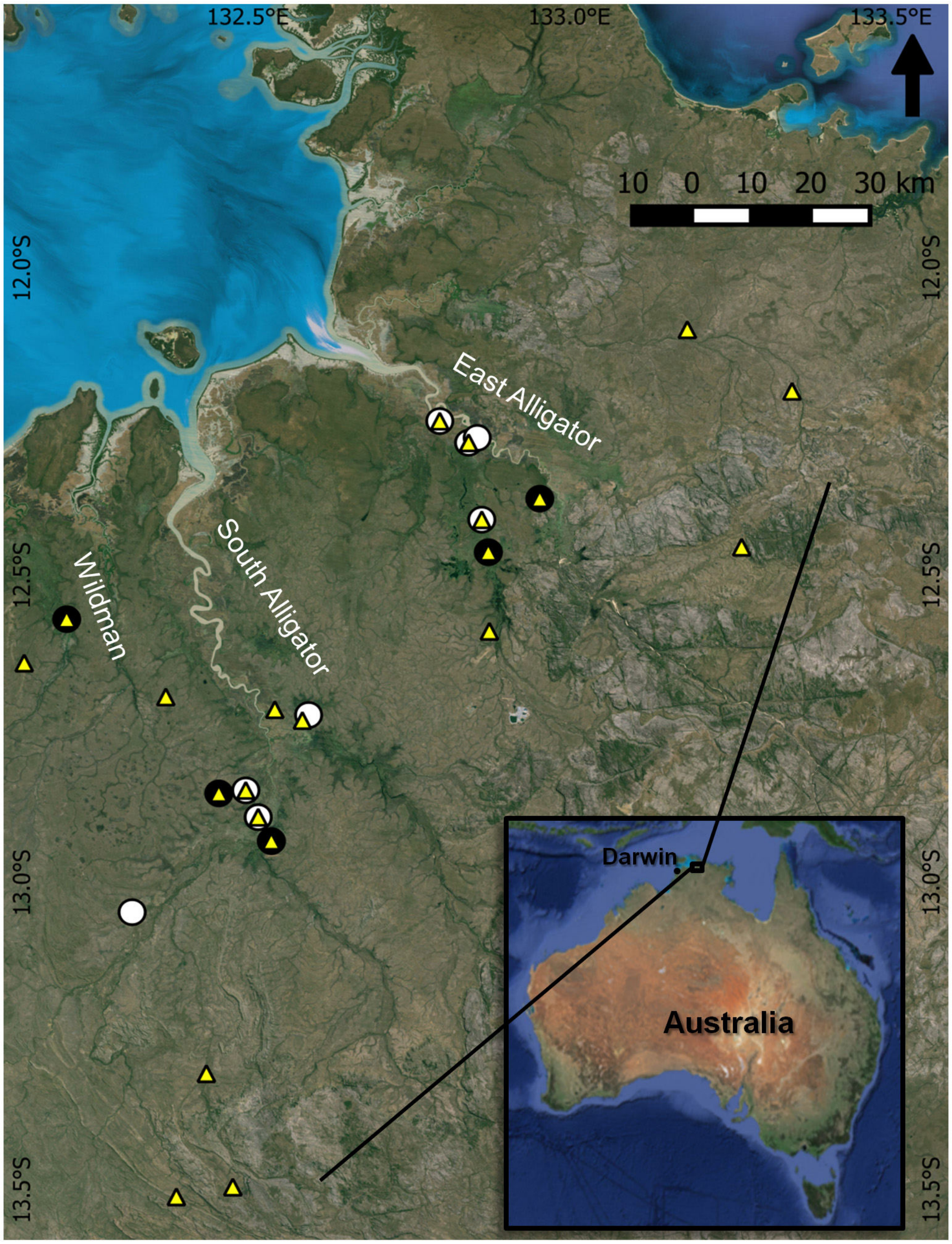

Fig 1. Study area. Location of mullet sampling sites during dry and wet seasons (black and white dots, respectively) in the Alligator Rivers region—Northern Territory, Australia. Yellow triangles represent locations where primary sources were collected.

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floodplains during the wet season (Fig 1). We concentrated sampling during the late-dry (October-November, 2013) and late-wet seasons (April-May, 2014).

Mullet were caught mainly using gill nets. Electrofishing was used in a few waterholes during dry-season sampling. Fish were measured (Standard length, ±1 mm), weighed (±1 g) and dissected to collect samples of muscle, liver, gonads and eggs, when present. We placed all tissue samples in labeled cryogenic vials and stored them immediately on ice for SIA and in liquid nitrogen for RNA:DNA analyses. All animals were euthanized using clove oil, and all efforts were made to minimize suffering. Only 42 of the 56 individuals collected were of adult size (standard length > 27 cm), and juveniles were not used in analyses.

The use of animals in this study was approved by Griffith University's Animal Ethics Committee in accordance with the Australian Government's code for the care and use of animals for scientific purposes. Permit Number: ENV/08/11/AEC "NABH-Northern Australia Biodiversity Hub".

We included data of other components of aquatic foodwebs, including biofilm, detritus and filamentous algae as possible primary resources for fish (S2 Table). These data were collected as part of a broader foodweb project between 2012 and 2014. The samples of these primary sources were collected in ten of the same sites where mullet were captured, and samples from 11 additional sites were also included in analyses (Fig 1). Specific methods used to collect end-member organisms are summarized in [49].

RNA:DNA laboratory processing

On arrival at the laboratory, samples were removed from liquid nitrogen and kept frozen at -80°C for a maximum of 15 days. We randomly took sub-samples weighing between 0.001 and 0.3g for analyses. We added 200μl of 0.5% Sarcosil-TE buffer (0.5% sarcosyl; 10mM Tris-HCl, pH 7.5; 1mMEDTA) and two sterile beads (3mm) to the samples in order to induce cell lysis by high-frequency oscillation. RNA and DNA content were quantified using a QubitTM flourometer and fluorescent dyes and standards from Qubit® dsRNA—DNA BR Assay Kits. RNA and DNA content were expressed as μg/mL. RNA:DNA represents the ratio of these concentrations.

SIA Laboratory processing

In the laboratory, all samples were kept frozen at -20°C for 2–4 weeks. We then dried the samples in an oven at 60°C for at least 24 h before grinding and homogenizing them with a mortar and pestle. Samples of 0.6–1.0 mg were used in the analyses.

Samples were combusted in a EuroEA 3000 (EuroVector, Italy) or Europa GSL (Sercon Ltd, Crewe, UK) elemental analyzer and the resulting N_2 and CO_2 gas were chromatographically separated and fed into an IsoPrime (Micromass,UK) or Hydra 20–22 (Sercon Ltd, Crewe UK) isotope-ratio mass spectrometer. This measures the ratio of heavy and light isotopes in a sample and compares them to a standard. Elemental ratios (C/N) are expressed in %C and %N by mass and isotope ratios (δ) as parts per mil (‰), defined as $\delta(‰) = (R_{sample}/R_{standard}—1)^{*}1000$, where R_{sample} and $R_{standard}$ are the isotope ratios of the sample and standard, respectively. Isotopic standards used were referenced to PeeDee Belemnite (PDB) for carbon, and atmospheric air for nitrogen [32]. Secondary standards of Ammonium Sulfate and Sucrose were used in each run. Acetanilide was used to cross reference elemental compositions of secondary standards.

Data analysis

We used exploratory bi-plots and regressions to examine relationships between length and body mass, and used regression residuals as an index of body condition. Because high C/N ratios in animal tissues are indicative of high lipid content [50–52], we used C/N as a secondary indicator of condition. Also, because high lipid levels can cause isotopic fractionation when C/N is higher than 4.0 [50], we performed chemical lipid extractions on a subset of 29 samples (10 muscle, 9 gonads and 10 liver) using a chloroform:methanol solution following the protocol from Bligh and Dyer [53]. After re-analyzing these samples for stable isotopes, we compared the resulting $\delta^{13}C$ values with those mathematically lipid corrected using C/N and the most common equations in the literature [51, 52, 54–57]. We used the slopes and fit (r^2) of the relationships to choose the most appropriate equation to correct the remainder of the samples. The equation from [51] yielded results that best matched values from our extracted samples, so we mathematically corrected samples with C/N ratios $\geq$4.0 using that equation (S1 Fig).

We explored the relationships between reproductive and somatic tissues in terms of RNA: DNA ratios and carbon and nitrogen stable isotopes using simple and multiple linear regressions. Specifically, we tested whether δ^{13}C and δ^{15}N of reproductive tissues (gonads) were predicted by somatic tissues (muscle and liver) and also whether long-term (muscle) is predicted by short-term turnover tissue (liver).

To test for differences between dry and wet seasons on carbon and nitrogen SI of mullet tissues (muscle, liver and gonads) and primary resources (detritus, filamentous algae and biofilm), we used one-way Analyses of Variance (ANOVA).

We used a two-way ANOVA to compare RNA:DNA means using season and tissue type as factors. We used Analyses of Covariance (ANCOVA) to test if gonadal RNA:DNA ratios are related to gonadal δ^{15}N and season.

We also included the site of capture nested within seasons as factor to control for confounding effects of different locations being sampled. R software [58] was used for all statistical analyses and graphics.

Results

We found no evidence suggesting that the site of capture influenced any of the patterns explained regarding body condition, RNA:DNA ratios and δ^{15}N of different tissues ($F<2.4$; $p>0.067$ in all cases). However, we found a significant influence of the site where mullet were caught on δ^{13}C of sampled tissues ($F = 2.8$; $p = 0.022$), suggesting site-specific δ^{13}C signatures being incorporated on mullet tissues. During the wet season, most individuals had immature or early developing gonads. During the dry season, most had early developing gonads, but four had ripe eggs. There was a significant positive correlation between standard length and body mass ($\log(y) = -5.13 + 3.18^{*}\log(x)$; $r^2 = 0.69$; $p<0.001$). During the wet season, most individuals were heavier for a given length, and conversely during the dry season they were lighter than expected for their length (S2 Fig). We used the residuals from the length—body mass regression as an index of condition.

Significant differences in body condition were found between seasons (t-test; $t = -8.91$, $df = 33$, $p< 0.001$), and all individuals had better body condition during the wet season. Although not quantified, we observed the presence of large mesenteric fat bodies comprising around one third of body volume in individuals during the wet season (Fig 2).

There was a strong positive, though non-linear, correlation between C/N ratios of muscle tissue and the regression residuals, suggesting that the two indicators were in good agreement, and C/N ratios were also higher during the wet season ($t = -2.86$, $df = 16.1$, $p = 0.011$) (Fig 3).

When data from wet and dry seasons were combined, body condition (BC) predicted RNA: DNA (R/D) in gonads ($R/D = 21.02–63.5\ BC$, $F = 15.8$, $r^2 = 0.36$, $p<0.001$). R/D was negatively related to BC, suggesting a trade-off between reproduction and somatic investment throughout the year. However, this relationship was not evident when analyzing each season separately (Dry season: $r^2 = 0.11$, $p = 0.159$; Wet season: $r^2 = 0.04$, $p = 0.222$) (Fig 3).

A two-way ANOVA on RNA:DNA values showed a significant interaction between season and tissue type ($F = 18.93$, $p<0.001$), suggesting that despite the lower body condition and lipid levels, most growth occurred in gonads during the dry season, when water level was at its lowest (Fig 4 and Table 1).

No significant differences in δ^{13}C and δ^{15}N of mullet tissues and primary resources (filamentous algae and biofilm) were found between dry and wet seasons. However, detritus δ^{15}N was 2.3‰ more enriched during the wet season (Table 2).

Fig 2. Adult individual of *Liza alata*. During the wet season, individuals of this species possess large mesenteric fat bodies (blue arrow) representing up to one third of the body volume.

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Carbon stable-isotope ratios ($\delta^{13}C$) of somatic and reproductive tissues were highly correlated. Liver $\delta^{13}C$ significantly predicted both gonad ($\delta^{13}C_G = -8.55 + 0.69\delta^{13}C_L$, F = 62.9, $r^2 = 0.65$, $p<0.001$) and muscle ($\delta^{13}C_M = -12.08 + 0.57\delta^{13}C_L$, F = 30.1, $r^2 = 0.46$, $p<0.001$) (S3A and S3B Fig, respectively). Gonad $\delta^{13}C$ was also predicted by muscle $\delta^{13}C$ ($\delta^{13}C_G = -5.91 + 0.79\delta^{13}C_M$, F = 49.1, $r^2 = 0.59$, $p<0.001$) (S3C Fig). Standardized regression coefficients from a multiple linear regression model ($R^2 = 0.72$, $p<0.001$) indicated that the variation in $\delta^{13}C$ from gonads was equally predicted by the variation in liver ($b_{standardized} = 0.43$, $p<0.001$) and that in muscle ($b_{standardized} = 0.43$, p = 0.003), but season had no significant effect ($b_{standardized} = -0.19$, p = 0.64).

Nitrogen stable isotopes from liver ($\delta^{15}N_L$) had a relatively weak relationship with gonads ($\delta^{15}N_G = 0.78 + 0.76\ \delta^{15}N_L$, F = 21.01, $r^2 = 0.43$, $p<0.001$) and muscle ($\delta^{15}N_M = 3.4 + 0.55\ \delta^{15}N_L$, F = 9.08, $r^2 = 0.22$, p = 0.005) (Fig 5A and 5B, respectively), but the relationship between muscle and gonad was stronger ($\delta^{15}N_G = -0.55 + 0.89\ \delta^{15}N_M$, F = 80.43, $r^2 = 0.74$, $p<0.001$). Gonads were consistently lower in $\delta^{15}N$ values relative to muscle, which caused the relationship to fall below the 1:1 line (Fig 5C). Standardized regression coefficients from a multiple regression model ($\delta^{15}N_G = -3.16 + 8.24_{\delta^{15}NM} + 3.62_{\delta^{15}NL} + 3.08_{Season}$, $R^2 = 0.85$, $p<0.001$) indicated that variation in $\delta^{15}N$ in gonads was better predicted by $\delta^{15}N$ in muscle ($b_{standardized} = 0.73$, $p<0.001$) than that in liver ($b_{standardized} = 0.32$, p = 0.001) or by season ($b_{standardized} = 0.4$, p = 0.004).

Analysis of covariance indicated that RNA:DNA ratios were significantly related to $\delta^{15}N$ in gonads (p = 0.04), and season (p = 0.02), but there was no interaction between season and $\delta^{15}N$ (p = 0.09).

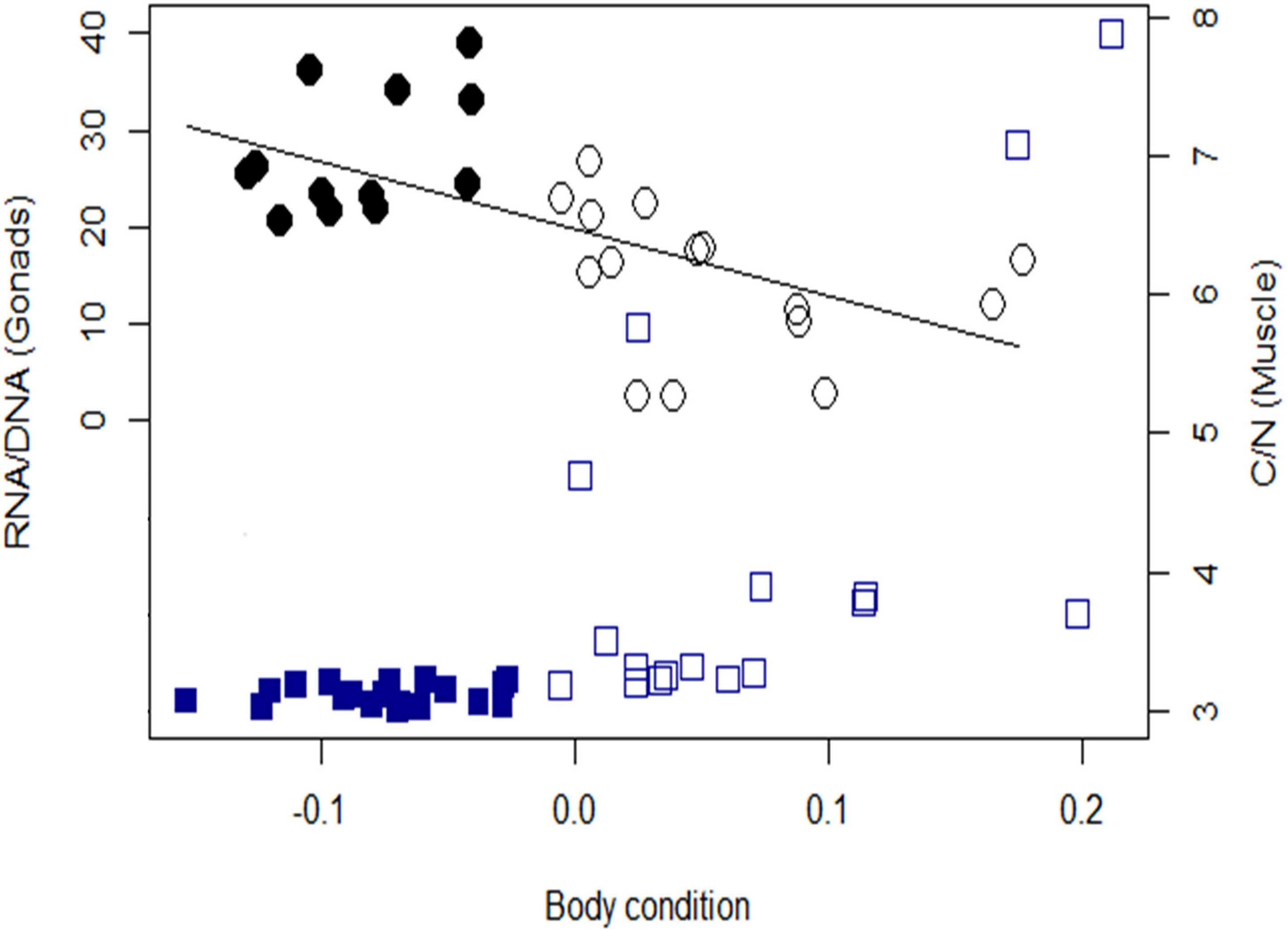

Fig 3. Relationships between body condition, reproductive investment and lipid content in somatic tissues. Regression residuals of standard length and body mass were used as surrogates of body condition (X axis). The Y axis represents RNA:DNA ratios in gonads, a proxy of reproductive investment (black circles). The Z axis represents C/N ratios, a proxy of lipid content in muscle tissue (blue squares). Individuals from dry and wet seasons are represented by solid and open symbols, respectively.

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Discussion

Using a novel combination of chemical indicators, we found evidence of temporal uncoupling between resource availability and allocation of energy to reproduction by *Liza alata* in the Alligator Rivers region. Fish were in better condition in the wet season when food availability peaks, yet were actively synthesizing gonads in the dry season. A trade-off between reproductive and somatic investment was evident and carbon and nitrogen stable isotopes suggested that the long-term diet was mainly contributing to reproductive growth.

We used the residuals from the length-mass relationship of mullet as an index of body condition. A strong correlation between a second indicator of condition, C/N ratios of muscle tissue, and the regression residuals of length and body mass suggests that residuals can appropriately be used as an index of body condition. The relationship between C/N and the regression residuals is likely nonlinear because, in addition to storing fat in muscle, this species also stores fat in specialized mesenteric fat bodies. Therefore, although they may be

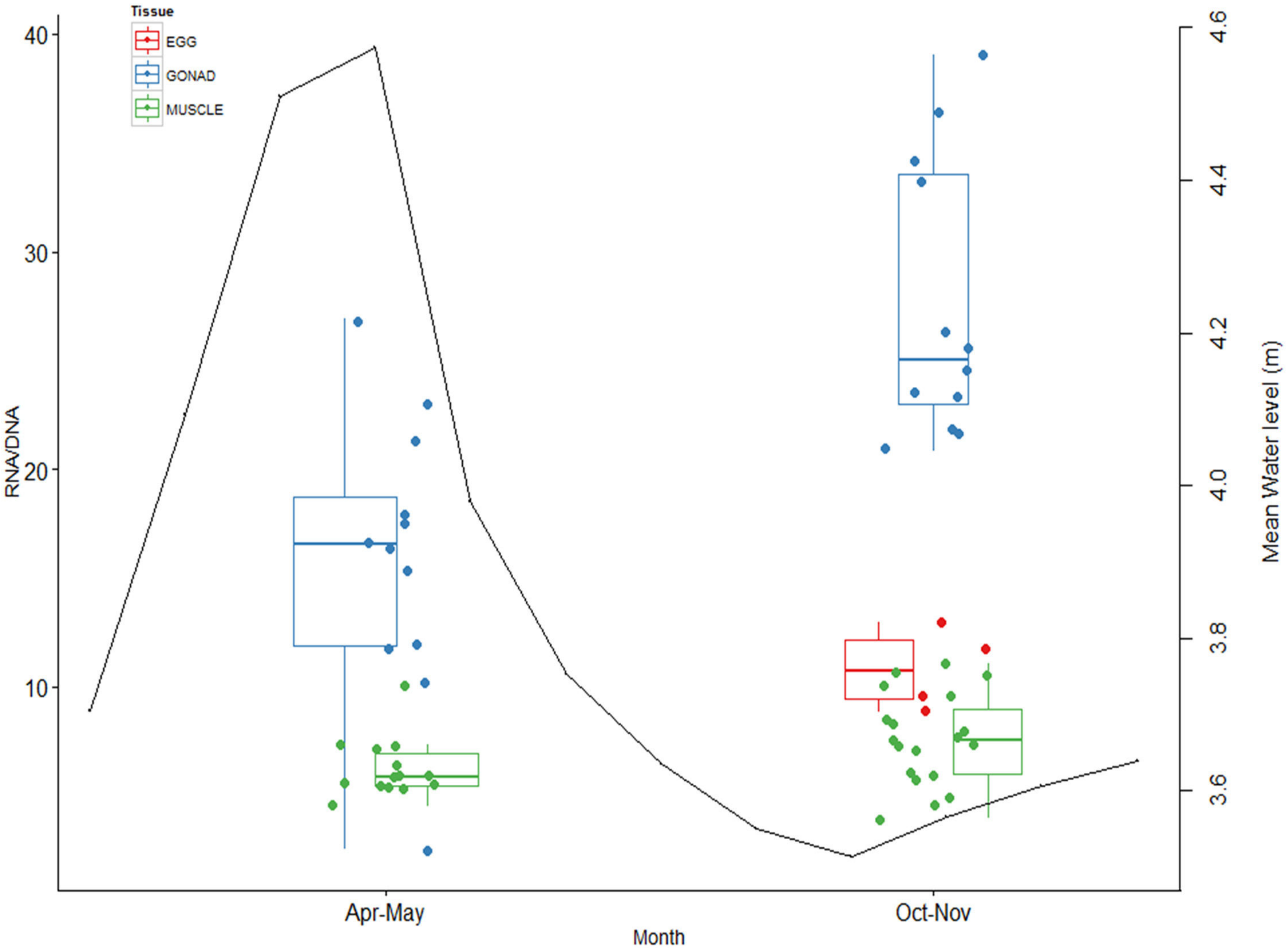

Fig 4. Somatic and reproductive growth. Growth of tissues is indicated by RNA:DNA ratios in relation to the flooding cycle (mean monthly water level data at the South Alligator River Data Warehouse, black line). Developed eggs, gonads and muscle tissues are represented by red, blue and green dots, respectively.

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Table 1. RNA:DNA values of *Liza alata* tissues.

	Dry		Wet	
Tissue	**Mean (n)**	**± SD**	**Mean (n)**	**± SD**
EGGS	10.83 (4)	1.92	-	-
GONAD	27.54 (12)	6.39	14.00 (12)	7.83
MUSCLE	7.61 (19)	2.11	6.22 (14)	1.27

Means and standard deviations (± SD) of RNA:DNA values from different tissues during dry and wet seasons.

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Table 2. Stable isotopes of C and N of *L. alata* and its primary sources.

	δ^{13}C (±SD)		F	p	δ^{15}N (±SD)		F	p
	Dry	Wet			Dry	Wet		
L. alata (M)	-29.02 (±1.81)	-29.83 (±2.85)	1.08	0.304	7.11 (±0.77)	6.91 (±0.99)	0.5	0.484
L. alata (G)	-28.44 (±2.03)	-29.32 (±2.28)	1.33	0.258	5.45 (±0.77)	5.82 (±1.06)	1.24	0.274
L. alata (L)	-28.68 (±2.35)	-30.25 (±2.80)	3.46	**0.071**	6.50 (±1.04)	6.45 (±0.71)	0.03	0.854
Detritus	-31.26 (±0.66)	-30.28 (±3.37)	1.06	0.322	1.39 (±1.31)	3.71 (±2.53)	4.39	**0.058***
Filam. algae	-28.47 (±5.15)	-35.69 (±1.33)	3.71	**0.072**	2.95 (±2.20)	2.4 (±2.74)	0.11	0.746
Biofilm	-27.03 (±2.97)	-26.19 (±3.46)	1.37	0.244	4.33 (±2.97)	3.71 (±2.0)	0.61	0.436

Mean and Standard deviation (±SD) values of carbon (δ^{13}C) and nitrogen (δ^{15}N) stable isotopes from mullet tissues (M = muscle, G = gonad, L = liver) and their main primary sources available during dry and wet seasons. F and p values correspond to results from a One-Way ANOVA testing differences on δ^{13}C and δ^{15}N between seasons.

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confounded by reproductive tissue or stomach fullness, indices based on length-weight relationships appear more sensitive than muscle C/N ratios for assessing fish condition.

Although mullet in this study showed evidence of gonadal growth during both dry and wet seasons, RNA:DNA ratios imply that higher growth occurred during the dry season. This was unexpected because the dry season is the period of the year when water level is at its lowest, as is primary productivity and food availability in the Alligator Rivers region [7]. Given the high prevalence of mesenteric fat bodies and the higher lipid content of muscle (higher C/N) in fish captured during the wet season, this suggests that mullet store most of their energy for reproductive and somatic growth as fat when resources are more abundant and they can attain a favorable body condition. The fat is then re-mobilized to the gonads during the dry season, reducing condition but increasing gonad mass in preparation for spawning in the early-wet season.

Although RNA:DNA values of muscle tissue were slightly higher during the dry season, this tissue grew significantly less relative to gonads during both seasons. Slow growth rates, a common characteristic of related species within the Mugilidae [59], might be causing this pattern. Some Mugilids attain about 75% of their maximum size in their first 3–4 years of life, with greatest mean annual growth increments during the first year and decreasing markedly after age 3–5 [60]. All mature mullet analyzed in this study had standard lengths between 27 and 42 cm. It is plausible that these individuals were growing slowly and with limited food consumption rates outside of the wet season.

Furthermore, low values of RNA:DNA found in developed eggs might be the result of increasing volumes of lipid granules during pre-ovulatory stages, typical of other Mugilidae species [61]. Throughout oogenesis, early oocytes are rich in protein and RNA within the yolk nucleus. Subsequently, with the approach of the breeding season, during the vitellogenic period, lipid droplets accumulate in the cytoplasm [61]; this might explain such low values of RNA:DNA in developed eggs relative to gonads.

Life-history theory states that organisms have a limited resource budget and thus, allocation to reproduction arises as a trade-off against somatic growth or survival. The "Principle of Allocation" [62] predicts negative correlations between reproduction and somatic growth [9–14]. Somatic investment is usually used as a surrogate of body condition (e.g. fat content or body mass per length) [14]. The strong negative correlation between body condition and RNA:DNA in mullet gonads in this study provides evidence of a trade-off between reproduction and somatic investment. Although expected, this trade-off has rarely been measured in natural conditions, mainly because individual variation in resource acquisition exceeds that of resource

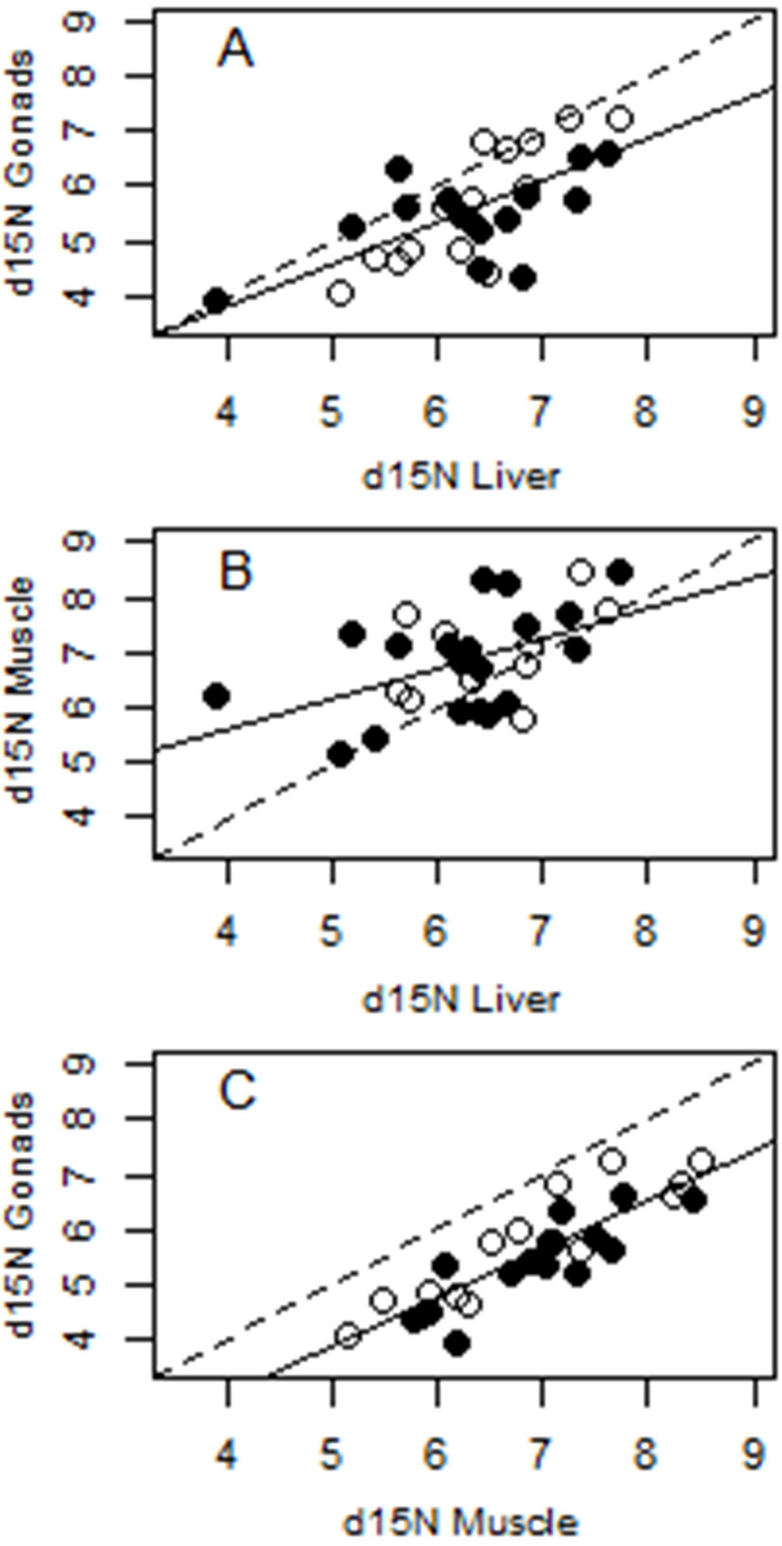

Fig 5. Relationships between $\delta^{15}N$ values in somatic and reproductive tissues of *Liza alata*. The dashed line represents the 1:1 expected relationship. The solid line represents the least-squares linear regressions of: A) liver vs. gonads, B) liver vs. muscle and, C) muscle vs. gonads. Individuals from dry and wet seasons are represented by solid and open symbols, respectively.

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allocation [15]. Estimates of reproductive investment in most studies under natural conditions rely on values such as total clutch mass, the number of young in a clutch and frequency of clutches (see [14]). However, there are difficulties associated with quantifying fecundity or reproductive effort of fish based on counting eggs [8] because there could be confounding effects, such as downregulation of secondary oocytes by atresia which may reduce the final number of eggs ovulated [63–66]. Since RNA:DNA ratios are molecular measures of instantaneous growth of specific tissues, such as gonads, they are likely to be useful estimates of reproductive investment. This can overcome the difficulties of estimating reproductive effort, especially in capital breeding fishes.

Because capital breeders [67–69] use stocks of energy in their body to sustain reproduction, a positive correlation between reproductive investment and pre-breeding body stores is expected [14]. This is true in the case of mullet in this study. Low values of gonadal RNA:DNA during the period when body condition is at its highest suggest that the wet season is the time of the year when mullet store most energy. On the other hand, elevated values of gonadal RNA: DNA ratios during the dry season suggest that these energy stocks are allocated to reproduction months after they were acquired. In combination, these lines of evidence suggest that *L. alata* is a capital breeding fish which shows a temporal uncoupling of resource ingestion, energy storage and allocation to reproduction.

Further evidence for capital breeding is provided through carbon and nitrogen stable isotope analyses. Isotopic equilibrium depends on turnover rates of tissues, and recent dietary sources are more rapidly reflected in fast-turnover tissues such as liver [37–40]. Diamond mullet in this study showed strong correlations of δ^{13}C values in reproductive (gonads) and somatic tissues (liver and muscle), suggesting that both short- and long-term diet could be contributing to gonadal growth. If gonads were being synthesized using only energy stored months before when mullet had access to resources from floodplains, then we would expect δ^{13}C values of gonads to be poorly correlated with those of liver and more correlated with tissues showing slower turnover rates, such as muscle. However, we also found a significant positive correlation between δ^{13}C values for liver and muscle tissues. This strong correlation may be due to overall isotopic similarity between wet and dry season habitat resources. Although filamentous algae showed a slight increase in δ^{13}C values which might explain the slight increase in liver δ^{13}C values during the wet season, we found no significant differences between dry and wet season δ^{13}C values of other potential mullet resources, such as detritus and biofilm.

Results of δ^{15}N analyses were more consistent with expectations for a capital breeder. Although gonad δ^{15}N values showed significant correlations with liver values, a stronger correlation was found with muscle tissue, as revealed by a significantly greater slope and r^2. Higher δ^{15}N in muscle tissue that turns over more slowly than in fast-turnover liver tissue might be a reflection of the significantly higher δ^{15}N found in detritus in the wet season. Detritus is an important resource for this species and during the wet season, mullet forage in more productive floodplains than during the dry season. It has been observed that high denitrification processes in wetland areas cause primary consumers to have higher δ^{15}N isotopic values than in areas with less wetland coverage [70].

The low correlations between δ^{15}N of muscle and gonads (likely produced during wet season) with liver (produced in less productive areas during the dry season) suggests that N-bearing proteins in gonads may be more readily mobilized from protein obtained during the wet season than sourced from a maintenance diet during gonadal formation in the dry season. Although there was a strong correlation between gonads and muscle tissue, gonads showed consistent depletions in ^{15}N relative to muscle. Furthermore, we found that instantaneous reproductive growth (gonadal RNA:DNA) was negatively correlated with δ^{15}N values during the dry season. Both of these patterns are consistent with results obtained by [71] for humans, who demonstrated that $\delta^{15}N$ values of hair can become low due to anabolic processes, such as those occurring during gestation. The authors hypothesized that higher retention of urea might be helping to re-incorporate N to the metabolic pool for protein synthesis. However, fish excrete N in the form of ammonia which is highly toxic, which makes it unlikely that mullet would be retaining and recycling excretory N. In general, N cycling is complex and can lead to enrichment or depletion depending on the tissue and physiological state of the organism (see review by [72]), and the amino acid profile of different tissues can also affect $\delta^{15}N$ because essential amino acids such as phenylalanine exhibit no fractionation relative to the diet whereas others such as glutamic acid exhibit strong fractionation [73]. Detailed physiological explanation for ^{15}N depletion in mullet gonads is beyond the scope of this study, but a subject worthy of future investigation. To our knowledge, this is the first record of ^{15}N depletion in fish tissues related to energy allocation to reproduction.

Although the sites where mullet were captured during the wet season were not exactly the same as those during the dry season because of lack of access or water availability, respectively, it is important to stress that the patterns found relating somatic and reproductive growth and $\delta^{15}N$ of mullet tissues were not affected by these differences. On the other hand, $\delta^{13}C$ values of mullet tissues were significantly influenced by the site of capture, this suggests that site-specific $\delta^{13}C$ signatures are being incorporated on mullet tissues. This is an expected result given that $\delta^{13}C$ values of primary aquatic producers depend mainly on CO_2 difusion rates and local isotopic composition of the dissolved inorganic carbon pool (DIC) [74].

The temporal and spatial uncoupling between energy acquisition and allocation to reproduction of this common fish has important implications for the preservation of the natural hydrological regimes of floodplain areas. Diamond mullet typically inhabit waterholes during the dry season and move into floodplains during the wet season where they may obtain most of their energy. Subsequently, as flood waters recede, some individuals migrate back to waterholes and others eventually migrate to saltwater to spawn [23]. Despite the low primary productivity found in remnant waterholes during the dry season [7], findings from this study emphasize the importance of these habitats as zones where reproductive allocation takes place. More importantly, wet season habitats such as floodplains are critical in providing most energy for growth and reproduction. Therefore, the maintenance of natural hydrological regimes would enhance the capacity for fishes of this region to maintain viable populations.

Supporting Information

S1 Fig. Relationships between lipid-extracted and mathematically-corrected $\delta^{13}C$ data using published lipid-correction equations. The X axis represents mathematically-corrected ($\delta^{13}C'$) minus uncorrected ($\delta^{13}C$) values. The Y axis represents chemically-extracted ($\delta^{13}C_{ext}$) minus uncorrected ($\delta^{13}C$) values. Gonad, liver and muscle tissues are represented by black dots, triangles and squares, respectively. The dashed line represents the expected 1:1 relationship. The solid line represents a least squares regression of $\delta^{13}C_{ext}—\delta^{13}C$ on $\delta^{13}C' - \delta^{13}C$. The correction equations used were as follows: **(A)** [47]: $\delta^{13}C' = \delta^{13}C - 2.98^{*}\log(C/N) + 3.09$; **(B)** [50]: $\delta^{13}C' = \delta^{13}C + (6-(22.2/C/N))$; **(C)** [51]: $\delta^{13}C' = (\delta^{13}C^{*}C/N + 7.08^{*}(C/N-3.7)) / C/N$; **(D)** [52]: $\delta^{13}C' = \delta^{13}C + (0.322^{*}\ C/N) - 1.175$; **(E)** [48]: $\delta^{13}C' = \delta^{13}C - 3.32 + (0.99^{*}\ C/N)$; **(F)** [53]: $\delta^{13}C' = \delta^{13}C + (6.3^{*}\ ((C/N—4.2) / C/N))$.
(TIF)

S2 Fig. Linear regression between log standard length and log body mass of *Liza alata*. Individuals captured during dry and wet seasons are represented by solid and open symbols, respectively.
(TIF)

S3 Fig. Relationships between δ^{13}C values in somatic and reproductive tissues of *Liza alata*. The dashed line represents the 1:1 expected relationship. The solid line represents the least-squares linear regressions of: A) liver vs. gonads, B) liver vs. muscle and, C) muscle vs gonads. Individuals from dry and wet seasons are represented by solid and open symbols, respectively.
(TIF)

S1 Table. Data of underlying findings. Raw data of the individuals of *L. alata* from which we draw the conclusions presented in the manuscript. Abreviations of attributes are as follows: sl_mm = standard length in milimeters, mass_g = mass in grams, %c = percentage of carbon, %n = percentage of nitrogen, le = lipid-extracted samples, d13c = δ^{13}C, d15n = δ^{15}N, r_d = RNA:DNA ratio, M = muscle, L = liver, G = gonad, EGG = eggs. Sex: F = female, M = male, U = unknown.
(XLS)

S2 Table. Primary sources data of underlying findings. Raw data of the primary sources (biofilm, detritus and filamentous algae) from which we draw the conclusions presented in the manuscript. Abreviations of attributes are as follows: d13c = δ^{13}C, d15n = δ^{15}N, %c = percentage of carbon, %n = percentage of nitrogen.
(XLS)

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Author Contributions

Conceived and designed the experiments: FV SEB DV TDJ. Performed the experiments: FV DV RW. Analyzed the data: FV WEM. Contributed reagents/materials/analysis tools: FV DV RW SEB. Wrote the paper: FV TDJ WEM.

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Capítulo 2

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Stable isotope and spatial analyses reveal resource partitioning among sympatric Amazonian crocodilians

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Running headline: Resource partitioning in Amazonian crocodilians

Summary

1. Although most crocodilians are considered generalist opportunistic predators that feed on any source of protein available in the environment, Amazon crocodilians show broad differences in the proportions of aquatic or terrestrial prey they consume. It is believed that these differences may be a reflection of macrohabitat use. However, it is unknown to what extent they represent interspecific differences in foraging behavior or are a function of macrohabitat selection.

2. The Amazon River basin is the only region known to sustain four crocodilian species in sympatry, which attain high densities in some regions. As top predators, their impact upon foodwebs should be substantial, but the degree to which crocodilians differ in their food sources, and potentially avoid competitive exclusion, is not well understood in waterbodies where they occur syntopically.

3. Carbon stable-isotope data (δ^{13}C) of crocodilians and their potential prey were used to assess differences in reliance on terrestrial versus aquatic resources. These data were then placed in a spatial context using classified maps that reflect macrohabitat classes (headwater streams, mid-order flooded-forest streams and *várzea* floodplains) to elucidate whether dietary differences are explained by macrohabitat selection or are more likely a reflection of foraging behavior.

4. Evidence for differences in basal resources supporting these crocodilians was found. Mean δ^{13}C values were highest in *Paleosuchus trigonatus* (Schneider's smooth-fronted caiman), intermediate in *Caiman crocodilus* (Spectacled caiman) and *Paleosuchus palpebrosus* (Cuvier´s smooth-fronted caiman) and lowest in *Melanosuchus niger* (Black caiman).

5. A progressive depletion in δ^{13}C values occurred from headwaters to floodplains which most likely reflects a progressive increase in autochtonous inputs in lower reaches of streams. The shift from terrestrial to aquatic resources sustaining these sympatric predators mirrors their spatial distribution along this ecotone. However, when taking into account habitat characteristics for pairs of syntopic individuals of distinct species, significant differences in δ^{13}C suggest that *P.*

trigonatus and *P. palpebrosus* have different prey bases. Thus, species differences in diet result from behavioral differences and foraging strategies in addition to macrohabitat selection.

Key-words. Aquatic food webs, crocodilian trophic interactions, food partitioning, spatial foraging modes, terrestrial subsidies.

Introduction

The diversity of coexisting species within ecological communities is partly determined by the ways in which they partition available resources (Finke & Snyder 2008). Coexisting species must differ in their ecological requirements for a shared resource by at least some minimal amount to avoid competitive exclusion (Pianka 1974). Two common ways in which different organisms directly interact with one another are predator-prey and interspecific territorial interactions (Pulliam 2000). The latter implies that stronger competitors might displace others from priority areas. The Amazon basin is the only river drainage known to sustain four crocodilian species living in sympatry. Such a diversity of aquatic top predators, together with the enormous abundances reported in some regions of central Amazonia (Da Silveira 2002) should require the partitioning of the available prey base for their coexistence to be maintained.

Most crocodilians are considered generalist opportunistic predators that take advantage of any available source of animal protein (Pooley 1989). Amazonian crocodilians partition space, with each species occurring most frequently in characteristic habitats (Magnusson 1985); thus, it is expected that their diets will vary depending on the availability of different prey in each habitat. However, the extent to which habitat selection influences the foraging mode of Amazonian crocodilians is unknown (Magnusson, Silva & Lima 1987). Furthermore, all four species coexist in syntopy in some waterbodies in the Amazon basin (Marioni et al. 2013). Within the same macrohabitat, ecological theory suggests that individuals should partition food resources in order to coexist.

Crocodilians in general experience ontogenetic diet shifts starting from terrestrial and aquatic invertebrates when young, to more protein-rich diets composed mostly of fish and terrestrial vertebrates as they grow (Ross 1998; Radloff, Hobson & Leslie 2012). Amazonian crocodilians show this ontogenetic variation, but exhibit interspecific differences in diet as adults. Adult *Paleosuchus palpebrosus* (Cuvier 1807)*, Caiman crocodilus* (Linnaeus 1758) and *Melanosuchus niger* (Spix 1825) which are common in open-canopy waterbodies and floodplains have diets mostly composed of fish (Magnusson, Silva & Lima 1987; Da Silveira & Magnusson 1999). On the other hand, adult *Paleosuchus trigonatus* (Shneider 1801), which is most common in closed-canopy headwater streams, consumes many terrestrial vertebrates, but few fish (Magnusson, Silva & Lima 1987).

Differences in resource use have traditionally been assessed using surrogates, such as feeding behaviour, prey preferences (McDonald 2002) or habitat choice (MacArthur, Diamond & Karr 1972; Bearhop et al. 2004). However, time-integrated dietary patterns have been difficult to determine using these conventional analyses because of practical limitations, including lack of information on temporal integration of diets, assimilation rates and relative abundances of prey (Bearhop et al. 2004).

Over the past few decades, stable-isotope analysis (SIA) has become an important chemical tracing tool, able to overcome some of these limitations because it is based on the principle that tissues of consumers reflect isotopic signatures of their diet in a predictable way (DeNiro & Epstein 1978). Carbon stable-isotope ratios (^{13}C:^{12}C; $\delta^{13}C$) in aquatic plants depend on the source signatures of inorganic C (Keeley & Sandquist 1992) and vary substantially among primary producers with different photosynthetic pathways, but change little with trophic transfers (DeNiro & Epstein 1978; Peterson & Fry 1987; Post 2002; Layman et al. 2012). Analysis of $\delta^{13}C$ in predators and their prey allows the use of mixing models that estimate the proportions of prey contributions to consumer tissues and the underlying energy source sustaining higher predators (Tunney et al. 2012). Recently, Bayesian mixing-model theory has been incorporated in refined models (Stock & Semmens 2013), which explicitly take into account uncertainty in source values and prior information (Moore & Semmens 2008; Ward, Semmens & Schindler 2010) and allow the use of categorical and continuous covariates (Semmens et al. 2009; Francis et al. 2011; Parnell et al. 2013).

In aquatic systems, isotope approaches are only rarely integrated with spatial analyses to allow characterization of dietary patterns by consumers in different habitat types (Jardine et al. 2011; Villamarín et al. 2016). In the Amazon basin, remote-sensing images have been increasingly used to answer ecological questions over large geographical scales (Melack et al. 2004; Villamarín et al., 2011). Active sensors, such as RADAR remote instruments, have been particularly useful due to their capacity to detect water under canopy cover. Thus, they have been used to estimate the extent of wetlands and generate accurate classification maps of vegetation types and flooding states throughout the Amazon basin with a spatial resolution of 100m (Rosenqvist et al. 2000; Hess et al. 2003; 2012; 2015). This potentially allows quantification of available macrohabitat for crocodilians.

Here, we explore dietary patterns in four species of crocodilians across a wide area of the central Amazon. We used δ^{13}C data of the crocodilians and their potential prey, and placed them in a spatial context using maps that reflect macrohabitat classes to answer the following questions: 1) To what extent do Amazonian crocodilians rely on terrestrial or aquatic resources? and 2) To what extent are differences in reliance on terrestrial versus aquatic resources a function of macrohabitat selection?

Materials and Methods

Study region

This study was conducted in lotic waterbodies in the Central Amazon region (Fig. 1) and comprised three different hydrological sampling scales (Table 1).

The first scale covered first- to third-order pristine closed-canopy streams with headwaters that originate in the forests of the interfluve between the Purus and Madeira Rivers. Stream order follows Strahler´s modification of Horton´s scale (Petts 1994). In this region, streams are affected by local rainfall rather than the hydrological regimes of the main rivers. Most of these streams dry out completely during dry months (June-October, F. Villamarín, Pers. obs.). Most headwater streams in the Amazon basin are nutrient poor (Furch & Junk 1980; Furch 1986). The "black" waters of these streams are very poor in electrolytes, and low in pH and electrical conductivity. They support few submerged aquatic macrophytes and algal growth because of their low-light conditions (Junk et al. 2011) and are net heterotrophic as evidenced by low dissolved oxygen (Table 1).

We sampled 250m-stretches from 55 pristine headwater streams clustered in ten sampling sites. These clusters were distributed along an approximately 600 km transect throughout the interfluvial region of the Purus and Madeira Rivers (Fig. 1). This region is intersected by the Br-319 highway, a partially unpaved road that connects the cities of

Manaus and Porto Velho and allows sampling of the otherwise inaccessible network of headwater streams. The sampling sites are part of a research-module network of the Research Program in Biodiversity (PPBio, http://ppbio.inpa.gov.br/sitios/br319).

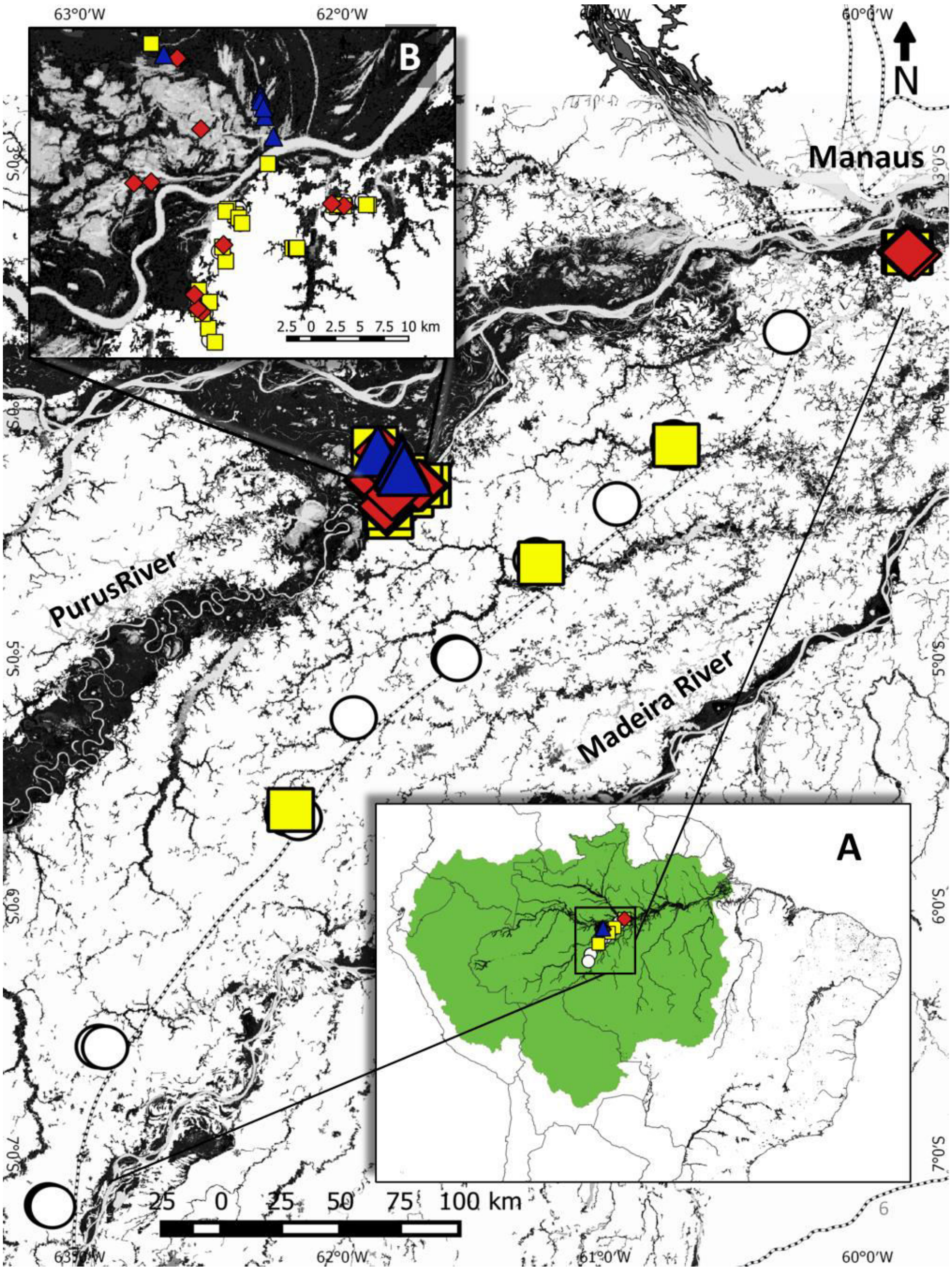

Fig. 1. Study region. Purus - Madeira interfluve. A) Northern South America showing the Amazon basin in green. B) Detail of the transition zone between non-flooded forests (white), flooded-forests (black) and *várzea* floodplains (gray). Symbols represent the four crocodilian species (*P. trigonatus* = o, *P. palpebrosus* = □, *C. crocodilus* = ◊, *M. niger* = Δ). The dashed line crossing the interfluve represents the mainly unpaved Br-319 highway.

Table 1. Physico-chemical characteristics of sampled waterbodies

	Headwater streams (n=55)	**Flooded-forest streams and ria-lakes (n=4)**	***Várzea* floodplains (n=5)**
	Mean (min-max)	Mean (min-max)	Mean (min-max)
Width (m)	4.21 (0 - 19.97)	865.7 (50 - 3400)*	62675 (55800 - 78000)*
Depth (m)	0.33 (0 - 1.14)	4.7 (1 - 13.4)	8.99 (2.68 - 19)
pH	4.51 (3.04 - 5.89)	5.59 (4.41 - 6.3)	6.46 (5.67 - 7.57)
Electrical conductivity (µS/cm)	13.96 (4.4 - 37.6)	12 (2 - 32)	36.4 (29 -48)
Dissolved oxygen (mg/l)	3.25 (0.75 - 5.17)	_	5.64 (0.62 - 9.27)
Temperature (°C)	25.27 (24.48 - 26.45)	26.6 (25 - 29)	31.41 (29.98 - 33.33)
Surrounding non-flooded forest in a 1 km radius (%)	96 (72 - 100)	59 (6 - 100)	0

*Distance between the nearest non-flooded forests based on classified maps (Hess et al. 2015)

The second hydrological sampling scale was comprised of third- to fifth-order flooded-forest streams and "ria-lakes" within four sub-basins, which originate within the Purus-Madeira interfluve, and flow into the Purus River (Fig. 1 B). These wetlands are subject to predictable monomodal pulses of inundation (Jardine et al. 2015), with flood amplitudes that are greatest near the confluence with nutrient-rich “white” waters of large rivers and decline rapidly upstream (Junk et al. 2011). Ria lakes may be temporarily influenced by sediment laden waters during highest water periods, but they are filled by black waters when river levels are low (Junk et al. 2011).

The third hydrological sampling scale of this study covered the western margin of the Purus River, where large extensions of white-water (sediment-laden) floodplains, locally known as "*várzeas"* extend for dozens of kilometers within the limits of the Piagaçu-Purus Sustainable Development Reserve (PP-SDR). *Várzea* floodplains receive water, sediments, and biological material from large parent rivers originating in the Andes and are subjected to long-lasting, monomodal and predictable flood pulses with high amplitudes (Junk et al. 2011). These are the most species-rich wetland forests in the world (Wittmann et al. 2006); they have mean flood periods of around 230 days per year (Junk et al. 2011) and have rates of net primary productivity of up to 33 Mg ha^{-1} yr^{-1} (Schöngart, Wittmann & Worbes 2010). Five waterbodies in *várzea* floodplains were sampled during crocodilian-monitoring activities carried out by the Crocodilian Conservation Program of the Piagaçu Institute.

Foodweb sampling

At each study site, primary producers were collected to characterize δ^{13}C at the base of the food web. Biofilm samples were obtained via toothbrush scrapes of submerged vegetation surfaces, such as leaves and twigs. Samples were placed in small ziplock bags with distilled water. In the field camp, the contents of the bags were transferred into capped cryogenic vials and stored in liquid nitrogen. Samples of stream water were collected and filtered in the field on glass-fiber filters (47-mm diameter, 0.6µm pore size). These samples represent fine particulate organic matter (FPOM) material in suspension as no phytoplankton growth is expected in these headwater streams. Leaf-litter samples were collected from the stream margins, rinsed with distilled water and placed in ziplock bags. In most cases, these samples were collected from outside the water column, as many of the streams were dry when sampled.

Potential crocodilian prey groups, such as terrestrial and aquatic invertebrates, were captured according to their availability. Terrestrial invertebrates, such as coleopterans, orthopterans and ants, were captured by hand. Aquatic invertebrates and fish were captured using dipnets, in a 50m-stretch of each stream. Small fish were stored whole and a small piece of white dorsal muscle was collected from larger fish. Claw samples from terrestrial vertebrates, such as *Dasyprocta* and *Cuniculus*, were obtained opportunistically from subsitence hunters in the area. While some samples of invertebrates and fish were preserved in ethanol for identification, all isotope samples collected from all trophic levels were kept frozen in liquid nitrogen for approximately one month before their return to the laboratory.

Crocodilians were captured using fyke nets in headwater streams and steel snares at night in other waterbodies. After measuring (snout-vent length, SVL), sexing and weighing the animals, a piece of claw and one or two tail scutes were collected from each individual. A small piece of muscle tissue was removed from the scutes and rinsed with distilled water to avoid contaminating the sample with blood. All tissue types were analysed for $\delta^{13}C$, but only claw tissue was used for mixing models. In the case of *M. niger*, only one sample of claw tissue was available, the remaining were muscle tissue samples. Thus, linear regressions were performed using muscle and claw tissues from the other three species to correct the values of muscle tissue in *M. niger* ($\delta^{13}C_{Claw} = -3.09*\delta^{13}C_{Muscle} + 0.86$; $r^2 = 0.63$; $p < 0.001$; df = 61). Tissues with C:N ratios > 4 were lipid-extracted using chloroform : methanol solution (Bligh & Dyer 1959).

SIA Laboratory processing

All samples were kept frozen at -20°C in the laboratory of water quality at INPA. Biofilm samples were sieved using a 300µm mesh to remove larger detrital material. The biofilm was then subsampled into a bulk fraction and a second sub-sample that was centrifuged using LUDOX®-TM50 colloidal silica following Hamilton, Sippel & Bunn (2005).

All samples were dried in an oven at 60°C for 24 to 48 h before grinding and homogenizing with a mortar and pestle. Samples were combusted in a EuroEA 3000 (EuroVector, Italy) or Europa GSL (Sercon Ltd, Crewe, UK) elemental analyzer and the resulting N_2 and CO_2 gases were chromatographically separated and fed into an IsoPrime (Micromass, UK) or Hydra 20–22 (Sercon Ltd, Crewe UK) isotope-ratio mass spectrometer. This measures the ratio of heavy and light isotopes in a sample relative to a standard. Isotope ratios (δ) are expressed in parts per thousand or per mil (‰), defined as δ (‰) = ($R_{sample}/R_{standard}$ - 1)*1000, where R_{sample} and $R_{standard}$ are the isotope ratios of the sample and standard, respectively. Isotopic standards used were referenced to PeeDee Belemnite (PDB).

Endmember isotopic signatures for mixing models

Unbalanced sample sizes of different prey organisms may overestimate the contribution of small prey that have low energy value but are easy to capture and underestimate that of larger, more energy-rich prey that are only opportunistically sampled. Therefore, prior knowledge about Amazonian crocodilian diets (Magnusson, Silva & Lima 1987) was used to create objective groupings representing aquatic and terrestrial endmember isotopic signatures (see Table S1 in Supporting Information). For this, published information on the mean number of prey individuals consumed per crocodilian per size class (Magnusson, Silva & Lima 1987) and mean mass per prey (Pérez 1992; this study) were used to estimate prey mass per crocodilian per size class. Crocodilians were grouped into six size classes: <20cm, 20.1 to 30cm, 30.1 to 40cm, 40.1 to 50cm, 50.1 to 60cm, and >60cm SVL, and the weighted prey mean (g/crocodilian/size class) based on the number of crocodilians per size class in our sample was calculated. This allowed estimation of the proportional mass of each prey type within terrestrial and aquatic groups. Random samples of δ^{13}C values of prey were taken in proportion to the prey mass to create distributions of aquatic and terrestrial endmember isotopic signatures. All δ^{13}C values of these prey came from our sample set with the exception of *várzea* fish that were obtained from Forsberg et al. (1993).

Endmember δ^{13}C mean and SD were used to run a one-isotope, two-source model in MixSIAR (Stock & Semmens 2013).

Model parameterization

The MixSIAR mixing model parameterization included three chains, a chain length of 100,000, burn in of 50,000, and thin of 50. Both residual and process error were included (Parnell et al. 2013), and results are reported as medians with 95% credible intervals (95% CrIs). To compare the posterior estimates of source contributions among crocodilian diets, pairwise Bhattachayya Coefficients were calculated (BC; Bhattachayya 1943), which indicate overlap between two Dirichlet distributions (Rauber, Braun & Berns 2008, Bond & Diamond 2011).

Spatial analysis

To further assess occupation of aquatic versus terrestrial habitats and how this macrohabitat use influenced diet, the proportion of non-flooded forest "terrestrial habitat" surrounding each sampled individual was estimated. A classified image map of wetland extent, vegetation type, and dual-season flooding state of the entire lowland Amazon basin was used (Hess et al. 2015). The classified image was derived from the Global Rain Forest Mapping Project (GRFM) Amazon mosaics (Rosenqvist et al. 2000; Siqueira et al. 2000) acquired during October-November 1995 and May-June 1996(see Hess et al. 2015 for details of classification procedures). From this map, spatial analysis R packages *sp* and *raster* (Bivand, Pabesma & Gómez-Rubio 2005; Hijmans 2015) were used to calculate the proportion of non-flooded forest present in a 1km-radius around each of the captured crocodilians. Linear-regression models were then used to estimate the influence of the proportion of non-flooded forests and SVL of the crocodilian on δ^{13}C values. Finally, pairs of individuals of different species captured within a maximum distance of 200 m of each other were identified and compared using paired t-tests on δ^{13}C values to determine if they had similar prey bases. For this test, the mean proportion of non-flooded forests around these pairs of individuals were re-calculated only for plotting purposes. All statistical analyses and graphics were run using R software (Team R. Core 2014).

Results

In the first hydrological scale, a total of 55 first- to third-order headwater streams were sampled. Within these headwater sites, 30 *P. trigonatus*, eight *P. palpebrosus* and six *C. crocodilus* individuals were captured. Further downstream, in the second hydrological scale, four sub-basins comprising 3rd to 5th -order flooded-forest streams and ria lakes that flow into the Purus River were sampled, and 15 *P. trigonatus* individuals, 28 *P. palpebrosus* individuals and six *C. crocodilus* individuals were captured. Temperature was relatively low in both the headwater streams and flooded-forest streams due to the dense canopy cover, and their waters were black coloured, very poor in electrolytes, and had low pH (Table 1). Only in ria lakes, just upstream of where they flowed into the main river, did the canopy open considerably. In the third hydrological scale, on the opposite margin of the river, *várzea* floodplains are periodically flooded by the nutrient-rich waters of the Purus River. Electrical conductivity, pH and temperature were higher in these waterbodies (Table 1). In these floodplains, one *P. palpebrosus* individual, four *C. crocodilus* individuals and nine *M. niger* individuals were captured.

Samples of bulk biofilm, suspended FPOM and C3 leaf litter showed mean δ^{13}C (± 1SD) values of -31.53‰ (± 1.44‰), -29.55‰ (± 0.85‰) and -33.10‰ (± 1.58‰), respectively. These three sources were statistically different (ANOVA: $F_{2,75} = 13.52$; $p < 0.001$) but their distributions overlapped considerably. Post-hoc testing showed significant pairwise differences among all sources ($p < 0.027$ in all cases). Bulk biofilm samples showed no statistical differences from centrifuged samples (-31.48 ± 2.68‰) (t-test: $t = -0.11$, $df = 78.8$, $p = 0.915$).

The variation in crocodilian δ^{13}C values were significantly influenced by differences among species (ANOVA: $F_{3,92} = 42.45$, $p <0.001$, Fig. 2). These differences were related to SVL nested within the species ($F_{4,92} = 4.02$; $p = 0.005$) but not to sex ($F_{5,92} = 0.99$, $p = 0.429$). Larger individuals had higher δ^{13}C values compared with smaller individuals.

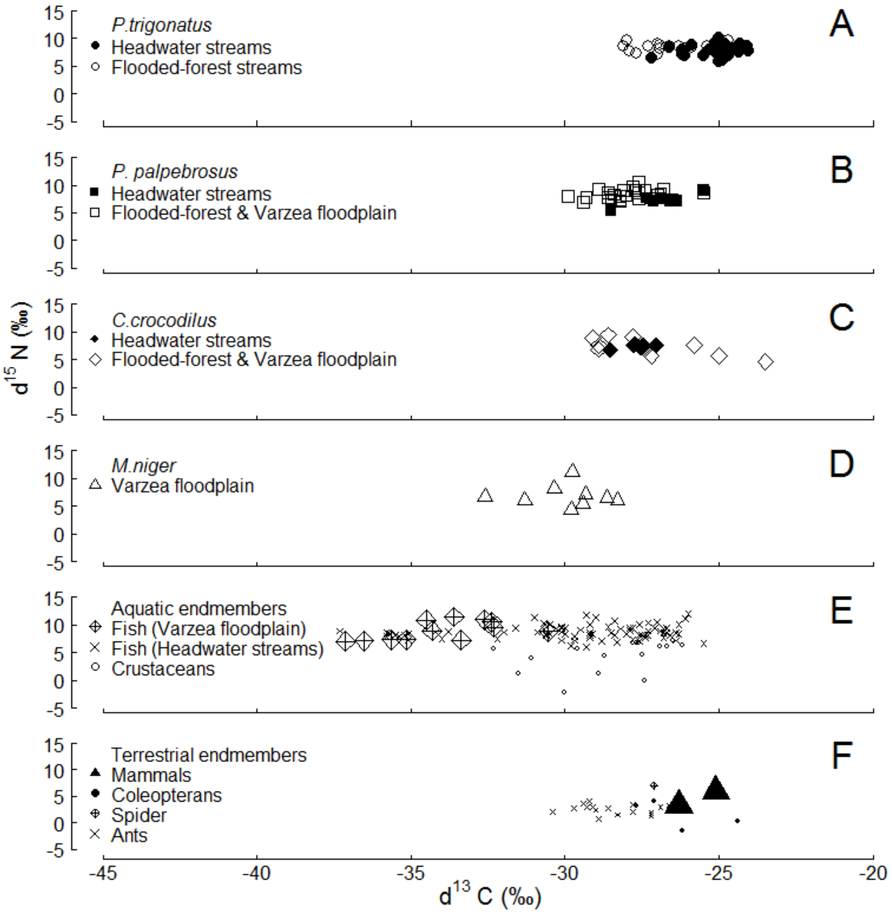

Fig. 2. Isospace of crocodilians and endmembers. Graphic representation of isotopic composition of the four caiman species (A-D), aquatic (E) and terrestrial (F) endmembers. The size of endmember symbols is broadly proportional to their relative importance in caiman diets.

Table 2. Estimates of aquatic and terrestrial proportional contributions in Amazonian crocodilian diets

Species	% Terrestrial Median (95% CrI)	% Aquatic Median (95% CrI)
P. trigonatus	58 (47 - 68)	42 (33 - 54)
P. palpebrosus	30 (17 - 41)	70 (59 - 83)
C. crocodilus	34 (14 - 48)	66 (52 - 86)
M. niger	21 (2 - 35)	79 (65 - 98)

Aquatic and terrestrial prey end-members sustaining all four crocodilian species showed significant differences in δ^{13}C values (t-test: $t > 14$; $df > 108$; $p < 0.001$ in all cases; Fig. 3 and Table S1). Despite leaf litter having lower δ^{13}C than biofilm, terrestrial prey were enriched in ^{13}C relative to aquatic prey.

Because fish and terrestrial vertebrates are much heavier than invertebrates, their contributions to aquatic and terrestrial endmember signatures were higher than 70 and 99%, respectively (Table S1). Differences among species in relative prey importance, based on prior information, meant that source values were slightly different depending on the species. Though terrestrial endmember δ^{13}C values did not differ among species (Table S1), aquatic endmembers composing *M. niger* diet were more depleted in ^{13}C than those of the other three crocodilians (Fig. 3).

MixSIAR models converged satisfactorily. Out of 13 chains, the Gelman diagnostic yielded only one chain >1.05. For the Geweke diagnostic, only two variables were outside ±1.96 in one chain. For all individuals, independent of species, terrestrial resources made a median proportional contribution of 36%, against 64% from aquatic inputs. However, these proportional contributions shifted among species. *P. trigonatus* had the highest median proportional contribution from terrestrial resources (58%), as opposed to the other species in which terrestrial inputs progressively decreased. *C. crocodilus*, *P. palpebrosus* and *M. niger* had 34, 30 and 21% respectively (Table 2). Overall, Bhattachayya´s coefficients were near 0.5 for all species pairs (Table 3), suggesting approximately 50% overlap of diet. Of all species pairs, *C. crocodilus* and *P. palpebrosus* showed the highest diet overlap (BC = 0.55) and *P. trigonatus* and *M. niger* had the lowest overlap (BC = 0.51) (Table 3).

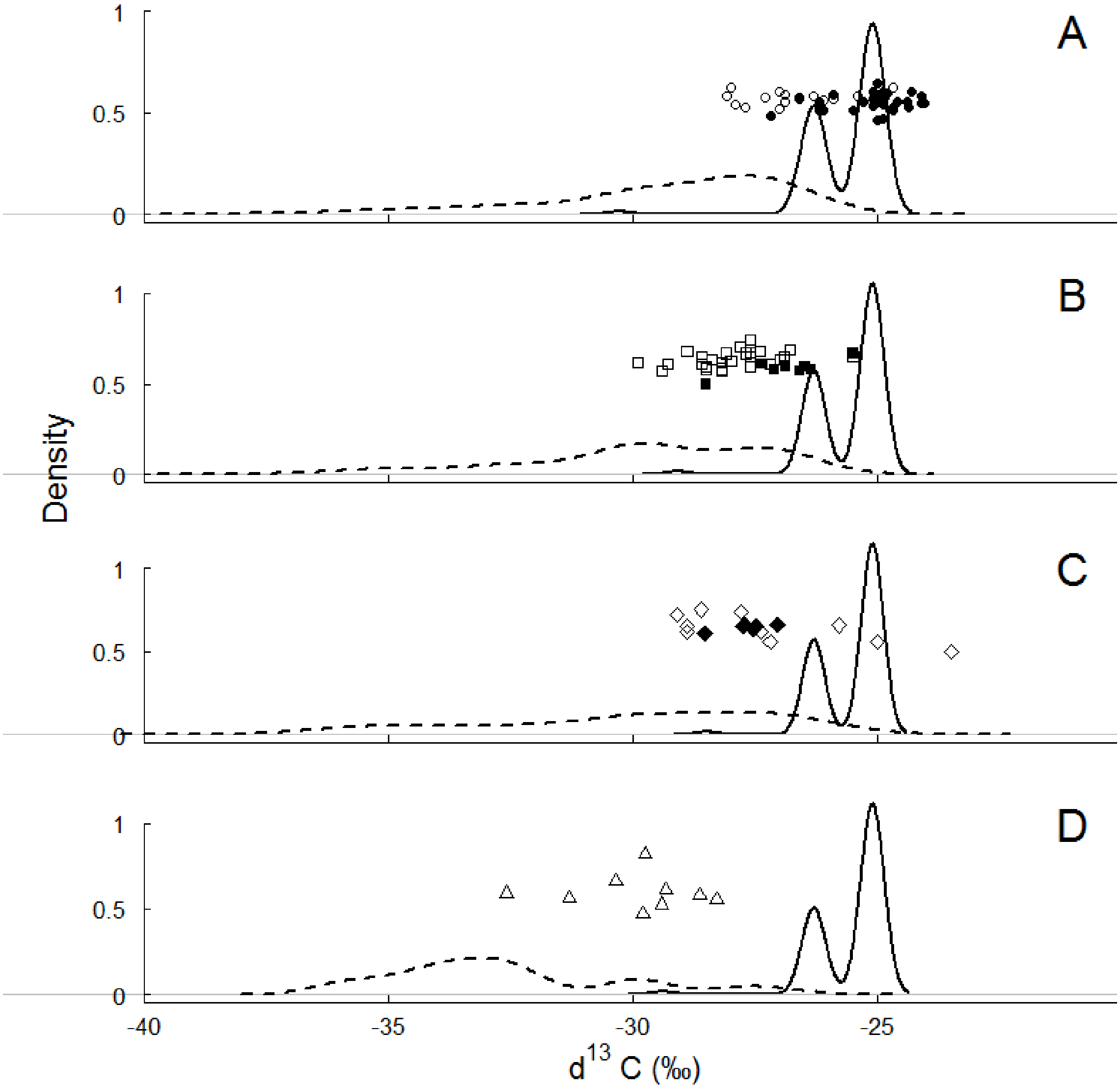

Fig. 3. Endmembers´ δ^{13}C distributions. Kernel density plots showing δ^{13}C distributions of terrestrial and aquatic endmembers (dashed and solid lines, respectively) for *P. trigonatus* (A, o), *P. palpebrosus* (B, □), *C. crocodilus* (C, ◊), and *M. niger* (D, Δ).

Table 3. Pairwise comparisons of Bhattacharyya coefficients showing medians, lower (LCL) and upper confidence limits (UCL)

	M.niger	***P.palpebrosus***	***P.trigonatus***
	Median (LCL-UCL)	**Median (LCL-UCL)**	**Median (LCL-UCL)**
C.crocodilus	0.54 (0.31-0.78)	**0.55** (0.37-0.74)	0.54 (0.5-0.59)
M.niger		0.54 (0.29-0.80)	**0.51** (0.39-0.63)
P.palpebrosus			0.54 (0.47-0.60)

Multiple-regression models (δ^{13}C = -29.79 + 0.02_{SVL}+ 3.18_{NFF}; df = 42, R^2 = 0.37, $p < 0.001$) indicated that variation in *P. trigonatus* δ^{13}C was predicted by both length (SVL; $p < 0.001$) and proportion of non-flooded forest (NFF; $p < 0.001$). This relationship was weaker for *C. crocodilus* (df = 13, R^2 = 0.33, p = 0.07), for which δ^{13}C values were influenced only by SVL (p = 0.052) but not by the proportion of non-flooded forest (p = 0.470). For the other two species, the relationships were not statistically significant (*P. palpebrosus*: df = 33, R^2 = 0.1, p = 0.17 and *M. niger*: df = 6, R^2 = -0.13, p = 0.68).

Individuals of *P. trigonatus* were captured together with *P. palpebrosus* at 15 locations and with individuals of *C. crocodilus* at four locations, but not with individuals of *M. niger*. *P. palpebrosus* individuals were captured sharing space with *C. crocodilus* in nine locations but only in one location with *M. niger*. *C. crocodilus* and *M. niger* were captured together in seven locations.

Values of δ^{13}C of syntopic individuals of different species were significantly different for the congeners *P. trigonatus* and *P. palpebrosus* (Paired t-test: t = 4.13, df = 14, p = 0.001, Fig. 4), but not for other species pairs: *P. trigonatus* and *C. crocodilus* (t = -1.19, df = 3, p = 0.319); *P. palpebrosus* and *C. crocodilus* (t = -1.31, df = 8, p = 0.228); *C. crocodilus* and *M. niger* (t = -0.86, df = 6, p = 0.422).

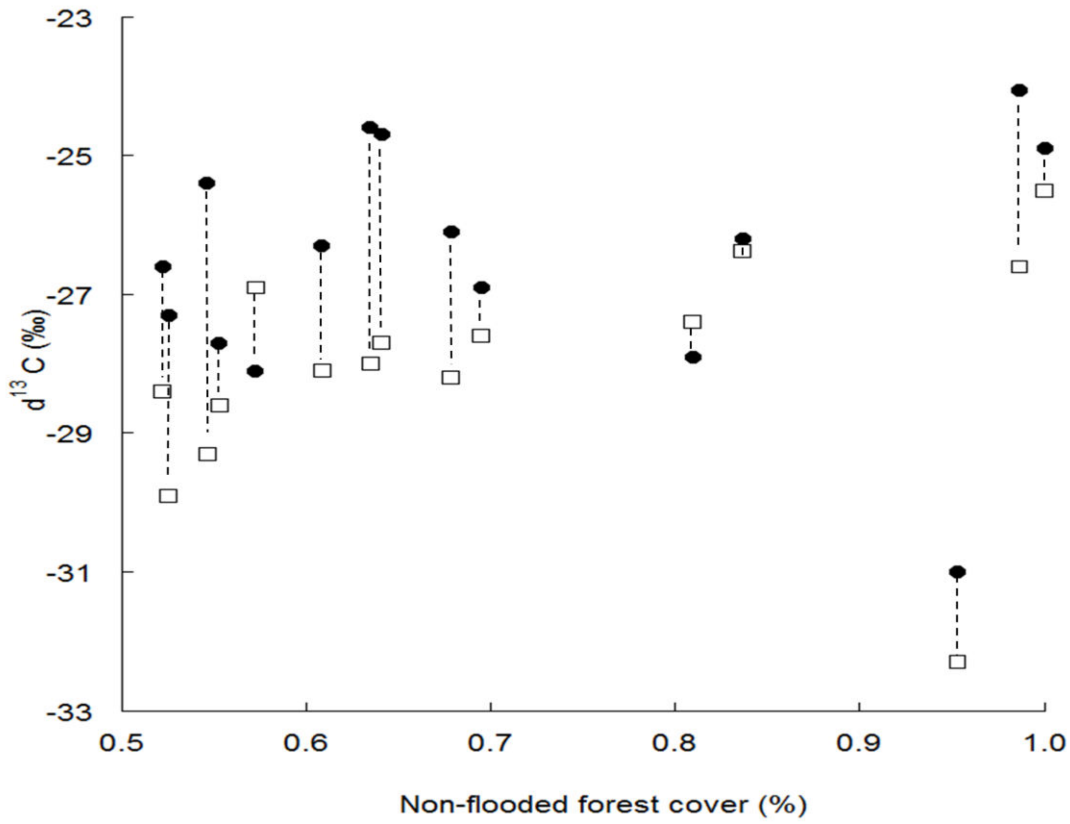

Fig. 4. Interspecific isotopic comparisons. Pairwise comparisons of δ^{13}C values between syntopic individuals of *Paleosuchus trigonatus* (●) and *P. palpebrosus* (□).

Discussion

Combining stable-isotope and spatial analyses, we found evidence of differences in resources supporting Amazonian crocodilians in an ecotone comprising headwater streams, mid-order flooded-forest streams and *várzea* floodplains. Mean δ^{13}C values were highest in *P. trigonatus*, intermediate in *C. crocodilus* and *P. palpebrosus* and lowest in *M. niger*. A progressive depletion in δ^{13}C values occurred from headwaters to floodplains, which reflects a shift from terrestrial to aquatic resources and mirrors the spatial distribution of these sympatric predators along this ecotone. However, when taking into account habitat characteristics for pairs of syntopic individuals, significant differences in δ^{13}C suggest that *P. trigonatus* and *P. palpebrosus* have different prey bases, so species differences probably result from behavioral differences and foraging strategies other than macrohabitat selection.

Most crocodilians are considered generalist, opportunistic predators (Pooley 1989) that take advantage of any source of ingestible animal protein of adequate size. Amazonian crocodilians partition space, each species occurring most frequently in characteristic habitats (Magnusson 1985), so it is expected that their diets will vary depending on the availability of different prey in each habitat (Magnusson, Silva & Lima 1987).

Paleosuchus trigonatus and the African dwarf crocodile, *Osteolaemus tetraspis*, which may contain three cryptic species (Eaton et al. 2009), are the only extant crocodilians known to thrive in headwater streams under closed-canopy forests (Magnusson 1985). In the streams we studied, terrestrial resources represented almost 60% of carbon inputs contributing to *P. trigonatus* tissues. This is expected for a species that lives in headwater streams where large terrestrial prey are readily available and may provide greater sources of organic matter than small fish and crustaceans. The primary carbon sources sustaining these terrestrial vertebrates are most likely derived from C3 trees. Vertebrates comprised more than 99% of the biomass of terrestrial endmembers in the diet of *P. trigonatus* in this study. Most carbon inputs probably originate from tree fruits and seeds, as they represent the main food sources for medium-sized forest-floor herbivores, such as species of *Dasyprocta* and *Cuniculus* (Dubost & Henry 2006). Non-photosynthetic parts of trees, such as reproductive and woody-stem parts, usually show higher $\delta^{13}C$ values than leaves (Martinelli et al. 1998; Medina, Sternberg & Cuevas 1991; Blumenthal et al. 2015). Furthermore, depleted values of leaf litter may be caused by intake of respired CO_2 in the forest understory (Medina & Minchin 1980). These factors may explain why our C3 leaf litter was ^{13}C-depleted relative to terrestrial prey. Fruits and flowers had mean $\delta^{13}C$ values of -26.5 ± 1.6‰ in a closed-canopy tropical African forest (Blumenthal et al. 2015). Although we do not have estimates of fruit $\delta^{13}C$ values from our study area, these values match well with those of keratin tissue from *Cuniculus* and *Dasyprocta*.

Headwater streams are loaded by inputs of leaf litter from C3 trees, with highest litterfall rates of >10 Mg ha^{-1} yr^{-1} occurring in lowland tropical regions (Naiman, Decamps & McClain 2010). Allochthonous resources, whether from trees or other sources, enter streams as coarse particulate organic matter (CPOM) that degrades to fine particulate organic matter (FPOM) and accumulates on underwater surfaces. When deposited, FPOM particles mix with live algae, algal detritus and detritus of terrestrial or aquatic vascular plant origin (Hamilton, Sippel & Bunn 2005). This conglomerate of allochthonous and autochthonous autotrophs, known as biofilm, is common in streams and could represent a second pathway by which terrestrial carbon reaches crocodilians via fish consumption.

Although they have little biomass, periphyton within the biofilm most likely represents the only autochthonous autotrophs in the shaded environments of forest streams, as no phytoplankton growth is expected. In these conditions, contributions of micro-algae and allochthonous resources are often very difficult to distinguish using stable isotopes as both resources often overlap in $\delta^{13}C$ values in headwater streams (France 1995; Finlay 2004; Jardine, Kidd & Cunjak 2009). This occurs as the result of isotopic overlap and the contamination of the autochthonous samples with allochthonous detritus (Jardine, Kidd & Cunjak 2009). The first problem is difficult to deal with, but the latter can potentially be overcome by centrifuging biofilm samples with colloidal silica and physically separating micro-algae from detritus (see Hamilton, Sippel & Bunn 2005). In this study, even after centrifuging biofilm in colloidal silica, we found no significant differences in $\delta^{13}C$ values between bulk and centrifuged samples, with few exceptions. Since bulk biofilm samples consist mostly of allochthonous FPOM, most likely almost no micro-algae was present in our samples, a reflection of what is expected in unproductive closed-canopy streams (Fisher & Likens 1973).

When there is relatively poor distinction of isotopic signatures between sources, other information can be used to parameterize MixSIAR models (Moore & Semmens 2008; Soto et al. 2016). We were able to estimate the relative importance of carbon inputs from aquatic versus terrestrial resources in the isotopic composition of caiman tissues by using δ^{13}C values of potential prey and prior information of their relative mass contributions within terrestrial and aquatic categories to caiman diets.

Caiman crocodilus and *P. palpebrosus* are often found occupying mid-order stream channels and flooded-forest habitats in syntopy. Some *P. trigonatus* may also be found sharing the same habitats. More autochthonous primary production is expected in the lower reaches than in the headwaters as stream channels widen progressively and the quantity of light reaching streams increases (Naiman et al. 1987). If this is the case in these streams, the increasing amounts of autochthonous carbon entering the foodweb are expected to be progressively incorporated in higher trophic levels, such as fish. Furthermore, fish size increases downstream as the waters become deeper (Schlosser 1982; Harvey & Stewart 1991). Crocodilian species living in the mid-reaches of these streams apparently benefit more from such prey than from terrestrial vertebrates. Consequently, isotopic inputs from aquatic resources are greater for both *P. palpebrosus* and *C. crocodilus* than for *P. trigonatus*.

Further downstream, *várzea* floodplains are extremely productive habitats (Junk, Bayley & Sparks 1989; Schöngart, Wittmann & Worbes 2010) where *M. niger* reaches its highest densities (Da Silveira 2002). In these floodplains *M. niger* divides the space with the syntopic *C. crocodilus*, sharing foraging areas (Marioni et al. 2008) and partitioning nesting sites (Villamarín et al. 2011). Considerable amounts of fish in the diet of adult *M. niger* are responsible for their almost exclusive reliance on aquatic resources. Floodplain fish included in this study are ^{13}C depleted (δ^{13}C mean ±SD = -33.91 ±1.25; Forsberg et al. 1993) since they are mainly supported by carbon from phytoplankton which has mean δ^{13}C values that vary between -33.3‰ (Araújo-Lima et al. 1986; Forsberg et al. 1993) and -40‰ (Mortillaro et al. 2015).

Overall, the progressive depletion of δ^{13}C values in caiman tissues from headwaters to floodplains reflect increasing reliance on aquatic resources as the proportion of non-flooded forest decreases. In headwater streams where poor light conditions predominate, *P. trigonatus* relies on allochthonous resources whereas in more open mid-order streams, *C. crocodilus* and *P. palpebrosus* are more reliant on aquatic sources, which fits the predictions of the River-Continuum Concept (RCC; Vannote et al. 1980). On the other hand, the autochthonous resources that support *M. niger*, a species rarely found outside the limits of *várzea* floodplains in the study area, are most likely produced within floodplain lakes. This agrees with the Flood Pulse Concept (FPC; Junk, Bayley & Sparks 1989) which predicts that primary productivity of floodplains is enhanced by predictable hydrological pulses. This is further supported by the observation that the Purus River is ranked number one out of 90 rivers from the Neo-tropics and Australia in terms of hydrological rhythmicity, and biota inhabiting rhythmic rivers are expected to derive more of their biomass from outside the river channel (Jardine et al. 2015). Thus, carbon resources from floodplains that sustain *M. niger* are most likely obtained by exploiting prey derived from a predictable flood pulse.

Although significant differences in δ^{13}C of caiman tissues suggest different proportions of primary resources sustaining these species, this may underestimate resource partitioning. Differences in diets could be a simple reflection of spatial distributions of these sympatric species (Magnusson, Silva & Lima 1987). Bhattachayya´s coefficients derived from posterior distributions of MixSIAR models showed overall overlaps in carbon inputs of around 50% among pairs of Amazonian crocodilian species. However, since mixing models did not take into account spatial distributions, this is not by itself strong evidence of resource partitioning.

When taking into account the proportion of non-flooded forest surrounding the location of pairs of syntopic individuals of different species, we found significant differences in δ^{13}C between *P. trigonatus* and *P. palpebrosus*. This suggests partitioning of prey resources between these closely related species that is not caused simply by macrohabitat selection. Evidently, even in locations where aquatic prey are more readily available, *P. trigonatus* exploits more terrestrial prey than *P. palpebrosus*. This might be facilitated by its terrestrial habits, as it is commonly found inside terrestrial retreats up to 90 m from the streams (Magnusson & Lima 1991). Morphological adaptations may be related to higher efficiency in consuming certain groups of prey. Analysis of skull shape shows divergences between the two species of *Paleosuchus* within morphometric space. In fact, *P. palpebrosus* appears closer to *M. niger* and *C. crocodilus* than to its congener, *P. trigonatus*, within skull morphospace (Pierce, Angielczyk & Rayfield 2008). This observation is in agreement with our findings on interspecific isotopic patterns which mirror the spatial distribution of Amazonian crocodilians. However, the interpretations of Pierce, Angielczyk & Rayfield (2008) suggesting higher ingestion of fish and terrestrial prey by long and broad-snouted crocodilians, respectively, are not supported by our data set. In fact, Amazonian crocodilians show the opposite pattern: *P. trigonatus* with a long narrow skull seems to be more adapted for preying on medium-sized terrestrial animals, while *P. palpebrosus* with a broader skull is evidently ingesting more fish, as do the other two species. These contrasting sources of evidence suggest that the relationship between morphological adaptations and foraging habits is complex and further studies that include morphological and dietary analysis, taking into account habitat characteristics, are needed in order to make broad generalizations.

Considerable knowledge has been gathered regarding the role of animals in the cycling and translocation of nutrients in terrestrial, marine, and freshwater ecosystems (e.g. Sirotnak & Huntly 2000; Vanni 2002; Rosenblatt & Heithaus 2011). The only Amazonian crocodilian that is common in nutrient-poor headwater reaches, *P. trigonatus*, may incorporate nutrients of terrestrial origin into the aquatic biocenosis by the consumption of mid-sized terrestrial vertebrates. However, the low primary productivity in the streams due to light limitation probably limits the importance of caimans as trophic integrators of aquatic and terrestrial habitats in these streams. On the other hand, individual specializations by mobile individuals may lead to habitat connections that may be maintained only by a subset of the population (Rosenblatt & Heithaus 2011). Our data set suggests that smaller *P. trigonatus* occupy downstream reaches with lower proportions of non-flooded forests. If these individuals show greater movements to downstream reaches of streams, they may be incorporating terrestrial nutrients into the aquatic biocenosis more effectively than larger sedentary individuals. Our findings provide evidence for interspecific differences in the origin of the resources sustaining these large aquatic predators. However, the extent to which particular movement tactics and the importance of nutrient translocations by these species in Amazon aquatic ecosystems are completely unknown and a promising ground for further investigation.

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Data accesibility

Raw data underlying the analyses will be available at the Research Program in Biodiversity data repository site (PPBio, https://ppbio.inpa.gov.br/repositorio/dados).

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Prior information on contributions of prey items composing Amazonian crocodilian diets for endmember isotopic groupings.

Capítulo 3

Villamarín, F.; Jardine, T.D.; Bunn, S.E.; Marioni, B. & Magnusson, W.E. Stable-isotope analyses reveal ontogenetic shifts in trophic position of Amazonian crocodilians**.** Manuscrito em preparação para *Journal of Animal Ecology*

Stable-isotope analyses reveal ontogenetic shifts in trophic position of Amazonian crocodilians

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Running headline: Trophic position in Amazonian crocodilians

Summary

1. The trophic position of top predators in a food chain strongly influences food-web structure because it reflects the number of steps that energy takes from primary producers to tertiary consumers. Crocodilians are likely to influence the structure of food webs because of their pronounced ontogenetic shifts in diet. Amazonian crocodilians show interspecific differences in diet. However, it is unknown to what extent those differences are reflected in ontogenetic shifts in trophic position.
2. Most studies use fish as top predators to estimate food-chain-length, thus it is not well understood to what extent trophic position of crocodilians may create significant divergences in food-chain-length estimates.
3. Here, we use stomach-content analysis of *P. trigonatus*, literature data on diet for the other three species, δ^{15}N values of all four Amazonian crocodilian species, as well as their potential prey to answer the following questions: 1) To what extent do diet trajectories of Amazonian crocodilians reflect changes in TP?; 2) After calculating TP of all consumers in the food web, to what extent the use of piscivorous fish TP values underestimate crocodilians as top predators?
4. The use of nitrogen stable-sotopes (δ^{15}N) of Amazon crocodilian tissues provided evidence of ontogenetic shifts in their trophic position (TP). Overall, progressive shifts in TP found in *P. palpebrosus* and *C. crocodilus* showed a positive linear relationship with their increase in length. *P. trigonatus* also showed increases in TP related to their increase in length, but the relationship was curvilinear, suggesting a plateau at maximum TP in mid-sized individuals. These observations are in accordance with published literature about ontogenetic diet shifts in crocodilians and are supported by stomach content analyses and δ^{15}N values from potential prey items in this study.
5. TP of adult *P. palpebrosus* was significantly higher than that of the piscivorous fish *Hoplias malabaricus* by 0.31 trophic levels. These findings suggest that the inclusion of true top predator organisms may yield significant divergences in food-chain length estimates.

Keywords. Aquatic food webs, baseline organisms, δ^{15}N, stomach-content analyses, trophic fractionation.

Introduction

Trophic position (TP) of a top predator is an important component of foodweb structure because it is a measure of the steps that energy takes between primary producers and tertiary consumers. Thus, this term is a synonym of food chain length, considered one of the most fundamental ecosystem attributes (Elton 1927, Lindeman 1942, Hutchinson 1959, Pimm 1982).

As top predators, crocodilians are likely to influence the structure of food webs. Because of their pronounced ontogenetic shifts in diet, some species of large crocodiles may be trophic links in diverse food webs throughout their lives, from freshwater, brackish, marine and adjacent terrestrial food webs (Radloff, Hobson & Leslie 2012; Hanson et al. 2015). Depending on the species, crocodilians may increase in length by 6- to more than 20-fold during their lifespan. As a result, they experience ontogenetic diet shifts starting from terrestrial and aquatic invertebrates when young, to more protein-rich diets composed mostly of fish and terrestrial vertebrates as they grow larger (Ross 1998). Amazonian crocodilians also show ontogenetic diet trajectories that lead to interspecific differences as adults. Stomach content analysis have shown that juvenile *Paleosuchus palpebrosus, Caiman crocodilus* and *Melanosuchus niger* have diets mostly composed of terrestrial and aquatic invertebrates, shifting progressively to fish when adults (Magnusson, Silva & Lima 1987; Da Silveira & Magnusson 1999). *Paleosuchus trigonatus*, on the other hand, broadly switches from terrestrial invertebrates as a juvenile to terrestrial vertebrates when adult (Magnusson, Silva & Lima 1987). While traditional stomach content analyses have shed light about these ontogenetic trajectories in the diet of Amazonian crocodilians, it is unknown to what extent these diet shifts are coupled to changes in TP.

During the last few decades, nitrogen stable-isotope ratios (δ^{15}N) have been broadly applied to estimate TP (DeNiro & Epstein 1981, Minagawa & Wada 1984, Peterson & Fry 1987; Post 2002). However, differences in nitrogen sources and biogeochemical processes may cause great variability in δ^{15}N at the base of the food chain among sites (Cabana & Rasmussen 1996). Thus, a baseline correction method has been developed in order to compare food webs among different systems. This method consists in 'leveling off' the isotopic signature of all consumers using that of known long-lived herbivores (Cabana & Rasmussen 1996; Vander Zanden & Rasmussen 1999, Post 2002). Another essential parameter used to estimate consumer TP is trophic fractionation, defined as the isotopic transformations that produce variations in the relative abundance of the heavy and ligth isotopes between the consumer and its diet. When applied to entire food webs with multiple trophic pathways and many species, a mean trophic fractionation of 3.4‰ is commonly applied (Post 2002). Many food-web studies define top predators as being the species or taxon with the highest δ^{15}N values; therefore fish have been considered the top predators in most aquatic food webs (Vander Zanden & Fetzer 2007). However, crocodilians show lower δ^{15}N values than expected for a predator at the top of a long food chain (Radloff, Hobson & Leslie 2012; Hanson et al. 2015). Furthermore, recent analyses have shown that the trophic-fractionation values may be significantly lower in alligatorid crocodilians than in most other consumers (Rosenblatt & Heithaus 2013; Marques et al. 2014). Thus, it is unknown to what extent raw δ^{15}N values and an assumed trophic fractionation value of 3.4‰ for all consumers may underestimate crocodilians as top predators.

Here, we use stomach-content analysis of *P. trigonatus*, literature data on diet for the other three species, δ^{15}N values of all four Amazonian crocodilian species, as well as their potential prey to answer the following questions: 1) To what extent do diet trajectories of Amazonian crocodilians reflect changes in TP?; 2) After calculating TP of all consumers in the food web, to what extent the use of piscivorous fish TP values underestimate crocodilians as top predators?

Material and Methods

This study was conducted in lotic waterbodies in the Central Amazon region. These water bodies included pristine closed-canopy headwater streams, third- to fifth-order flooded-forest streams and "ria-lakes". These waterbodies originate within the forests of the interfluve between the Purus and Madeira Rivers. At the western margin of the Purus River, white-water (sediment-laden) floodplains, locally known as "*várzeas*" were also sampled within the limits of the Piagaçu-Purus Sustainable

Development Reserve (PP-SDR). Information about the study area and physico-chemical characteristics of waterbodies is detailed in Villamarín et al. (chapter 2).

Stomach content analysis

Crocodilians were captured using baited fyke nets in headwater streams and steel snares at night in other waterbodies. A total of 28 *P. trigonatus* individuals had their stomach flushed with modifications suggested by Webb et al. (1982). Since most individuals were captured using fyke nets during the night, stomach contents were usually removed the next morning, a few hours after capture. All stomach contents were preserved in 70% alcohol. After measuring snout-vent length (SVL), sexing and weighing the animals, they were released at their capture site. All prey within stomach contents were identified to the lowest taxonomic level possible and grouped in nine categories that broadly reflect trophic groups: herbivorous aquatic and terrestrial invertebrates, predatory aquatic and terrestrial invertebrates, amphibians, reptiles, birds, fish and mammals. *P. trigonatus* diet was analyzed based on the frequency of occurrence (%) of each prey group, defined as the number of stomachs where each prey group was present out of the total number of stomachs analyzed. All *P. trigonatus* individuals were grouped in four size classes based on their SVL in cm (class I: 20 - 40; class II: 41 - 60; class III: 61 - 80; class IV: >80).

Stable-isotope sample collection and analyses

After retrieving data on snout-vent length (SVL), sex and mass, a piece of claw and one or two tail scutes were collected from each of the captured crocodilians for stable-isotope analysis (SIA). A small piece of muscle tissue was removed from the scutes and rinsed with distilled water to avoid contaminating the sample with blood. We had differing numbers of muscle and keratin (claw) tissue samples for each species. Overall, δ^{15}N values of keratin tissue showed a strong pairwise correspondence with muscle tissue δ^{15}N values ($\delta^{15}N_{keratin} = 1.06 + 0.87*\delta^{15}N_{Muscle}$; $F_{1,61} = 155.5$; $r^2 = 0.72$; $p < 0.001$; Fig 1). Thus, we only used data from the tissue type with the largest number of samples per species. In the case of *M. niger*, we included both types of tissue samples in the analyses corresponding to different individuals due to low sample size. Comprehensive food web sampling and laboratory procedures for SIA are presented in Villamarín et al. (chapter 2).

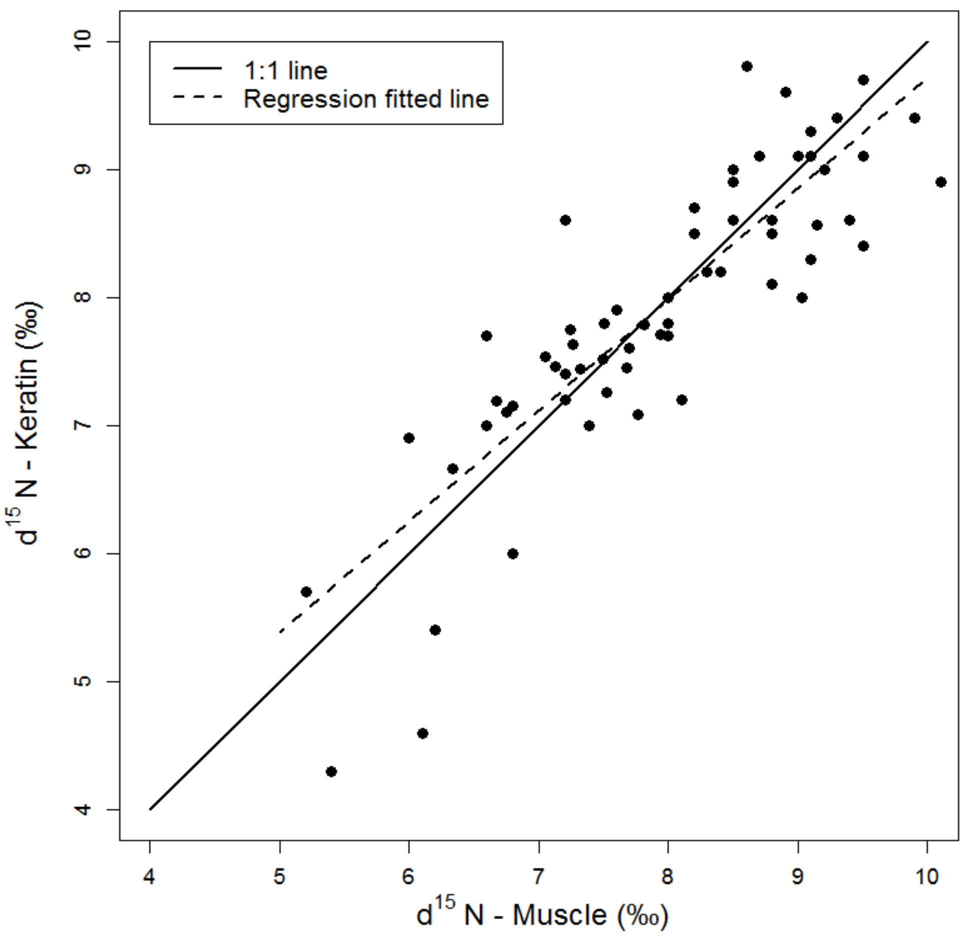

Fig. 1. Relationship between δ^{15}N values of muscle and keratin tissues.

Trophic position calculations for crocodilians and other consumers

Raw $\delta^{15}N$ values of an organism provide little information about its absolute trophic position within the food web. Thus, we calculated trophic position of crocodilians based on the following equation modified from Post (2002):

$$TP = \lambda\,(\delta^{15}N_{croc} - [\delta^{15}N_{terr.base} * \alpha + \delta^{15}N_{aq.base} * (1 - \alpha)])\,/\,\Delta_n,$$

where $\delta^{15}N_{croc}$ is the nitrogen isotope ratio of the crocodilian; $\delta^{15}N_{terr.base}$ and $\delta^{15}N_{aq.base}$ are nitrogen isotope ratios of terrestrial and aquatic baselines, respectively; α is the proportional contribution of carbon from terrestrial origin (derived from Villamarín et al. this study, chapter 2); λ is the trophic position of the organisms used to estimate $\delta^{15}N_{terr.base}$ and $\delta^{15}N_{aq.base}$ (e.g., $\lambda = 2$ for primary consumers); and Δ_n is the trophic fractionation, or trophic enrichment in $\delta^{15}N$ per trophic level.

$\delta^{15}N_{terr.base}$ and $\delta^{15}N_{aq.base}$ were derived from the mean value of all terrestrial and aquatic primary consumers available in this study (2.39‰ and 3.87‰, respectively). Trophic fractionation of ^{15}N in crocodilians (around 1.2‰, Rosenblatt & Heithaus 2013; Marques et al. 2014) have been found to be much lower than most organisms studied (3.4‰; Vander Zanden et al. 1999; Post 2002; Vander Zanden & Fetzer 2007). Thus, we used a conservative Δ_n value of 2.5‰ for crocodilians which is a mean fractionation value also reported elsewhere (Vanderklift & Ponsard 2003, Jardine 2016).

Since we did not have information on the relative contributions of terrestrial vs. aquatic carbon resources supporting other consumers in this study, we used the following equation to calculate TP for all the other organisms in the food web (Post 2002; Vander Zanden & Fetzer 2007):

$$TP = \lambda\,(\delta^{15}N_{cons} - \delta^{15}N_{base})\,/\,\Delta_n,$$

where $\delta^{15}N_{cons}$ is the nitrogen isotope ratio of the consumer under consideration; $\delta^{15}N_{base}$ is the nitrogen isotope signature of the terrestrial or aquatic baselines, according with the organism in consideration; and Δ_n is 3.4‰.

All analyses and graphics were performed using R software (Team R Core, 2014).

Results

Stomach-content analysis of *P. trigonatus*

We analyzed stomach contents from 28 *P. trigonatus* individuals between 23.8 and 99.2 cm SVL. We only found one individual smaller than 40cm SVL. Although not always full, all stomachs had some remains of at least one prey type. With the exception of mammals, all prey groups occurred most frequently in stomachs of mid-sized individuals, class III. Of these, vertebrates most frequently found in this size class were reptiles, fish, birds and to a lesser degree, amphibians. All four groups of invertebrates, terrestrial and aquatic, herbivorous and predators were also most frequently present in size class III individuals. The only prey group most frequently found in stomachs of the largest individuals, class IV, were mammals (11% of all stomachs), although they were also present in stomachs of class II and III. Terrestrial invertebrates both herbivorous and predators were the only groups present in all four size classes (Fig 2).

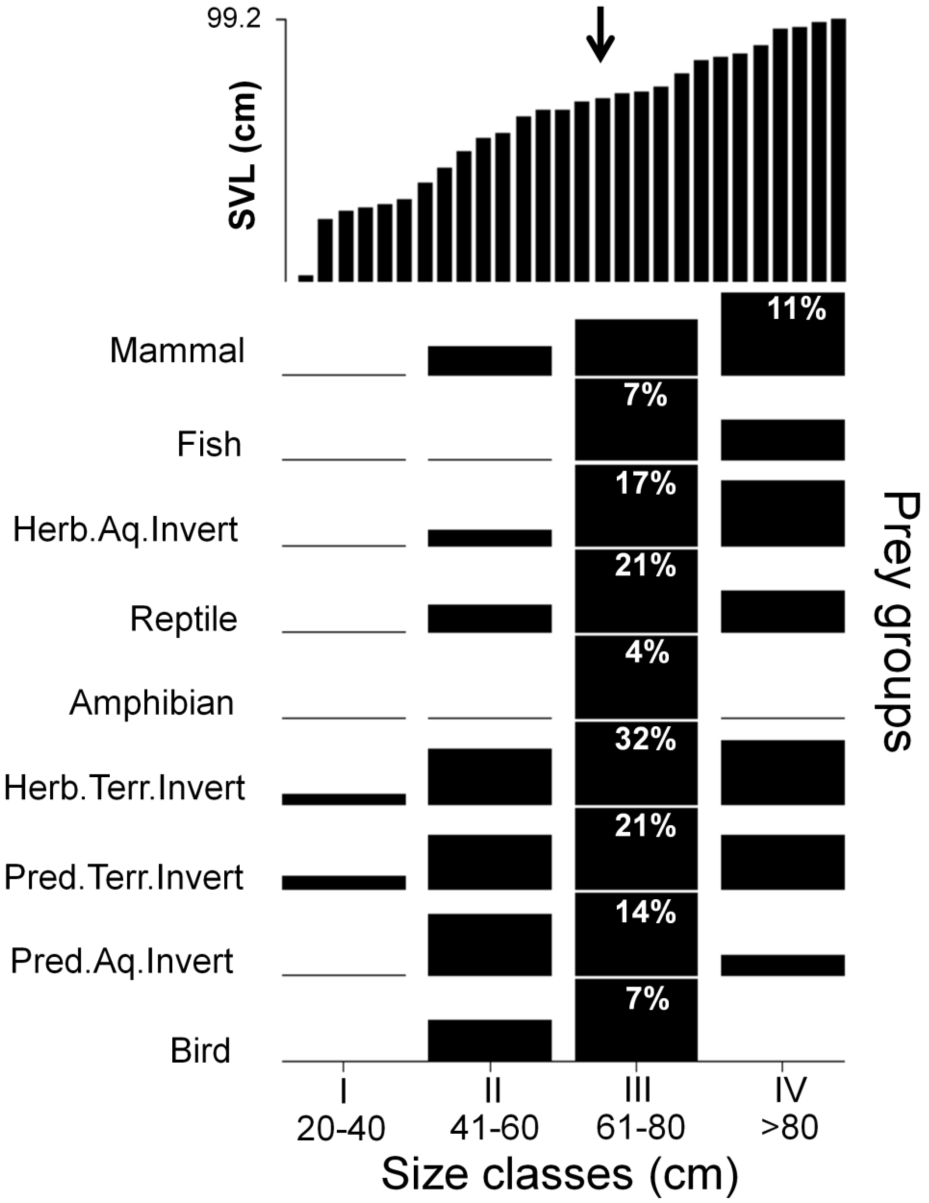

Fig. 2. Stomach content analyses showing the frequency of occurrence of prey groups in the stomachs of *P. trigonatus* individuals. Percentage numbers represent the percentage of stomachs containing each prey group from the total number of stomachs analysed and are shown only in the size class were the prey group was most frequently found. The black arrow shows the approximate minimum reproductive SVL. Abreviations are as follows: SVL (cm) = snout-vent length measured in centimeters; Herb = herbivorous; Pred = predators; Aq = aquatic; Terr = terrestrial; Inv = invertebrates.

Nitrogen stable-isotope ($\delta^{15}N$) analysis and TP of crocodilians

A total of 121 individuals of Amazonian crocodilians corresponding to all four species (31 *C. crocodilus*, 36 *P. palpebrosus*, 9 *M. niger* and 45 *P. trigonatus*) were included in the analyses.

Excluding samples of *M. niger* because most individuals were juveniles and subadults, the variation in crocodilian $\delta^{15}N$ values were not significantly influenced by differences between species (ANOVA: $F_{2,102} = 0.9$; $p = 0.410$), nor sex ($F_{4,102} = 1.45$; $p = 0.224$); however, they were significantly influenced by SVL nested within the species ($F_{3,102} = 23.29$; $p < 0.001$).

After correcting $\delta^{15}N$ values to reflect crocodilian TP, all species showed positive relationships between TP and SVL. *C. crocodilus* (TP = 4.44 + 0.05*SVL; $F_{1,29} = 25.94$; $r^2 = 0.47$; $p < 0.001$), *P. palpebrosus* (TP = 3.18 + 0.014*SVL; $F_{1,34} = 38$; $r^2 = 0.53$; $p < 0.001$) and *M. niger* (TP = 1.35 + 0.025*SVL; $F_{1,7} = 4.01$; $r^2 = 0.36$; $p = 0.085$) showed linear increases of TP as a function of their increase in SVL. *P. trigonatus* also showed a positive effect of SVL over TP, but the relationship was quadratic producing a parabolic curve (TP = 2.34 + 0.05*SVL - 0.0003*SVL^2; $F_{2,42} = 9.42$; $r^2 = 0.31$; $p < 0.001$) and suggesting a plateau at maximum TP for mid-sized individuals (Fig 3).

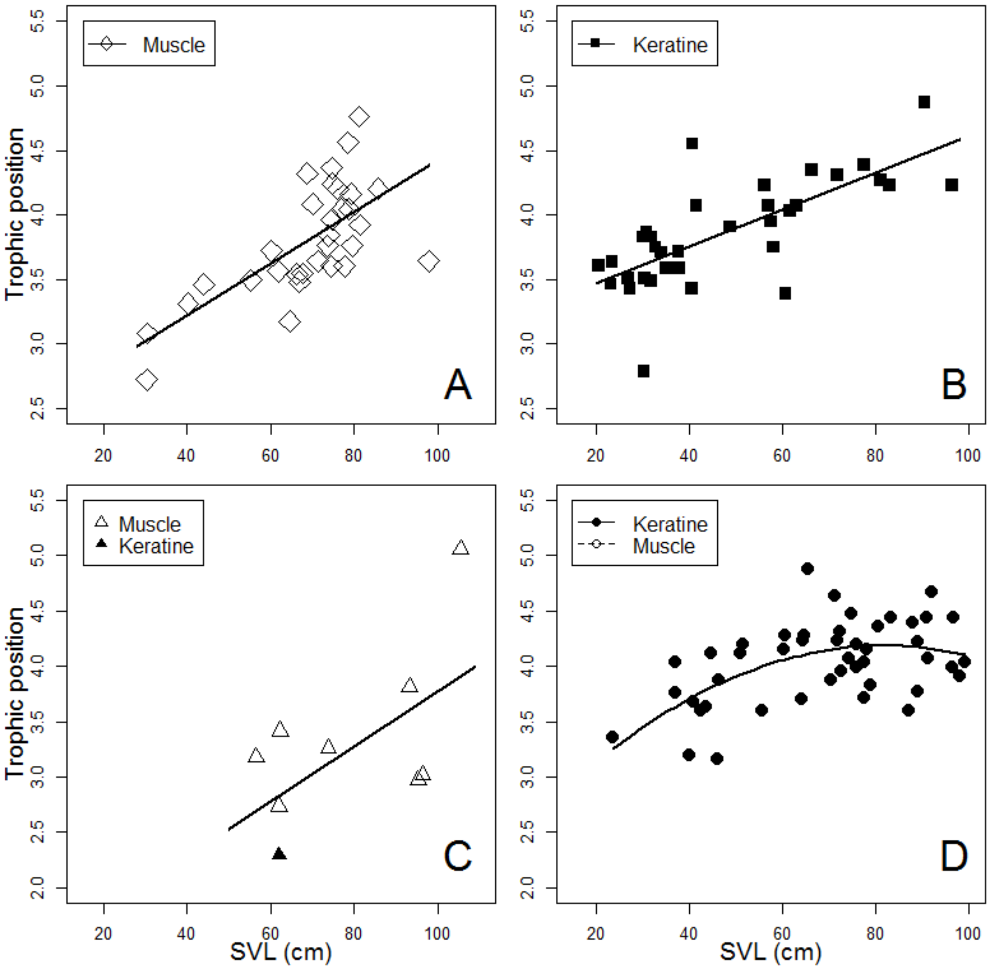

Fig 3. Increase in trophic position as a function of increments on snout-vent length (SVL) in the four species of Amazonian crocodilians (*C. crocodilus*, A; *P. palpebrosus*, B; *M. niger*, C; and *P. trigonatus*, D).

Nitrogen stable-isotope analysis (δ^{15}N) and TP of the food web

Excluding crocodilians, a total of 431 individual organisms corresponding to four trophic groups (autotrophs: 169; herbivores: 42; omnivores: 95 and carnivores: 126) were analyzed. Mean δ^{15}N values of primary aquatic (3.87‰) and terrestrial (2.39‰) consumers were slightly different (t-test: $t = 2.04$; $df = 8.56$; $p = 0.073$). These values were used as aquatic and terrestrial baselines to estimate TP, and a trophic fractionation value of 3.4‰ (Post 2002).

Overall, crocodilians showed δ^{15}N values lower than most families of predator fish and were comparable with those of omnivorous invertebrates and fish (Fig. 4A). Adult individuals of *C. crocodilus*, *P. palpebrosus* and *P. trigonatus* had δ^{15}N values significantly lower than the piscivorous fish *H. malabaricus* (t-test; $t = -8.31$; $df = 12.53$; $p < 0.001$). After estimating TP, these three crocodilians occupy a higher position in the food chain (Fig 4B). However, only adult individuals of *P. palpebrosus* were significantly higher than *H. malabaricus* by 0.31 trophic levels ($t = 2.94$; $df = 15.88$; $p = 0.009$). Adults of both *C. crocodilus* ($t = 0.86$; $df = 24.47$; $p = 0.397$) and *P. trigonatus* ($t = 1.16$; $df = 20.64$; $p = 0.26$) were not statistically different from this species of piscivorous fish. *M. niger* was not included in these analyses due to low sample size of adult individuals.

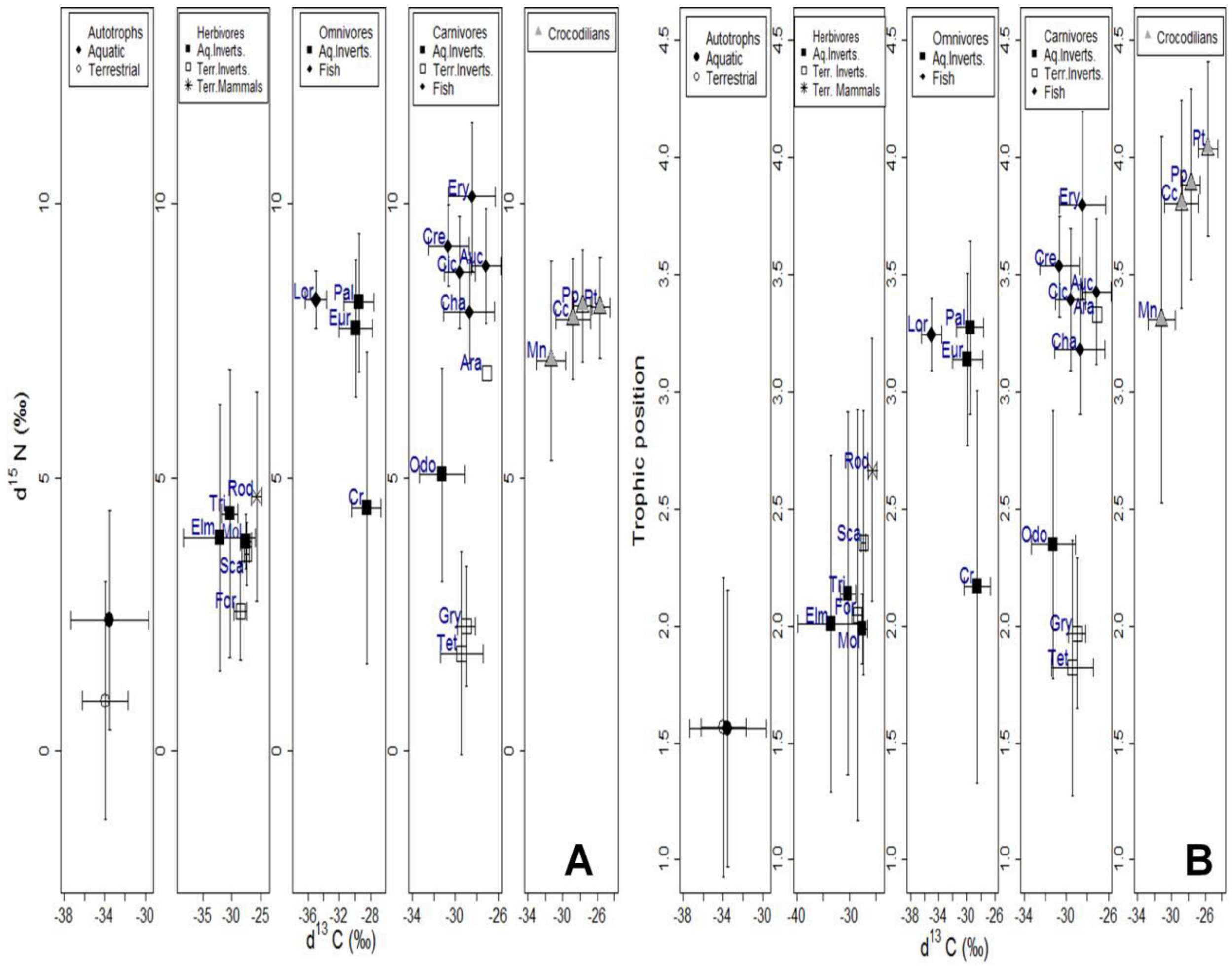

Fig. 4. Aquatic and terrestrial foodwebs isospace. Distribution of means and standard deviations of main trophic groups in relation to δ^{15}N (A) and trophic position (B). Black and hollow symbols represent aquatic and terrestrial organisms, respectively. Abreviations are as follows: **Herbivores**: **Elm** = Elmidae, **For** = Formicidae, **Mol** = mollusc (Ampullariidae), **Rod** = Rodentia, **Sca** = Scarabaeidae and **Tri** = Trichoptera. **Omnivores**: **Cr** = crab (Trichodactylidae), **Eur** = Euryrhynchidae, **Pal** = Palaemonidae and **Lor** = Loricariidae. **Carnivores**: **Ara** = Araneae (Mygalomorphae), **Auc** = Auchenipteridae, **Cha** = Characidae, **Cic** = Cichlidae, **Cre** = Crenuchidae, **Ery** = Erythrinidae, **Gry** = Gryllidae, **Odo** = Odonata and **Tet** = Tettigonidae. **Crocodilians**: **Cc** = *Caiman crocodilus*, **Mn** = *Melanosuchus niger*, **Pt** = *Paleosuchus trigonatus*, **Pp** = *P.palpebrosus*.

Discussion

The use of nitrogen stable-sotopes (δ^{15}N) of Amazon crocodilian tissues provided evidence of ontogenetic shifts in their trophic position (TP). Overall, progressive shifts in TP found in *P. palpebrosus* and *C. crocodilus* showed a positive linear relationship with their increase in SVL. *P. trigonatus* also showed increases in TP related to their increase in SVL, but the relationship was non-linear, suggesting a plateau at maximum TP in mid-sized individuals. These observations are in accordance with published literature about ontogenetic diet shifts in crocodilians and are supported by stomach content analyses and δ^{15}N values from potential prey items in this study.

Depending on the species, crocodilians may increase in length by 6- to more than 20-fold throughout their lives. As a result, they are expected to experience ontogenetic diet shifts starting from terrestrial and aquatic invertebrates when young, to more protein-rich diets composed mostly of fish and terrestrial vertebrates as they grow larger (Ross 1998; Radloff, Hobson & Leslie 2012). This general trend may be facilitated by ontogenetic changes in bite force (Erickson, Lappin & Vliet 2003). According to traditional stomach content analyses, the four species of Amazonian crocodilians show different ontogenetic diet trajectories that lead to interspecific differences as adults. Juvenile *P. palpebrosus, C. crocodilus* and *M. niger* have diets mostly composed of terrestrial and aquatic invertebrates with diets changing gradually to fish when adults (Magnusson, Silva & Lima 1987; Da Silveira & Magnusson 1999). In habitats where these three species are common such as open-canopy waterbodies and floodplains, large fish are abundant, leading to their reliance mainly on aquatic carbon resources (Villamarín et al. Chapter 2). On the other hand, *P. trigonatus* occurs in closed-canopy headwater streams where large fish are uncommon, except for *Hoplias malabaricus* and a few species of Heptapteridae, Sternopygidae and Gymnotidae (Zuanon et al. 2015). Juvenile *P. trigonatus* diet is mostly composed of terrestrial invertebrates. However, the ingestion of reptiles, amphibians and birds is more frequent in medium-sized individuals. These prey items become less frequent as they grow to the largest size class and terrestrial mammals become the most common item for most individuals (Magnusson, Silva & Lima 1987; this study). Consequently, *P. trigonatus* derives most of its carbon from terrestrial resources (Villamarín et al. this study, chapter 2).

The linear increase in TP as a function of SVL observed in *P. palpebrosus* and *C. crocodilus* may result from progressively less ingestion of invertebrates coupled with greater fish consumption as they grow. Almost all groups of invertebrates, both aquatic and terrestrial, sit very low in the trophic chain in our study. The only exceptions are spiders (Mygalomorphae) and omnivorous shrimps (Palaemonidae and Euryrhynchidae) that are slightly higher on the food chain, with a TP > 3. Although this TP of shrimps fits well with that expected for prey of medium-sized crocodilians, very few records of this group have been found to be eaten by Amazonian crocodilians (Magnusson, Silva & Lima 1987). Fish, on the other hand, are located higher on the food chain. Most fish included in the analyses correspond to carnivorous species (*i.e.* Erythrinidae, Crenuchidae, Auchenipteridae, Cichlidae and Characidae). However, even species such as *Ancestridium discus* (Loricariidae) that have traditionally been considered algivorous, but lately found to be omnivorous - detritivorous (de Ávila Lacerda 2007), appear relatively high on the food chain. Thus, the lower TP of juvenile *P. palpebrosus* and *C. crocodilus* and higher TP of adults may reflect ingestion of invertebrates and fish, respectively.

Other studies involving ontogenetic trophic shifts in crocodilians found similar results but with slight differences from this study. Introduced *C. crocodilus* in Puerto Rico, showed increasing δ^{15}N values as a function of increasing SVL (Bontemps et al. 2016). However, the relationship for that species was quadratic, with δ^{15}N values of adults that were lower than mid-sized individuals, similar to our observations for *P. trigonatus* in this study. This parabolic trend differs from the linear relationship in *C. crocodilus* in this study. The authors show a considerable proportion of adult *C. crocodilus* consuming terrestrial mammals in the Puerto Rican population and we speculate that a mixture of fish and terrestrial herbivorous mammals might be responsible for that trend. Similarly, the largest size class of saltwater crocodiles (*Crocodylus porosus*) from Australia, are thought to assimilate a mixed diet composed mainly of fish from freshwater and marine habitats and terrestrial herbivorous mammals, leading to lower δ^{15}N values in largest individuals (Hanson et al. 2015). Although we have few samples from the largest size class of *C. crocodilus*, their mostly piscivorous diets in the Amazon basin and their strong reliance on aquatic carbon resources do not give us reason to think that largest individuals would sit lower on the trophic chain than smaller individuals. Furthermore, *C. crocodilus* and *P. palpebrosus* have very similar diets. A large enough sample size of large *P. palpebrosus* which attain a maximum SVL about 25% smaller than the former species is probably good evidence to consider large individuals of both species relying mostly on prey sitting high on the trophic chain.

Melanosuchus niger is the largest species of Amazonian crocodilian, with adult males reaching up to 4 to 5m in TL (≈ 200 - 250 cm SVL) and estimated minimum reproductive length of around 2 m TL (≈ 100 cm SVL; Thorbjarnarson, 2010). Our samples included only one individual over reproductive size and the remainder were sub-adults and juveniles. If the diet of juveniles is composed mainly of terrestrial and aquatic invertebrates, this may explain why *M. niger* has a low mean TP in our study. However, the positive trend between SVL and TP in this species suggests that *M. niger* may show the same ontogenetic trends seen in *C. crocodilus* and *P. palpebrosus*.

Of the four Amazonian species, adult *P. trigonatus* has the most divergent diet, ingesting more terrestrial mammals compared with medium-sized individuals. Since terrestrial herbivorous rodents in our study (mainly *Cuniculus* and *Dasyprocta*) occupy a relatively low TP of around 2.5, larger *P. trigonatus* individuals show a lower TP than medium-sized individuals. Although we do not have estimates of δ^{15}N values from snakes, lizards or frogs which are important items in the diet of medium-sized *P. trigonatus*, we can assume that their TP is higher than terrestrial herbivorous rodents due to their predatory habits. Species of ground-dwelling and aquatic snakes (*Bothrops* spp. and *Helicops angulatus*, respectively) and frogs (*Leptodactylus pentadactylus*), which are common in riparian systems in the study area, are all predator species and potential prey for *P. trigonatus*. Furthermore, enriched δ^{15}N values cause both snakes and frogs to sit high in the food chain of another tropical riparian system (Krupfer et al. 2006).

Crocodilians show lower δ^{15}N values than expected for a predator at the top of a long food chain (Radloff, Hobson & Leslie 2012; Hanson et al. 2015; this study). When using δ^{15}N to calculate TP, two key parameters are appropriate baseline δ^{15}N values and trophic fractionation estimates (Cabana & Rasmussen 1996; Post 2002). Baseline δ^{15}N values must be able to integrate temporal and spatial isotopic changes to adequately reflect those of larger consumers; thus, long-lived herbivores have commonly been used as baselines in foodweb studies (Cabana & Rasmussen 1996; Vander Zanden & Rasmussen 1999; Post 2002). We used mean δ^{15}N values of available primary consumers as the system´s aquatic and terrestrial baselines (Cabana & Rasmussen 1996; Vander Zanden & Fetzer 2007). This approach typically yields results in which baseline variation is not a major source of error in TP estimates (Vander Zanden & Fetzer 2007). However, a possible confounding factor contributing to the low δ^{15}N values of crocodilians relative to their prey is low trophic fractionation of ^{15}N in crocodilians. Crocodilians phylogenetically related to Amazonian species show ^{15}N trophic fractionation values of around 1‰ (Rosenblatt & Heithaus 2013; Marques et al. 2014), whereas less closely related species had a higher value of ~3‰ (Hanson et al. 2015). Trophic fractionation is the most sensitive parameter in TP estimates (Post 2002) because fractionation values sit in the denominator of the equation to calculate TP; thus, any reduction in fractionation values would yield significant increases in TP. Although some studies show divergences in trophic fractionation values for diverse organisms (McCutchan et al 2003), when applied to entire food webs with multiple trophic pathways and many species, a mean trophic fractionation of 3.4‰ is commonly applied (Post 2002). We applied this value to correct for trophic fractionation in most consumers but since crocodilians appear to show lower trophic fractionation we used a slightly more conservative value of 2.5‰ for crocodilians, a mean value also reported elsewhere (Vanderklift & Ponsard 2003). Applying this value to crocodilians places them high in the foodchain as expected for a top predator.

Trophic position of a top predator is an important component of food-web structure because it is a measure of the number of energy transfers between primary producers and tertiary consumers. Thus, this term is a synonym of food chain length . Comparative analyses of food-chain length have revealed that most aquatic foodwebs around the world show a maximum length of three to five trophic levels (Vander Zanden & Fetzer 2007) and our results fit well with this global pattern. However, most studies have conventionally used fish as top predators to estimate food-chain length, despite the fact that aquatic mammals and crocodilians in aquatic systems may potentially sit above fish as top predators. For example, when marine mammals have been included in the analyses, food-chain length estimates increased by 0.6 trophic levels relative to estimates that use marine fish as top predators (Vander Zanden &

Fetzer 2007). In our study, TP of adult *P. palpebrosus* was significantly higher than that of the piscivorous fish *Hoplias malabaricus* by 0.31 trophic levels. These findings suggest that the inclusion of true top predator organisms may yield significant divergences in food-chain length estimates, which should be appropriately balanced with the logistical difficulties of including non-fish top predators, such as crocodilians in future studies.

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Data accesibility

Raw data underlying the analyses will be available at the Research Program in Biodiversity data repository site (PPBio, https://ppbio.inpa.gov.br/repositorio/dados).

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Síntese geral

O presente estudo apresenta evidências espaço-temporais dos diversos mecanismos em que a energia que sustenta os grandes consumidores ectotérmicos flui através das teias alimentares em dois tipos de ecossistemas aquáticos tropicais. Utilizando ferramentas isotópicas e bioquímicas, mostramos como a energia é alocada para reprodução em um peixe australiano. Conseguimos estimar, também, as diferenças interespecíficas nas proporções em que os recursos terrestres e aquáticos sustentam as quatro espécies de crocodilianos amazônicos e como as mudanças ontogenéticas na dieta influenciam a posição trófica desses grandes predadores.

O estudo apresenta informações sobre a alocação da energia para reprodução em *Liza alata*, um peixe herbívoro-detritívoro do Território Norte da Austrália. Combinando dados sobre a razão de RNA:DNA e isótopos estáveis de carbono (δ^{13}C) e nitrogênio (δ^{15}N) de tecidos somáticos e reprodutivos descobrimos um desacoplamento espacial e temporal entre a obtenção de recursos alimentares e a posterior alocação reprodutiva nessa espécie de peixe catádromo. As evidências encontradas sugerem que, apesar de *L. alata* possuir melhor condição corporal durante a época cheia (quando os recursos são mais abundantes nas planícies de inundação), a maior parte da alocação reprodutiva acontece durante a época seca, quando os recursos são escassos e os peixes apresentam pior condição corporal. Achamos fortes evidências de um compromisso entre o investimento reprodutivo e somático, embora esse tipo de compromisso seja raramente encontrado em condições naturais (Van Noordwijk e de Jong, 1986; Glazier, 1999) devido a limitações na disponibilidade de energia (Gadgil e Bossert, 1970; Calow, 1970; Partridge et al., 1991; Stearns, 1992). Baseado nas informações dos isótopos estáveis, observamos que as gônadas crescem principalmente durante a época seca e apresentam síntese de longo prazo, mostrando valores isotópicos muito correlacionados com os valores de músculo, um tecido com uma taxa de substituição lenta. Em combinação, essas linhas de evidência sugerem que *L. alata* usa os excedentes de energia estocados de épocas anteriores para abastecer seu investimento reprodutivo, uma estratégia chamada de *'capital breeding'*. Há um desacoplamento temporal entre a ingestão de recursos, o estoque de energia em corpos lipídicos mesentéricos e a posterior alocação dessa energia para reprodução.

O desacoplamento temporal e espacial entre a aquisição de energia e sua alocação para reprodução nessa espécie de peixe tem importantes implicações para a preservação dos regimes hidrológicos naturais das planícies de inundação. *L. alata* tipicamente habita lagoas durante a época seca e migra para dentro das florestas alagadas durante a época cheia, onde obtém a maior parte dos seus recursos. Subsequentemente, alguns indivíduos voltam para as lagoas e outros migram até o oceano para desovar no início da vazante (Bishop et al., 1980). Apesar da baixa produtividade encontrada nessas lagoas durante a época seca (Pettit et al., 2011), as descobertas desse estudo enfatizam a importância desses hábitats como áreas onde ocorre a alocação reprodutiva. Mais importante, os hábitats formados durante a cheia, como as florestas alagadas, são críticos para proporcionar a maior parte da energia para crescimento e reprodução dessa espécie. Portanto, a manutenção dos regimes hidrológicos naturais é importante para garantir a capacidade dos peixes dessa região de manter suas populações viáveis.

Por outro lado, no contexto dos ecossistemas lóticos da Amazônia central, as quatro espécies de crocodilianos amazônicos são predadores de topo de cadeia que exercem um impacto substancial nas teias alimentares aquáticas e terrestres. O uso de isótopos estáveis de carbono ($\delta^{13}C$) possibilitou a identificação da proporção em que os recursos terrestres e aquáticos sustentam esses predadores. Por outro lado, isótopos estáveis de nitrogênio ($\delta^{15}N$) permitiram entender como acontecem as mudanças ontogenéticas do nível trófico em função da mudança de dieta e qual a posição trófica deles nas teias alimentares aquáticas e terrestres.

Encontramos evidências de diferenças nos recursos basais sustentando as quatro espécies de crocodilianos amazônicos. Valores de $\delta^{13}C$ foram baixos em *Melanosuchus niger*, intermediários em *Caiman crocodilus* e *Paleosuchus palpebrosus* e altos em *Paleosuchus trigonatus*. Uma progressiva depleção de valores de $\delta^{13}C$ ocorreu das cabeceiras até a várzea que reflete um incremento progressivo na ingestão de recursos autóctones nos trechos mais baixos dos riachos. A mudança de recursos terrestres para aquáticos sustentando essas espécies simpátricas de predadores espelha a sua distribuição espacial ao longo desse ecôtone. No entanto, quando se leva em consideração as características do hábitat em pares de indivíduos sintópicos de espécies diferentes, discrepâncias significativas na assinatura isotópica sugerem que *P.*

trigonatus e *P. palpebrosus* consomem diferentes presas basais. Portanto, as diferenças na dieta resultam de divergências comportamentais e estratégias de forrageio além da seleção de macrohábitat.

Encontramos também uma relação positiva entre o comprimento dos crocodilianos e a posição trófica que ocupam. Essa relação foi linear para *M. niger*, *C. crocodilus* e *P. palpebrosus*, mas curvilinear em *P. trigonatus*, sugerindo que os indivíduos de comprimento médio dessa espécie ocupam níveis tróficos mais altos. Análises de conteúdos estomacais de *P. trigonatus* e dados na literatura sugerem que esse padrão pode ser explicado porque os indivíduos de comprimentos médios consomem com maior frequência vertebrados predadores, como cobras, lagartos e sapos, enquanto os indivíduos maiores consomem principalmente mamíferos herbívoros de médio porte, como pacas e cutias. Por outro lado, a relação linear entre o comprimento e a posição trófica das outras três espécies pode ser explicada pelo aumento progressivo no consumo de peixes. Os peixes nesses estudo ocuparam, em sua maioria, níveis tróficos altos o que pode estar refletindo essa forte relação.

A maioria dos estudos têm usado convencionalmente peixes como predadores de topo de cadeia para estimar o comprimento das cadéias tróficas aquáticas (Vander Zanden e Fetzer 2007). Porém, geralmente mamíferos aquáticos e crocodilianos podem se posicionar acima dos peixes como predadores de topo. Nesse estudo, a posição trófica de adultos de *P. palpebrosus* foi significativamente maior do que a do peixe piscívoro *Hoplias malabaricus* por 0.31 níveis tróficos. Essa descoberta sugere que a inclusão de organismos verdadeiramente de topo de cadeia como crocodilianos pode gerar divergências significativas nas estimativas do comprimento das cadeias alimentares, o que deveria ser apropriadamente ponderado com as dificuldades logísticas de incluir predadores como crocodilianos em futuros estudos sobre a estrutura das teias alimentares.

As informações apresentadas nesse estudo trazem aspectos inovadores sobre a biologia e ecologia dos consumidores estudados, retratando o funcionamento trófico dos ecossistemas onde ocorrem em relação aos recursos que os sustentam.

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Anexo A

Table S1. Prior information on contributions of prey items composing Amazonian crocodilian diets for endmember isotopic groupings

Appendix A

Table S1. Prior information on contributions of prey items composing Amazonian crocodilian diets for endmember isotopic groupings

Crocodilian species	Prey habit	Prey group	Mean prey mass (g)	Number of individual prey, per crocodilian, per size class*						Weighted mean (g/caiman)	Proportion of prey within aquatic and terrestrial diets (%)	Prey mean δ 13C (±SD)
				<20	**20-30**	**30.1-40**	**40.1-50**	**50.1-60**	**>60**			
P. Trigonatus	Aquatic	Shrimp	0.1	0.4	0.2	0.4	0.2	0.2	0.3	0.028	0.04	-29.39 (2.61)
		Crab	16.3	0	0	0.2	0.7	0.2	0.2	4.46	6.48	
		Mollusc	48	0	0	0	0.5	0	0.2	10.56	15.35	
		Fish	340.7	0	0	0	0.1	0	0.2	53.76	78.13	
	Terrestrial	Invertebrate	1.5	6	1	2	3	1	1.5	2.57	0.05	-25.58 (0.75)
		Vertebrate	6750	0.4	0.3	0.4	0.5	0.2	1	5580	99.95	
P. palpebrosus	Aquatic	Shrimp	0.1	0.5	1	0	0.2	0	_	0.024	0.002	-29.78 (2.54)
		Crab	16.3	0	0	1.6	0.2	0.5	0.3	10.66	0.98	
		Mollusc	48	0	1.5	1.7	7	7	0.5	122.85	11.25	
		Fish	1822.49	0.05	0.5	0.3	0.85	0.5	0.7	958.04	87.77	
	Terrestrial	Invertebrate	1.5	3.5	4.5	2.5	3	1	0.5	3.08	0.25	-25.56 (0.68)
		Vertebrate	6750	0.2	0	0	0.42	0.25	0.33	1233.24	99.75	
C. crocodilus	Aquatic	Shrimp	0.1	0	0.1	0.2	0	_	_	0.018	0.001	-30.07 (2.98)
		Crab	16.3	0.1	0.5	0.5	0.2	0.8	0.4	9.89	0.83	
		Mollusc	48	0.1	2	2.1	0.8	0.4	4	99.9	8.38	
		Fish	1822.49	0.1	0.2	0.2	0.45	0.8	0.9	1083.1	90.79	
	Terrestrial	Invertebrate	1.5	9.5	6	7	3.5	2	1.5	2.39	0.15	-25.53 (0.64)
		Vertebrate	6750	0.1	0.05	0.06	0.2	0.8	0.7	1573.6	99.85	
M. niger	Aquatic	Shrimp	0.1	_	0	_	_	0	0	0	0	-32.55 (2.41)
		Crab	16.3	_	0	_	_	0	0.5	5.29	0.41	
		Mollusc	48	_	0.2	_	_	0	4.5	63	4.85	
		Fish	1822.49	_	0.2	_	_	0.5	1	1230.18	94.74	
	Terrestrial	Invertebrate	1.5	_	5.5	_	_	0.5	2	0.84	0.04	-25.52 (0.68)
		Vertebrate	6750	_	0.4	_	_	0	0	2160	99.96	

*Magnusson et al.1987