Universidade do Estado do Rio de Janeiro

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Priscila de Oliveira Cunha

Fish as bags of macro and micronutrients: factors that influence fish excretion and body stoichiometry

Rio de Janeiro

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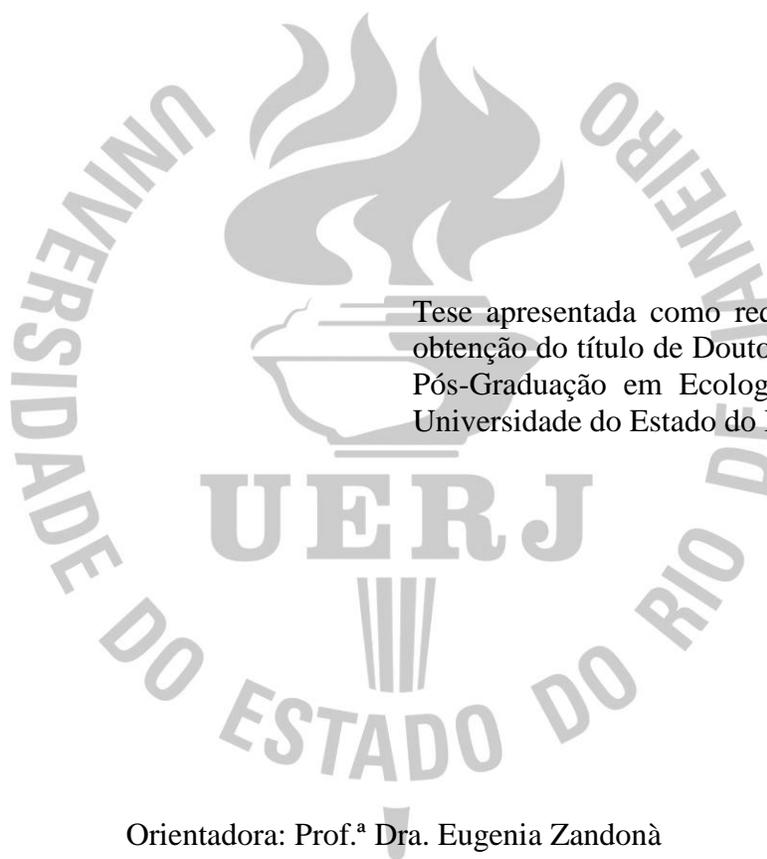
Tese apresentada como requisito parcial para obtenção do título de Doutora, ao Programa de Pós-Graduação em Ecologia e Evolução, da Universidade do Estado do Rio de Janeiro.

Orientadora: Prof.ª Dra. Eugenia Zandonà

Coorientadores: Prof. Dr. Adriano Caliman

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Aprovada em 19 de novembro de 2021.

Orientadora:

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Rio de Janeiro

2021

DEDICATÓRIA

À minha família, amigos e todos que participaram dessa caminhada.

AGRADECIMENTOS

PhD é a sigla usada para Doutor em Filosofia, então tomo a liberdade de filosofar um pouquinho aqui nos agradecimentos. Ao longo dessa tese, eu falo basicamente sobre peixes. Peixes e suas características, peixes e suas interações com outros seres vivos, peixes e suas interações com seu ambiente, e como tudo isso pode afetar o ciclo de nutrientes de um riacho. Pra nós, humanos, não é muito diferente. Nossas próprias características e nossas interações uns com os outros e com a sociedade em que vivemos afetam o nosso ciclo da vida, e podem fazer parte de algo maior. O que esse "algo maior" significa é um grande mistério e ficamos com a liberdade de escolher em que acreditar.

Na minha vida, algumas interações foram determinantes para esse doutorado. Há 32 anos, nasci. Desde esse instante, conto com a torcida incondicional dos meus pais e dos meus avós, e 4 anos depois, minha irmã se juntou a esse time, de quem sempre recebi todo apoio e incentivo possíveis. Durante a minha infância, as férias de verão foram na casa dos meus avós em Friburgo, brincando no jardim, observando as lagartas se transformarem em borboletas, os girinos se transformarem em sapos, apreciando o canto dos pássaros, comendo jabuticaba do pé, tomando banho de cachoeira, criando pintinhos, patos e preás. Nasceu ali o amor e curiosidade pela natureza, que determinaram a escolha da minha profissão. Por isso, começo agradecendo aos que acompanharam tudo desde o início, meus pais, irmã e avós, pelo apoio durante todo esse trabalho.

Durante a faculdade de Biologia na UERJ, vi um anúncio de estágio no laboratório de Ecologia de Rios e Córregos (LERC) e me candidatei. Consegui o estágio e tive a sorte de começar a trabalhar com o Tim (Timothy Moulton). Uma pessoa com conhecimentos diversos, sempre com perguntas interessantes, que ajudou a moldar minha maneira de pensar sobre questões científicas e sobre o que significa ser cientista. E é também um super anfitrião, sempre organizando as melhores festas (junto com a Rosário, claro!). Tim, obrigada por ter aberto as portas do laboratório pra mim e ter contribuído com seu ponto de vista em todos os momentos da minha formação acadêmica. Obrigada também por ter revisado este trabalho e por ter aceito ser parte da banca.

Quando ingressei no LERC, o Vini (Vinicius Neres de Lima) já era a pessoa mais popular de lá. Com razão, pois não existe pessoa mais paciente e prestativa, que sempre faz o possível pra ajudar a todos. Um grande coração, um grande cientista e também um grande talento para a

psicologia, pois para mim foi uma espécie de psicólogo várias vezes! Sua ajuda com as análises estatísticas foi indispensável para conclusão desse doutorado. Obrigada por compartilhar seu conhecimento e sempre arranjar tempo para uma conversa.

Algum tempo depois, a Eugenia Zandonà se juntou ao laboratório. Sua orientação, disponibilidade para ensinar, os contatos com outros pesquisadores que me possibilitou fazer, e suas críticas sempre construtivas foram cruciais para minha formação profissional. Além disso, Eugenia é aquela pessoa com quem podemos conversar sobre tudo, desde artigos científicos até trabalho de parto ou marcas de roupas... Ela foi a terceira pessoa pra quem contei que estava grávida e de quem recebi um apoio imenso, que me tranquilizou e fortaleceu muito. Não existem palavras para expressar o significado disso. Seu exemplo de pesquisadora, professora, mãe são uma inspiração pra mim e muitas outras mulheres. Sem dúvidas, uma das grandes referências que tenho. Sou muito grata pela nossa parceria que já dura anos, e mais ainda pela relação de amizade que construímos.

Uma das melhores partes de toda essa experiência no doutorado foi, sem dúvidas, a oportunidade de trabalhar com pessoas incríveis. Além das 3 pessoas citadas acima, todos os colegas que fazem ou fizeram parte do LERC me ensinaram o quanto o trabalho de pesquisa colaborativo é recompensador e apaixonante. Beatriz Ferreira, Bruna Silva, Jefferson Ribeiro Amaral, Bruno Gorini, Fernanda Motta, Élida Ferreira, Karoline Lacerda e Juliana Argento foram grandes companheiros de dias no campo e suas adversidades. Bia ainda esteve muito presente nas análises de nutrientes no laboratório, e Jefferson me ensinou a fazer análises estatísticas no R. Meus co-orientadores, Adriano Caliman e Peter McIntyre, contribuíram muito para este trabalho, ajudando a conduzir a tese, trazendo novas perspectivas e perguntas interessantes a serem incorporadas. Rafael Guariento e Nicholas Marino também acrescentaram muito ao trabalho, compartilhando seu conhecimento e trazendo ótimas sugestões. Muito obrigada a todos!

Outra experiência incrível foi ter realizado o doutorado sanduíche nos EUA. I must thank Peter McIntyre for making this exchange period possible, for his guidance and all the support during my time at Cornell University. My thanks to Alexander Flecker for generously sharing his laboratory space and equipment, and to Keeley McNeill for her patience and kindness while teaching me chemical analysis. It was a pleasure to work with you all. To Debora Carvalho and Diessy Kipper, thank you for your friendship and all the fun moments we shared together.

Agradeço à agencia de fomento Capes pela bolsa de doutorado e doutorado sanduíche. Ao

E a vida, como metáfora de um rio, tudo traz, tudo leva, tudo lava. Menos o amor. O amor é uma verdade à prova do tempo.

Cris Guerra

RESUMO

A excreção de nutrientes e estequiometria corporal de consumidores podem ser influenciadas por vários fatores. Neste trabalho, testamos como 12 espécies de peixes de um córrego tiveram suas taxas de excreção de nutrientes afetadas pelo seu tamanho corporal, temperatura, estequiometria corporal e dieta (grupos alimentares como proxy). Nós aprofundamos a investigação sobre a magnitude do efeito da temperatura sobre as taxas de excreção de peixes de água doce através de uma meta-análise com 15 estudos e 40 tamanhos de efeito. Por fim, medimos o conteúdo corporal de 19 peixes para os elementos cálcio (Ca), fósforo (P), sódio (Na), potássio (K), enxofre (S), magnésio (Mg), manganês (Mn) e zinco (Zn). Testamos como a estequiometria corporal foi influenciada pelo habitat das espécies, suas guildas tróficas e ordens taxonômicas, e cada par de elementos foi testado para correlações. Nossos resultados mostram que o tamanho corporal é o principal fator determinando as taxas de excreção de nitrogênio (N) e fósforo (P) e, contrário às expectativas, a temperatura, estequiometria corporal e dieta não foram estatisticamente significativos no nosso estudo. Entretanto, experimentos futuros devem tentar incluir medidas de ingestão e egestão para aumentar a compreensão do efeito da dieta e estequiometria corporal nas taxas de excreção. Através da meta-análise, vimos que aumentos na temperatura levam a aumentos nas taxas de excreção, mas a magnitude do efeito é extremamente variável entre estudos. O tempo de aclimatização e status alimentar respondem juntos por 22% da variação, no entanto a maior parte desta permanece inexplicada e é muito provavelmente relacionada a identidade das espécies e suas características fisiológicas. São necessários mais experimentos usando métodos padronizados para explicar melhor a influência da temperatura na excreção de nutrientes. Finalmente, vimos que elementos com funções similares são correlacionados, especialmente cálcio e fósforo devido ao seu papel na formação dos ossos. Peixes tropicais apresentaram maior variação em sua composição corporal, o que pode estar relacionado com um nicho estequiométrico mais especializado ou com um maior tamanho amostral (12 espécies tropicais versus 7 espécies temperadas), e foram basicamente direcionadas por diferenças no conteúdo de Ca e P. A composição corporal dos peixes diferiu de acordo com as ordens taxonômicas, ressaltando a influencia da filogenia. A estequiometria corporal dos peixes também variou entre guildas tróficas, mas devido à presença de peixes com armadura óssea.

ABSTRACT

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Consumer nutrient excretion rates and their body stoichiometry can be influenced by a series of factors. Here, we tested how 12 species of fish from a freshwater stream had their excretion rates affected by body size, temperature, body stoichiometry and diet (feeding group as proxy). We further investigated the magnitude of the temperature effect on freshwater fish excretion through a meta-analysis conducted with 15 studies and 40 individual effect sizes. Finally, we measured 19 fish species whole-body content of calcium (Ca), phosphorus (P), sodium (Na), potassium (K), sulfur (S), magnesium (Mg), manganese (Mn) and zinc (Zn). We tested how body stoichiometry was influenced by the species habitat, trophic guild and taxonomic order, and each pair of nutrients was tested for correlation. Our results show that body size is the primary driver of the excretion rates of nitrogen (N) and phosphorus (P) and, contrary to our expectations, temperature, N:P body stoichiometry and diet were not statistically significant in our study. However, further experiments should try to include measurements of ingestion and egestion rates to increase understanding on how diet and body stoichiometry affects nutrient excretion. Through our meta-analysis, we saw that temperature increase leads to an increase in fish nutrient excretion, but the magnitude of the effect is extremely variable between studies. The acclimatization time and feeding status together answer for 22% of this variation, however most of it is still unexplained and it is most likely related to the species identity and their physiological characteristics. More experiments using standardized methods are needed to better explain what can influence the temperature effect on nutrient excretion. Finally, we found that elements with similar functions were correlated with each other, especially calcium and phosphorus, due to their role in bone formation. Tropical fish presented higher variation in their body composition, which could be due to a more specialized stoichiometric niche or to a bigger sample size (12 tropical species versus 7 temperate species), and was mostly driven by differences in Ca and P content. Fish body composition differed between taxonomic orders, highlighting the influence of phylogeny. Fish body stoichiometry also differed between trophic guilds, but mostly driven by armored fish species.

Key words: Ecological stoichiometry. Metabolic ecology. Animals. Nitrogen. Phosphorus. Ionome. Temperature. Freshwater.

LISTA DE FIGURAS

LISTA DE TABELAS

LISTA DE ABREVIATURAS E SIGLAS

ASFA -   Aquatic Science and Fisheries Abstracts

CND -   Consumer- driven nutrient dynamics

ES -   Ecological Stoichiometry

INMET -   Instituto Nacional de Meteorologia

MTE -   Metabolic Theory of Ecology

REGUA -   Reserva Ecológica de Guapiaçu

SUMÁRIO

INTRODUÇÃO

Nos últimos anos, o interesse da comunidade científica a respeito da influência dos animais na ciclagem de nutrientes em ecossistemas aquáticos aumentou consideravelmente (Atkinson et. al 2016). Estes consumidores participam do processo de ciclagem de nutrientes de diversas maneiras. A forma direta é através da ingestão, eliminação e transporte de nutrientes entre habitats, e a forma indireta é alterando a abundância de presas e a produção, distribuição e biomassa dos produtores primários (Vanni 2002)

Atuando de forma direta na ciclagem de nutrientes, os consumidores obtêm os nutrientes necessários para seu crescimento, reprodução e atividades metabólicas a partir de sua dieta e eliminam o restante através da excreção de urina ou egestão de fezes (Sterner and Elser 2002). Os nutrientes excretados encontram-se na forma dissolvida e podem ser prontamente absorvidos pelos produtores primários, enquanto os nutrientes egestados encontram-se na forma orgânica e precisam ser mineralizados por microorganismos para se tornarem disponíveis para absorção pelos produtores primários (Allan and Castillo 2008).

Diversos estudos demonstram que a excreção por consumidores é uma fonte significativa de nutrientes em ambientes aquáticos (Benstead et al. 2010, McIntyre et al. 2007, Vanni et al. 2002). No entanto, há uma grande variabilidade entre espécies e ecossistemas no que diz respeito aos fatores que afetam a excreção e sua importância para o sistema, e as causas desta variabilidade precisam ser melhor esclarecidas (Atkinson et al. 2016).

Dentre os fatores que podem afetar as taxas de excreção de um consumidor estão o seu tamanho corporal (Alves et al. 2009, Hall et al. 2007), sua estequiometria corporal (Sterner and Elser 2002, Vanni et al. 2002), sua dieta (Pilati and Vanni 2007, Elser and Urabe 1999) e a temperatura do ambiente (Brown et al. 2004, Vanni 2002). Adicionalmente, o impacto da excreção de consumidores para o ecossistema também pode ser afetado de acordo com os hábitos de vida dos consumidores (diurno, noturno, em grupos ou solitário) (Oliveira-Cunha et al. 2018,Capps and Flecker 2013) pela presença de predadores (Guariento et al. 2018, Leroux and Schmitz 2015, Leroux et al. 2012.), a estrutura de tamanhos corporais da população ou comunidade (Fritschie and Olden 2016), a abundância (Benstead et al. 2010, El-Sabaawi et al. 2015) e diversidade de espécies (Capps et al. 2015, McIntyre et al. 2007.) e fatores abióticos como luz (El-Sabaawi et al. 2015, Moslemi et al. 2012), concentração de nutrientes da água (Spooner et al. 2013), profundidade e correnteza (McIntyre et al. 2008).

Devido à influência de tantos fatores sobre a excreção de nutrientes de consumidores, o papel destes na ciclagem de nutrientes do ecossistema torna-se dependente do contexto (Atkinson et al. 2016). Este fato representa um desafio ainda maior a um dos grandes dilemas em Ecologia, que é a busca por padrões que permitam generalizar processos ecológicos entre táxons e ecossistemas.

Por isso, para compreender melhor o funcionamento do ecossistema é necessário investigar que aspectos bióticos, abióticos e suas interações, influenciam a excreção de nutrientes de consumidores aquáticos, seu impacto no ecossistema e como isto acontece.

OBJETIVOS

O objetivo deste trabalho é investigar a influência das características dos consumidores e do ambiente sobre a excreção de nutrientes de peixes. Especificamente:

1) Testar a influência da dieta, da estequiometria corporal, do tamanho corporal dos indivíduos e da temperatura nas taxas de excreção de nitrogênio e fósforo de peixes para verificar qual teoria ecológica tem maior poder preditivo em relação às taxas de excreção: Teoria da Estequiometria Ecológica ou Teoria Metabólica da Ecologia.

2) Avaliar o tamanho do efeito da temperatura nas taxas de excreção de nutrientes de peixes de água doce através de uma meta-análise.

3) Testar o efeito da taxonomia e da dieta na composição corporal de peixes de água doce de ambientes tropicais e temperados.

1 BODY SIZE IS THE MAIN DETERMINANT OF NUTRIENT EXCRETION: EXPLORING METABOLIC THEORY AND STOICHIOMETRIC PARADIGMS IN A TROPICAL FRESHWATER FISH COMMUNITY.

Abstract

Discussions of the factors regulating nutrient excretion by consumers have focused on predictions from Ecological Stoichiometry (ES) and the Metabolic Theory of Ecology (MTE). ES posits that imbalances between the composition of the diet and the animal's body tissues should determine its nutrient excretion. Whereas, MTE states that metabolism, mostly influenced by body size and temperature, are the primary controls on nutrient excretion rates. Each framework has been supported by data, but they are rarely tested together. In this study, we measured excretion rates of nitrogen (NH_4) and phosphorus (SRP), body N:P stoichiometry, body size, and temperature for 12 species of fish from an Atlantic rainforest stream in Brazil (3 of these species are armored fish species with boney plate structures). We fitted 8 separate models reflecting different combinations of ES (body N:P, armor classification, and feeding group) and MTE (body size, temperature) variables. For both N and P excretion, only body size was included in the best model. Size scaling coefficients were lower than the MTE prediction of 0.75 for N (average coefficient=0.59, 95% CI = 0.45,0.73), and presented a relatively high variation between species. As for P, the size scaling coefficient of 0.75 was within the limits of the 95% confidence interval (average coefficient=0.56, 95% CI = 0.40, 0.77), and did not vary much between species.Contrary to expectations, excretion rates were not significantly influenced by diet (feeding group), body N:P or temperature. We also found, the armor classification performed better than body N:P. Therefore, we conclude that the ES framework has relatively little explanatory power on nutrient excretion compared to the scaling of metabolism with body size.

Key words: ecological stoichiometry, metabolic ecology, animals, nitrogen, phosphorus, biogeochemistry, freshwater.

Introduction

One of the challenges in ecological studies is finding patterns that allow for the generalization of ecological processes between species, taxa and ecosystems, and thus creating theories. When it comes to nutrient cycling, Ecological Stoichiometry (ES) and the Metabolic Theory of Ecology (MTE) are two ecological frameworks that try to answer the question: which factors affect the excretion rates of nutrients by consumers?

The ES is based on the premise that consumers are homeostatic, which means that they do not change their body composition according to their diet (Sterner and Elser 2002, Vanni 2002). Therefore, ES assumes that the ratios of excreted elements by a consumer depends on the imbalances between the ratios of elements present in their diet and body composition (Sterner and Elser 2002). This way, individuals with a nutrient rich diet show higher excretion rates compared to individuals with a nutrient poor diet. For example, individuals with a high N:P diet should excrete more N compared to individuals with a low N:P diet. Similarly, individuals with a high demand for a specific nutrient will present low excretion rates for this nutrient, for instance, vertebrate organisms that have a high phosphorus demand to build their bone skeleton (Lovell 1934, Vanni 2002, Hood et al. 2005).

The MTE states that metabolism determines all ecological processes, from individuals to ecosystems (Brown et al. 2004, Schramski et al. 2015). Metabolic rate is the rate at which an organism processes energy and materials. It determines the amount of food an animal ingests, the speed at which it converts energy and materials into other forms, their allocation into growth and reproduction and their elimination back to the environment (Brown et al. 2004). It is considered the most fundamental biological rate governing almost all biological activities. According to this theory, an organism's metabolic rate is affected by itsbody size and ambient temperature (Brown et al. 2004). The relation between metabolism and body size is a power function with a scaling coefficient of ¾, which means that for every 4 orders of magnitude increase in body size, metabolic rates increases by 3 orders of magnitude (West etal. 1997, Brown et al. 2004). Many studies show an allometric relation between an animal's body size and its nutrient excretion rate, which means that smaller animals excrete proportionately more nutrients compared to bigger animals (Alves et al., 2009; Hall et al., 2007; Vanni, 2002). Possibly, this allometric relation between nutrient excretion rate and body size is related to the individual's metabolism (Brown et

al. 2004). Some studies tested if the relation between body size and nutrient excretion rates were also, determined by a ¾ factor and found contrasting results. Allgeier et al. (2015) found evidence of a ¾ factor for N and P excretion of fish and macroinvertebrates in marine ecosystems, whereas Vanni and McIntyre (2016) found that the scaling was smaller than ¾ for both nutrients' excretion of invertebrates, fish, amphibians and turtles from marine and freshwater ecosystems. As for temperature, studies demonstrate that this is an important abiotic variable, because it affects the rates of metabolic reactions and physiological processes (Burel et al. 1996, Brown et al. 2004). According to MTE, an increase in temperature means an increase in metabolism and, therefore, an increase in nutrient excretion rates.

Both theories (ES and MTE) are based on basic principles of science, but ES concerns chemical elements and MTE concerns energy (Allgeier et al., 2015). Therefore, from the ES point of view, the drivers of nutrient excretion rates of consumers are diet and body stoichiometry, whereas, for MTE the drivers are body size and temperature.

Few studies have tried to explain the role of consumers in nutrient cycling by comparing ES and MTE in communities with high richness of species (McIntyre et al. 2007, Allgeier et al. 2015). Allgeier et al. (2015) analyzed 50 families of marine fish and invertebrates and Vanni and McIntyre (2016) analyzed 50 families of marine and freshwater fish, invertebrates, amphibians and turtles. Both studies concluded that MTE has a better predictive power of nutrient excretion rates than ES. In both cases, body size was more important than diet and body stoichiometry. However, in these studies, body size variation is significantly bigger than body stoichiometry variation (Barneche & Allen, 2015). As a consequence, it is possible that by reducing body size variation, by focusing on one taxonomic group for example, the predictive power between ES and MTE will not be so different. This way, both theories can be efficient in explaining nutrient excretion by consumers.

Several researchers have been defending the integration of both theories to enhance comprehension on which factors can affect consumer's nutrient excretion rates (Allen and Gillooly 2009, Allgeier et al. 2015, Vanni and McIntyre 2016). Despite that, few studies investigate both theories jointly. With that in mind, our goal is to compare the predictive power of ES and MTE on the nutrient excretion rates of a fish community, with relatively little body size variation (body size ranged from 0.02 to 22.0g in our study, 0.04 to 2,597g in Allgeier et al.

2015, and 1 ug to 500g in Vanni and McIntyre 2016), by testing models that consider the variables of both theories' (diet, body stoichiometry, body size and temperature) combined and separated.

We chose to study the fish community of an Atlantic Forest stream, because fish can act as a significant source of nutrients, since they are the most nutrient rich organisms in these ecosystems (Vanni 2002, McIntyre et al. 2008), they can transport nutrients between habitats(Glaholt and Vanni 2005, Vanni 2002), are abundant in many streams and present high variation in their body composition (McIntyre and Flecker 2010, Pough et al. 2005). For these reasons, they represent a good model for nutrient cycling studies. Also, in mountain Atlantic Forest streams there is a great annual temperature variation, with cold water during the winter and much warmer water during the summer season. Moreover, the fact that all species belong to the same ecological community and were exposed to the same methodological procedures and environmental conditions (i.e., temperature, water flow, depth, background nutrient concentration) allow us to eliminate potential noise in our results and to focus on the variables of interest.

Also, among our 12 studied species, 3 are armored catfish. This means that their bodies are covered by boney plates and, consequently, they present higher body P (up to approximately 3 times higher in our dataset) compared with non-armored catfish. Because this group differentiates from the rest in terms of body stoichiometry, we expect to find differences in their nutrient excretion rates. Moreover, classifying between armored and non-armored catfish could provide a useful model for estimating more precise nutrient excretion in cases where body composition data arenot available.

We expect that the model with best predictive power will include variables from both ES and MTE theories. We believe fish from higher trophic positions will excrete more nutrients (both N and P) than fish from lower trophic positions due to their nutrient rich diet (e.g. piscivore fish should excrete more nutrients than detritivore fish) of a given body size. We expect armored catfish species will excrete less P than the other fish species from the same size, due to their higher P demand for building their boney armor. We predict that temperature will show a positive relation with nutrient excretion, due to its effect on metabolism. And we expect to find an

allometric relation between body size and nutrient excretion with a scaling coefficient of approximately 0.75, in accordance with MTE.

Materials and Methods

Study site and species

      The study was conducted at Rio Guapiaçu, (22°26'08.1"S, 42°45'34.2"W), a fourth order stream located in the hydrographic complex Guapiaçu-Macacu, inside the Reserva Ecológica de Guapiaçu (REGUA), in Cachoeiras de Macacu, RJ, Brazil. The hydrographic complex c supplies water to approximately 2.5 million people in five cities (Helder1999). Here, we worked with the dominant fish assembly. In all, we sampled 12 species, 8 families and 4 orders of fish (Table 1) and estimated their absolute excretion rates ($\mu g\ L^{-1}$) of nitrogen, phosphorus and molar N:P.

Table 1- List of species, their feeding groups, body size measures (as estimated dry weight) and if they show a boney armor.

Order	Family	Species	Average body size (g)	Body size range (g)	Feeding group	Armored fish
Siluriformes	Callichtyidae	*Scleromystax barbatus*	0.91	0.31 – 2.05	Omnivore	Yes
Siluriformes	Heptopteridae	*Pimelodella lateristriga*	1.40	0.18 – 4.16	Invertivore	No
Siluriformes	Heptopteridae	*Rhamdia quelen*	2.59	0.19 – 22.01	Piscivore	No
Siluriformes	Heptoteridae	*Acentronichthys leptos*	0.42	0.39 - 0.45	Invertivore	No
Siluriformes	Loricariidae	*Ancistrus multyinis*	2.59	0.93 – 6.62	Detritivore	Yes
Siluriformes	Loricariidae	*Rineloricaria sp*	1.69	0.16 – 7.48	Omnivore	Yes
Siluriformes	Trichomicteridae	*Trichomycterus sp*	0.97	0.26 – 2.19	Invertivore	No
Characiformes	Characidae	*Bryconamericus ornaticeps*	1.48	0.14 – 5.30	Invertivore	No
Characiformes	Characidae	*Mimagoniates microlepis*	0.19	0.03 – 0.49	Invertivore	No
Characiformes	Crenuchidae	*Characidium vidali*	0.44	0.13 – 0.84	Invertívoro	No

Ciprinodontiformes	Poecilidae	*Phalloceros harpagos*	0.11	0.02 – 0.22	Omnivore	No
Synbranchiformes	Synbranchidae	*Synbranchus marmoratus*	2.19	0.42 – 10.01	Piscivore	No

Information on the feeding groups (detritivore, omnivore, invertivore and piscivore) was obtained on published literature (Mazzoni et al. 2010, Menezes et al. 2007, Oyakawa et al. 2006, Fogaca et al. 2003) and Fishbase (www.fishbase.se).

Nutrient recycling trials

Fish were collected through electrofishing (Smith Root, LR-24 Backpack Electrofisher) and left in the holding device inside the river for approximately 15 minutes to acclimatize. Then, the fish were placed individually in ziploc bags or transparent plastic boxes (Figure1)with a known volume of stream water, previously filtered with a net (200 μm)to remove solid particles. The volume of water varied from 400 ml a 5000 ml according to animal size. Plastic bags or boxes were placed at the river margins to preserve temperature and minimize animal's stress.

Figure 1- Fish incubation at Rio Guapiaçu.

After 60 minutes, water samples were collected from inside the incubations using a 60 ml syringe and samples were filtered with a glass filter (GF/F, 25mm, Whatman) contained in a filter holder (Swinnex, Millipore). Water samples from the stream were collected on all days of the experiment to serve as control. All samples were kept frozen until analysis in the laboratory. The majority of animals captured had their standard lengths (cm) measured and were released back

into the stream. The fish wet mass was estimated through a length-mass regression calculated for each species using data from previous collections. Then, we converted the wet mass to dry mass using a conversion factor of 0.23, which is themean conversion factor obtained from wet-dry mass regressions using some of the species in this study and others. Three to five individuals of each species were collected for measurements of wet and dry weight (g) and body stoichiometry (%N, %P, %C).Water temperature was measured once a day with a thermometer on some days of the experiment. For the days in which we were unable to measure temperature directly, we estimated it through a simple linear regression between air and water temperature (more details in the Supplementary Material). We understand that not having the direct measuring of temperature for each incubation can represent a source of error, however we believe our temperature estimatesrepresent the range of temperatures that naturally occur in this system. The experiment was conducted in three seasons (summer, fall and winter), during day (9am to 4pm) and night (8pm to 12am), between 2016 and 2018. Temperatures varied from 9.9 to 25.7 ºC (details on dates, time of day and temperatures on Table S1 of the Supplementary Material).

Nutrient analysis

Nitrogen was analyzed as ammonium (NH_4^+-N) using fluorimetric methods by Holmes et al. (1999) modified by Taylor et al. (2007). All fluorimetric measurements were made with a hand-help fluorimeter (Aquafluor, Turner Designs, Inc., Sunnyvale, CA, USA). Phosphorus was analyzed as soluble reactive (PO_4^{3-}-P) using Molybdenum Blue method by Gotherman et al. (1978). All colorimetric analyses were conducted on FIA- Flow Injection Analysis (Lachat Instruments Div.Zellweger Analytics, Inc., Milwaukee, WI, USA).

Statistical analyses

To compare the predictive power on nutrient excretion rates between ES and MTE paradigms, we tested models considering all variables from both theories (Model 1), their combinations (Models 4, 5, 6 and 7) and variables from just one of the theories (2, 3 and 8) (Table 2). We also tested the classification as Armored Fish instead of Body N:P (Models 1b, 2b, 4b and 6b) considering that, in cases where there was no information on body composition, this classification could be used instead.

Our predictor variables were: $\log_{10}$ body size (g dry mass), temperature (ºC), body N:P (molar), Armor classification (presence of boney armor or not) and feeding group (as a proxy for diet). We used linear mixing models with species as a random factor, with random slope and intercept.

Table 2- Models used to compare the predictive power of ES and MTE paradigms on fish excretion rates. Below each variable we indicate which theory it is referring to.

Model	$\log_{10}$ Body size (g) MTE	Temperature (ºC) MTE	Body N:P ES	Armor ES	Feeding group ES
1.ES + MTE	x	x	x	-	x
1b. ES + MTE	x	x	-	x	x
2. ES	-	-	x	-	x
2b. ES	-	-	-	x	x
3.MTE	x	x	-	-	-
4. ES + MTE	-	x	x	-	-
4b. ES + MTE	-	x	-	x	-
5. ES + MTE	-	x	-	-	x
6. ES + MTE	x	-	x	-	-
6b. ES + MTE	x	-	-	x	-
7. ES+ MTE	x	-	-	-	x
8. MTE	x	-	-	-	-

For model selection we used the AICc (Akaike Criterion for small sample sizes) as criterion. The model with the lowest AICc value was considered the best model to explain nutrient excretion by our fish species, however models with ΔAICc<2 are also valid models. If models presented similar AICc, we chose the one with the fewest variables to maximize its application in cases in which there were few available data. Models were generated using the package lme4 (Bates et al., 2015) and lmerTest (Kuznetsova et al., 2017) in statistical program R 3.6.3 (R Core Team, 2020).

Results

The model that best explained N, P and N:P excretion rates of fish was the simplest one, which considers only body size as a predicting variable (Model 8, Table 3).Conversely, a significant effect (p<0.05) of body size was observed for N (R^2=0.57) and P excretion (R^2=0.37), but not N:P (Table 4, Figure2).The full MTE model, including both body size and temperature (Model 3),performed poorly compared to the model with body size alone (Model 8, Table 3).

The second best model for explaining excretion rates of N, P and N:P (ΔAICc<2) used body size and the Armor classification (Model 6b) combining MTE and ES variables (Table 3), however, the Armor classification did not have a significant effect in excretion rates (p>0.05). The full MTE model (Model 3) performed far better than the full ES model (Models 2 and 2b) (Table 3).

Table 3- Comparisons among statistical models that estimate N, P and N:P excretion of fish through the predicting variables of the Ecological Stoichiometry (ES), Metabolic Theory of Ecology (MTE) and both (ES +MTE) paradigms.

Model	Variables	N Excretion			P Excretion			N:P Excretion		
		df	AICc	ΔAICc	df	AICc	ΔAICc	df	AICc	ΔAICc
1. ES +MTE	Body size, temperature, diet and Body N:P	17	128	17	17	168	23	17	191	26
1b. ES+ MTE	Body size, temperature, diet and Armor	14	120	10	14	158	12	14	182	18
2.ES	Diet and Body N:P	9	236	126	9	237	92	9	210	45
2b. ES	Diet and Armor	7	226	116	7	226	80	7	205	41
3.MTE	Temperature and Body size	10	116	6	10	154	9	10	172	7
4. ES +MTE	Temperature and Body N:P	10	240	130	10	235	89	10	210	46
4b. ES+ MTE	Temperature and Armor	7	233	123	7	227	82	7	202	38
5. ES +MTE	Temperature and diet	9	235	125	9	232	86	9	208	43
6. ES +MTE	Body size and Body N:P	10	116	6	10	155	9	10	173	8
6b. ES+ MTE	Body size and Armor	7	111	1	7	147	2	7	167	2
7. ES +MTE	Body size and diet	9	114	3	9	148	3	9	171	6
8.MTE	**Body size**	**6**	**110**	**0**	**6**	**145**	**0**	**6**	**165**	**0**

Legend: Models for N, P and N:P excretion including species as a random factor. Highlighted, the model chosen by the parsimonious principle (AICc).

Table 4- Statistical details for the selected model for N, P and N:Pin excreta.The * indicates a significative relationship between body size and nutrient excretion rate.

	Fixed effects	Estimate	SE	df	t-value	p
N excretion	Intercept	1.982	0.047	8.716	42.000	<0.001
	Log $_{10}$Body size (g)	0.597	0.053	8.223	11.230	<0.001*
P excretion	Intercept	0.768	0.055	14.151	14.087	<0.001
	Log $_{10}$Body size (g)	0.563	0.079	11.200	7.113	<0.001*
N:P excretion	Intercept	1.576	0.075	11.936	21.100	<0.001
	Log $_{10}$Body size (g)	-0.004	0.101	31.149	-0.040	0.968

Figure 2- Relationship between body size and excretion rates of N (a) and P (b) of the 12 studied species (symbols of different colors represent different species). Data are $\log_{10}$ -transformed.
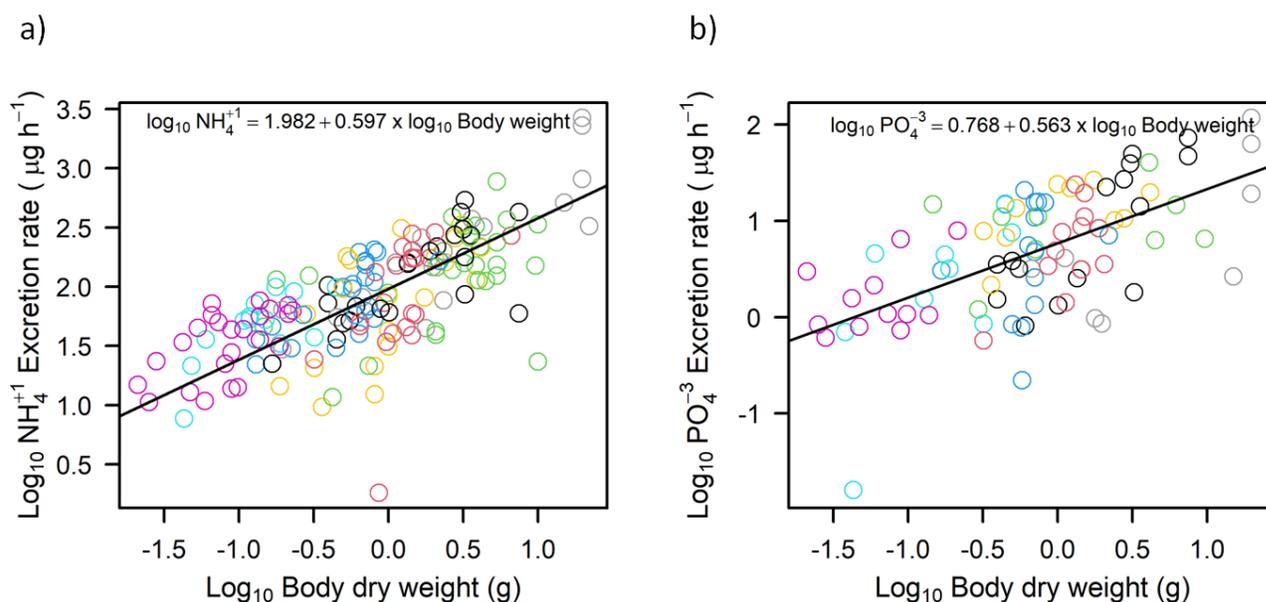
The models using the classification as Armored or Non- Armored fish (Models 1b, 2b, 4b and 6b) performed better in comparison to models using Body NP (1, 2, 4 and 6). Armored and non-armored fish significantly differed in their body composition (Figure 3). However, our results show that theydo not show any significant difference in their excretion rates for N and P (Figure 4).

Figure 3- Body composition of P (a) and N:P (b) of armored and non-armored fish.
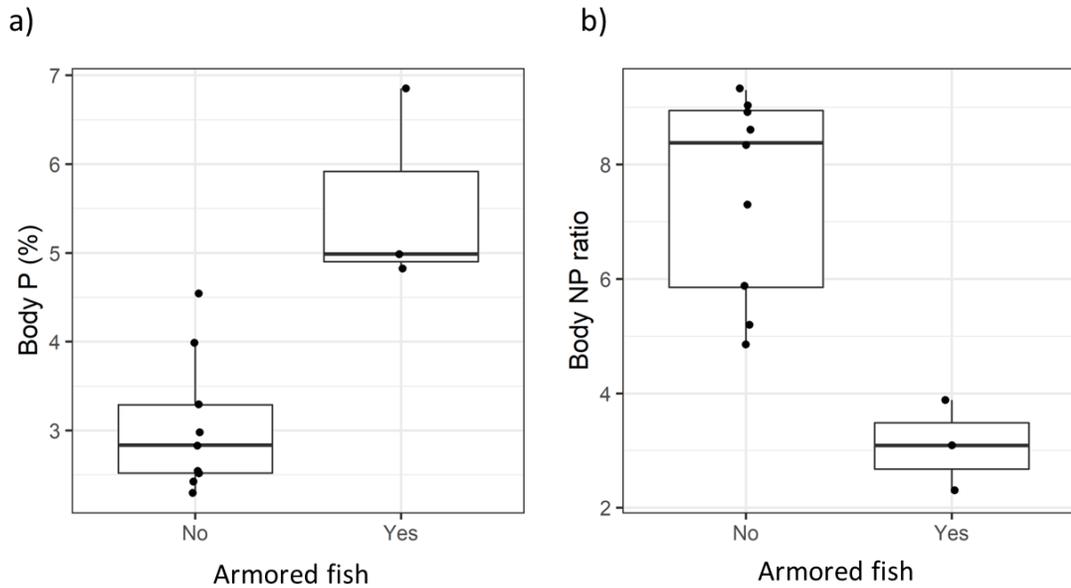
Figure 4- Excretion rates of N (a) and P (b) of armored and non-armored fish.
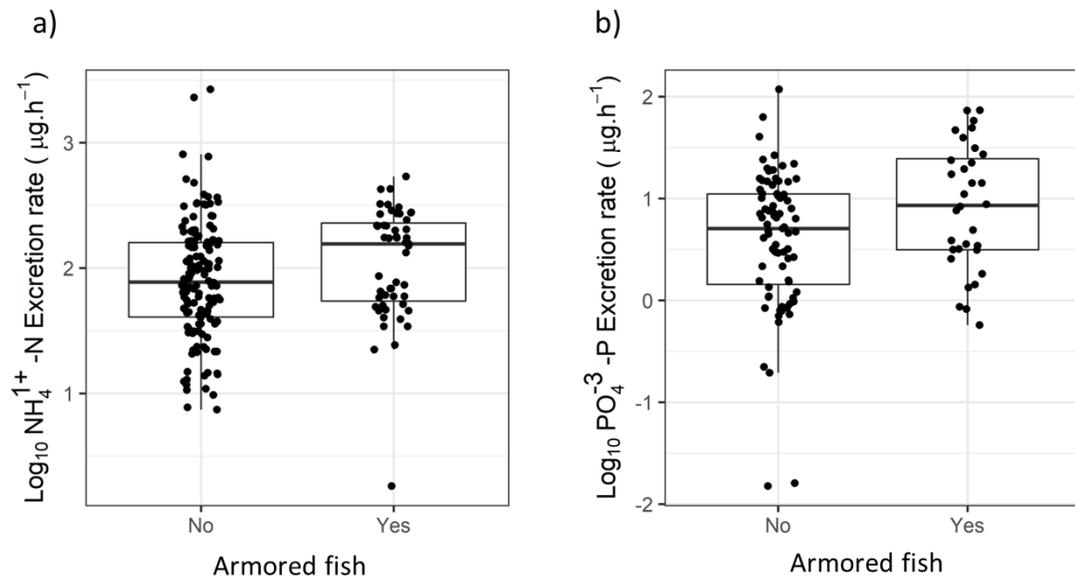
The scaling coefficients between excretion rates and body size were less than 0.75 for both N and P, and varied from 0.57 to 0.64 for N excretion (LLCI95%=0.45, ULCI95%=0.73) and from 0.55 to 0.57 for P excretion (LLCI95%=0.40, ULCI95%=0.77) among species (Table 5). The overall correlation coefficients (marginal R^2) of the regression analysis was 0.57 for N and

0.37 for P. When the correlation coefficients were corrected for fish species (conditional R^2) the coefficient for N increased to 0.64, indicating that differences between species explained some of the variation. On the other hand, the conditional R^2 for P was not different from the marginal R^2, which indicates that differences between species did not contribute to the relationship between P excretion and body size.

Table 5- Intercept and coefficient values from the relation between body size (g) and the excretion of N and P of all fish species.

Species	N excretion		P excretion	
	Intercept	Log_{10}Body size (g)	Intercept	Log_{10}Body size (g)
Acentronichthys leptos	2.00	0.59	0.77	0.56
Ancistrus multispinis	1.92	0.61	0.77	0.56
Bryconamericus ornaticeps	2.06	0.57	0.77	0.56
Characidium vidali	2.06	0.59	0.77	0.56
Mimagoniates microlepis	2.11	0.57	0.77	0.57
Phalloceros harpagos	2.11	0.57	0.77	0.56
Pimelodella lateristriga	1.89	0.62	0.77	0.57
Rhamdia quelen	2.04	0.60	0.76	0.55
Rineloricaria sp	1.99	0.60	0.77	0.57
Scleromistax barbatus	1.88	0.62	0.77	0.56
Synbranchus marmoratus	1.72	0.64	0.77	0.56
Trichomycterus SP	2.00	0.59	0.77	0.56

Discussion

Metabolic Theory of Ecology (MTE) and Ecological Stoichiometry (ES) are two common frameworks used independently to predict energy and nutrient budgets at various biological levels of organization (Sterner and Elser 2002, Brown et al. 2004). Here, we investigate how body size, temperature, diet and body composition affect nutrient excretion rates of a fish community, using both theories together to explain patterns in nutrient recycling. We found that body size had a strong influence on nutrient excretion of N and P, but we did not observe an effect of temperature on nutrient excretion. Also, the scaling coefficients for the relation between N excretion and body size were lower than the 0.75 coefficient predicted by MTE, but for P the

95% confidence interval encompassed the 0.75 value. The ES variables (diet, body N:P, "armor") were outperformed by body size, which indicates that the ES framework has relatively little predictable effect on nutrient excretion compared to therole of body size.

Similarlyto previous studies, our results show that body size affects the excretion of N and P by fish(Allgeier et al. 2015, Fritschie and Olden 2016, Schindler and Eby 1997, Vanni et al. 2002). Bigger fish excreted more nutrients per capita when compared to smaller fish, however they excreted less nutrients per mass. This result was expected since MTE states that there is an allometric relation between metabolism and body size, determined by a ¾ factor, (Brown et al. 2004). Therefore, according to the theory, the relation between nutrient excretion rates and body size should also have a scaling coefficient of around 0.75. In fact, Allgeier et al. (2015) found evidence of the ¾ factor regulating the relation between body size and nutrient excretion rates using data from marine fish and invertebrates. But in our study, the scaling coefficient for N differed from the theory, being on average 0.59, showing that the relation between nutrient excretion rates and body size is closer to a ½ factor than ¾. Our results are similar to Vanni and McIntyre (2016), who also found scaling coefficients lower than 0.75 for N and P (0.68 and 0.56, respectively). Biologically, these results mean that animals are excreting nutrients at a slower rate than would be expected. The reasons for these lower than expected coefficients are uncertain, since the uptake of nutrients is driven by metabolic rates and it is reasonable to believe the release of nutrients would be as well (Vanni and McIntyre 2016).

Delong et al. (2010) compared the scaling coefficients of body size and metabolism between prokaryotes, unicellular eukaryotes and metazoans. They found that the exponent goes from approximately 1 to ¾ with increasing body size, elucidating the metabolic rate dependence on I) the number of membrane-bound sites where ATP synthesis and proton pumping occur, and II) constraints of resource supply and vascular systems. Although MTE refers to metabolism instead of excretion, it is logical to assume the theory also applies to the case because many biological rates show ¾ power-scaling most likely due to metabolism (e.g individual development rate) (Vanni and McIntyre 2016). Therefore, it could be that our lower than ¾ power-scaling is related to fish growth and ingestion rates or to ontogenetic diet shifts.

Despite having a good range of temperature variation during experiments (from 9.9 to 25.7ºC), we saw that temperature did not affect nutrient excretion rates of fish, as expected according to MTE and seen in other studies (Tatrai 1986, Tsui et al. 2002, Zakes et al. 2007, Liu

et al. 2009, Currie et al. 2010, Vanni and McIntyre 2016,). This is a surprising result since fish are poikilotherms, which means their body temperature is determined by the external temperature of the water they inhabit and should have direct influence on metabolic rates, feeding rates and activity levels (Dockray et al. 1996). It could be that in the tropics, as the rate of temperature change is usually slow (Allgeier et al. 2015, Oliveira-Cunha et al 2018found a $1°$ C variation in 24h), fish can acclimate and perform metabolic compensation (Dockray et al. 1996). Consequently, because of fish acclimation, we see no apparent changes in nutrient excretion rates.

As for the effects of ES variables, diet and body stoichiometry, our results revealed counterintuitive patterns. Many studies have demonstrated that diet can directly influence fish nutrient excretion (Pilati and Vanni 2007, Moody et al. 2015). The nutritional quality of the diet of aquatic consumers progressively increases from detritivores, to omnivores, to invertivores and, finally, to piscivores (Cross et al. 2005). Therefore, according to ES, it is expected that piscivores present the highest nutrient excretion rates compared to detritivores, for example. However, similarly to other studies (Allgeier et al. 2015, Vanni and McIntyre 2016), our results do not reflect this pattern. Vanni and McIntyre (2016) attribute the lack of a diet effect to the absence of information on growth,ingestion and egestion, whereas Allgeier et al. (2015) questions how useful diet really is for predicting nutrient excretion rates. To be sure, we need future studies to investigate the importance of diet by measuring growth, ingestion, excretion and egestion rates across a range of feeding and taxonomic groups.

As expected, armored catfish species presented up to 3x more P in their body composition than the other fish species. Therefore, according to ES predictions, it was expected that they would excrete less P because of their higher P demand for building their boney plates. However, armored and non-armored fish did not differ in their P excretion rates. This could be because we sampled adult individuals, with bone skeletons already formed and taking up P just for their tissue maintenance, especially for their soft tissue. Perhaps if we had sampled individuals in different life stages we would see growing individuals with a higher P demand and consequent low P excretion. Surprisingly, differentiating between armored and non-armored fish proved to be more efficient than using actual data on body NP for our model selection. This classification seems to represent body elemental composition for our dataset, however, there is a need to test for a larger number of species. Classifying as armored and non-armored fish could be an

informative factor in lieu of direct body composition data, since armored fish present significantly higher body P than other species.

This work was built on the previous studies of Vanni and McIntyre (2016) and Algeier et. al (2015), where the authors combined MTE and ES to predict animals' nutrient excretion rates and found that body size was the variable with the strongest influence over nutrient excretion. However, in both cases, the range of body sizes (Vanni and McIntyre 2016: 1µg to 500g dry mass, Allgeier et al 2015: 0.04 to 2.597g) was much larger compared to the range of body NP. This fact led us to wonder if the dataset could be generating a bias for the MTE variables, and that perhaps by limiting the dataset to a single taxon and, consequently, reducing body size range (0.021 to 22.01g dry mass), the outcome could be different. Therefore, our model selection with 12 species dataset offers a strong test of the predictability of nutrient excretion without bias for body size. From our study, we can see that even with a smaller range of body size compared to body NP, body size is still the key variable driving N and P excretion.

Conceptual integration of MTE and ES has provided important insights that can be fundamental to understanding the mechanisms guiding nutrient cycling. Our evaluation leads to the conclusion that body size can explain far more variance in nutrient excretion rates than temperature, body stoichiometry and diet. Nonetheless, ES predictors should be further investigated in conjunction with growth, ingestion and egestion rates from the same organisms. This way, we will gain better comprehension on the mechanisms governing nutrient budgets of animals. Moreover, while our models provide useful estimates of nutrient excretion, collecting field data is imperative when very accurate measures of excretion are needed for a given species or ecosystem.

Supplementary Material

Details on our stream temperature estimates

The weather station in REGUA, the reserve where our site in Rio Guapiaçu is located, was out of order during our experiments, therefore we do not have air temperature measurements. To obtain an equation to estimate the air temperature in REGUA, we plotted the air temperature from a close weather station (located in Nova Friburgo) in the x axis, with the air temperature data from the weather station in REGUA in the y axis (Figure S1).Air temperature data for the weather station in Nova Friburgo was obtained from INMET (Instituto Nacional de Meteorologia).

We obtained the following equation: y= 1.1589x + 3.8237.

Where:

y= Air temperature in REGUA

x= Air temperature in Friburgo

Figure S1- Relationship between the air temperature at REGUA and in Friburgo.
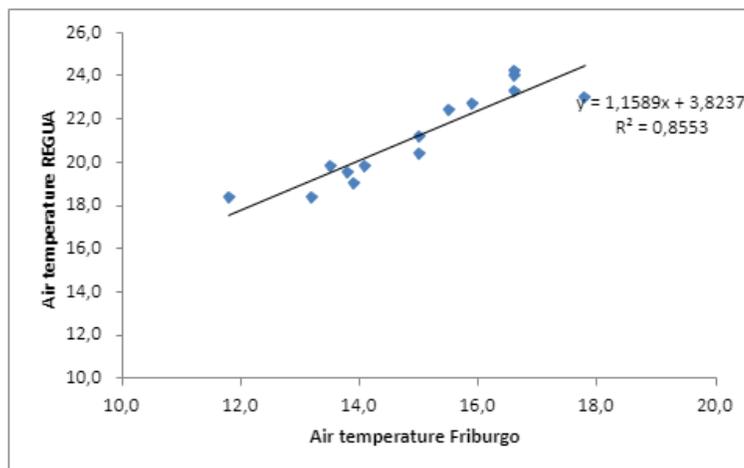
Then, we plotted the estimated air temperature in REGUA in the x axis with the water temperature we measured in the stream in the y axis (Figure S2). This way, we obtained an equation that allows the estimation of water temperature for the days we did not measure it.

We obtained the following equation: y= 1.1719x – 8.0599.

Where:

y= Water temperature in Rio Guapiaçu

x= Air temperature in REGUA

Figure S2- Graph showing the relation between the estimated air temperature in REGUA and the water temperature in Rio Guapiaçu.
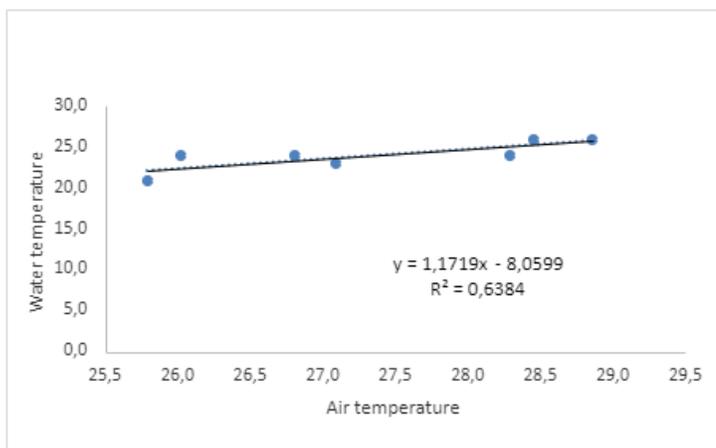
Table S1- Date, season of the year, average, maximum and minimum estimated water temperature during nutrient recycling trials.

Date	Season	Time of day	Average temperature (ºC)	Minimum and maximum temperature (ºC)
August 2016	Winter	Day	22.2	22.2
December 2016	Summer	Day	25.5	25.3 – 25.7
January 2017	Summer	Day	23.7	22.4 – 25.1
February 2017	Summer	Day	23.3	23.3
April 2018	Fall	Night	24.3	24.3
May 2018	Fall	Night	15.6	9.9 – 21.7

2 TEMPERATURE INFLUENCE ON NUTRIENT EXCRETION RATES OF FISH: A META-ANALYSIS.

Abstract

The Metabolic Theory of Ecology posits that temperature is a critical environmental variable for ecosystem processes, since metabolic rates of organisms increase exponentially with temperature, as do consequently, the rates of nutrient uptake, allocation to growth and reproduction, excretion and other ecological processes. Future predictions show that temperature will increase globally over the next years, therefore it is imperative to understand how temperature influences other biological rates, such as nutrient excretion rates. Here, we review, quantify and synthesize the effects of temperature on nutrient excretion rates of freshwater fish through a meta-analysis using 15 scientific papers and 40 individual effect sizes. Fish were used as our model organisms since the fish community can have an important effect on ecosystem function through their excretion of nutrients. Overall, our results show that fish excretion rates increase with temperature, but there is considerable variability between studies, which could be partly due to methodological differences. We analyzed the influence of the range of temperature tested, the acclimatization time and the fish being fed or starved before excretion measurements. At first, none of the moderators tested explained the variability between studies (SMD= 0.90, I^2=89%). However, after excluding two studies as a sensitivity test, we found that the acclimatization time and the fish feeding status (starved or fed) influenced how excretion was affected by temperature increase (SMD=0.87, I^2=83%). Studies where fish were exposed to longer periods of acclimatization to new temperatures showed lower differences in excretion rates between the lowest and highest temperature, and starved fish showed higher excretion rates than fish that were fed before the experiment. However, a considerable part of the variability among studies remains unexplained. To develop a more comprehensive understanding, future studies should investigate further the effects of temperature on nutrient excretion rates of fish, among species and across ecosystems, using standardized methods. At last, we suggest potential directions for future studies.

Key words: nutrient cycling, consumer-driven nutrient dynamics, warming, metabolic theory, nitrogen.

Introduction

The Metabolic Theory of Ecology (MTE) is a common framework applied to consumer-driven nutrient dynamics (CND) studies (Atkinson et al. 2016, Vanni and McIntyre 2016). Consumers take up nutrients through their diets, transform them to other forms, allocate them to their growth, reproduction and survival, and excrete the excess nutrients back to the environment (Vanni 2002). According to MTE, the pace in which all these events happen is determined by the consumer's metabolism (Brown et al. 2004). This theory states that individual metabolic rates are the fundamental factor governing all ecological processes, from individuals to the biosphere, and that individual metabolic rates are influenced by the organism's body size and by temperature (Brown et al. 2004).

Metabolism consists of a series of complex biochemical reactions that are catalyzed by enzymes. Temperature affects the rates of these biochemical reactions by altering the energy of molecular motion. Within the range of temperature for normal activity of each organism (between 0 to 40°C for most organisms), experimental studies show that enzymatic activity increases with temperature until an optimum activity temperature(Maffucci et al. 2020). This is because the increase in temperature increases the proportion of molecules with sufficient kinetic energy, speeding the process of achieving the activation energy necessary for the biochemical reactions of metabolism (Brown et al. 2004). Above the organism's normal operating temperature, enzymatic activity drops abruptly due to protein denaturation, and thus loss of function (Maffucci et al. 2020, Craig et al. 1996).Therefore, temperature is a critical environmental variable for ecosystem processes such as nutrient cycling, since it can influence organism metabolism and, consequently, animal nutrient excretion rates. Although developed to explain metabolic rates, MTE has been successfully applied to describe the relationship between animal nutrient excretion rates with body size and temperature (Morgan and Hicks 2013, Algeier et al. 2015).

Consumers can have an important role in the nutrient cycling of aquatic ecosystems (Allgeier et al. 2017, Vanni 2002). Several studies have shown that animal excretion can supply significant amounts of nutrients to their environment (Small et al. 2011, Atkinson et al. 2016). In recent years, studies on CND have dramatically increased, with the majority of studies focusing on fish (Atkinson et al. 2016). Fish have a considerable importance in modulating nutrient

cycling as they are the most nutrient rich taxa in aquatic environments (Vanni 2002, McIntyre et al. 2008) and can act as nutrient transporters between habitats and ecosystems due to their mobility (Glaholt and Vanni 2005). They also demonstrate high variability in their nutrient excretion rates between species (McIntyre and Flecker 2010, Pough et al. 2005).

However, the relative importance of animals' excretion to their environment is context dependent, which means that it can be influenced by both biotic and abiotic factors (Atkinson et al. 2016). Studies testing the effect of temperature on fish nutrient excretion have found contrasting results, where excretion increases with temperature, but also where excretion is unaffected by temperature increase (Currie et al. 2010, Perry et al. 2010, Dockray et al. 1996). These results could be due to using a short range of temperatures or related to a slow rate of temperature change, that in both cases would allow for a fast acclimation and no apparent changes to excretion. The allometric scaling between body size and consumer nutrient excretion rate has been well- documented, where smaller animals excrete proportionally more nutrients than bigger animals, although the scaling coefficient can be lower than the 0.75 predicted by MTE (Allgeier et al. 2015, Vanni and McIntyre 2016). Also, animals excrete more nutrients during their period of activity, when they are actively feeding (Oliveira-Cunha et al. 2018, Capps and Flecker 2013). Whiles et al. (2009) demonstrated the importance of feeding status by incubating fish that kept receiving food throughout the experiment and fish that were deprived of food for 4 hours and measuring their nitrogen excretion after 0, 1, 2 and 4h. They saw that fish nutrient excretion rates declined significantly with time, partly due to fasting.

There are many factors that can influence consumer nutrient excretion and warrant further investigation, but temperature is a critical one since it is changing rapidly all over the world, and it is also neglected in most excretion experiments. Over the past century, global temperature increased approximately $0.6^{\circ}C$ mostly due to an increase in carbon dioxide (CO_2) levels and other greenhouse gases in the atmosphere (IPCC 2001). Future predictions indicate that there will be a significant additional warming by the next century, which will undoubtedly affect aquatic ecosystems (Allan et al. 2005).For that reason, comprehending the effects of temperature on animal excretion of nutrients will help to understand the consequences of global warming to nutrient cycling. Freshwater ecosystems should be especially affected since they are smaller and more sensitive to dial, seasonal and annual temperatures, and consequently, freshwater

consumers excretion should vary more in comparison to saltwater consumers. Moreover, investigating the impact of global warming for freshwater ecosystems is crucial for the development of adequate management intervention and measures of conservation.

Here, we review, synthesize and conduct a meta-analysis using published literature data that will contribute to our understanding of the effect of temperature on nutrient excretion rates of freshwater fish. Next, to better comprehend the importance of temperature to nutrient cycling of freshwater ecosystems, we suggest possible directions for future studies.

Methods

Database

We conducted a meta-analysis of the published literature concerning the influence of temperature on freshwater fish excretion rates, from1960 until September 2017. We used the PICO method (Population, Intervention, Comparator, Outcome) (Higgins et al. 2019) for formulating our question: What is the effect of temperature on nutrient excretion rates of freshwater fish? With nutrient excretion rates of freshwater fish as the Population, higher temperatures as the Intervention, lower temperatures as the Comparator and the outcome being: fish nutrient excretion rates will be higher under higher temperatures.

We chose ASFA (Aquatic Science and Fisheries Abstracts) as our online database to conduct our search, since it covers the world's literature on science and conservation of freshwater environments. We found 1.511 records by using the following keywords in our search: (warming OR temperature) AND fish* AND freshwater AND (excretion OR excretion rate*), filtering the results for the English language and for Scholarly Journals.

We selected our references by using three inclusion criteria: 1) the study had to be experimental, 2) it had to study at least one species of freshwater fish and 3) it had to measure nitrogen and/or phosphorus excretion under different temperatures. Using the criteria above, we reached a total of 27 publications, but because of missing information on experimental or statistical details (e.g. sample size or type of error used), a total of 15 publications were included in the meta-analysis and all of them measured excretion rates of nitrogen only. In total, we quantified 40 individual effect sizes.

For each selected paper, we extracted information on 1) species identity, 2) body size measures, 3) acclimatization period, 4) acclimatization temperature, 5) feeding status (fed or starved), 6) control temperature, 7) control sample size, 8) control mean excretion rate and its respective standard deviation, 9) experimental temperature, 10) experimental sample size, 11) experimental mean excretion rate and its respective standard deviation, and 12) the rate of temperature change. To extract information from the paper's figures or graphs, we used the online program Web Plot Digitizer. We considered the lowest temperature as control and the highest as experimental temperature. The effect size represents the magnitude by which temperature increase affected fish nutrient excretion rates and it was calculated as Standardized Mean Difference (SMD) or Hedge's d, by the difference in excretion between the control and experimental treatments. The effect size was weighted by study precision. We used the variance inverse as the precision measure for the effect size.

Statistical analyses

We conducted a meta-analysis by running random effects models and mixed effects models using meta and metafor packages (Balduzzi et al. 2019, Viechtbauer 2010)in R software. The effect size was the dependent variable and the delta temperature (difference between the control and experimental temperature), body size (g), acclimatization time and feeding status (starved or fed) were the predictors. The overall effect size was estimated by fitting the models without predictors.

Results

Characterizing published literature

The studies included in this review were conducted in Europe (40%), North America (33.3%), Asia (20%) and Oceania (6.6%). The majority of them was published since 1991 (Figure 5), mostly in journals that focused on fisheries and aquaculture (46.6%; e.g. Aquaculture Research, Canadian Journal of Fisheries and Aquatic Sciences) and a few that focused on ecological aspects (13.3%; e.g. Environmental Biology of Fishes, Journal of Fish Biology). Our data show a decline in the number of studies during the last decade (Figure 5), which is curious since the consequences of global warming have been widely debated in this period.

All experiments were conducted under controlled laboratory conditions, with fish captured from lake ecosystems (60%) or obtained from aquariums (40%). Nitrogen (N) was measured as NH_3 (ammonia), NH_4 (ammonium) or urea. The range of temperatures tested varied from5 to 35°C. The information concerning the place where the fish were obtained, studied species, natural climate occurrence, range of temperatures tested and if the study found that temperature influenced excretion is summarized in Table1.

The studies reviewed encompassed 4 orders, 10 families and 15 species of fish (Table 6). Most species were adapted to Temperate climate (46.6%), followed by Subtropical climate (33.3%) and Tropical climate (20%) (information obtained from Fishbase). Many of the species studied present commercial value for the food industry and are exploited and widely commercialized, such as *Lepomis macrochirus* (Bluegill), *Esox lucius* (Northern pike), *Salvelinus alpinus* (Artic charr)and *Oncorhynchus mykiss* (Rainbow trout)(Kyle and Wilson 2007). In all studies, the experiments were conducted within temperature ranges in which the species occur in nature.

Figure 5- Number of papers published through decades, since 1961 until 2017.
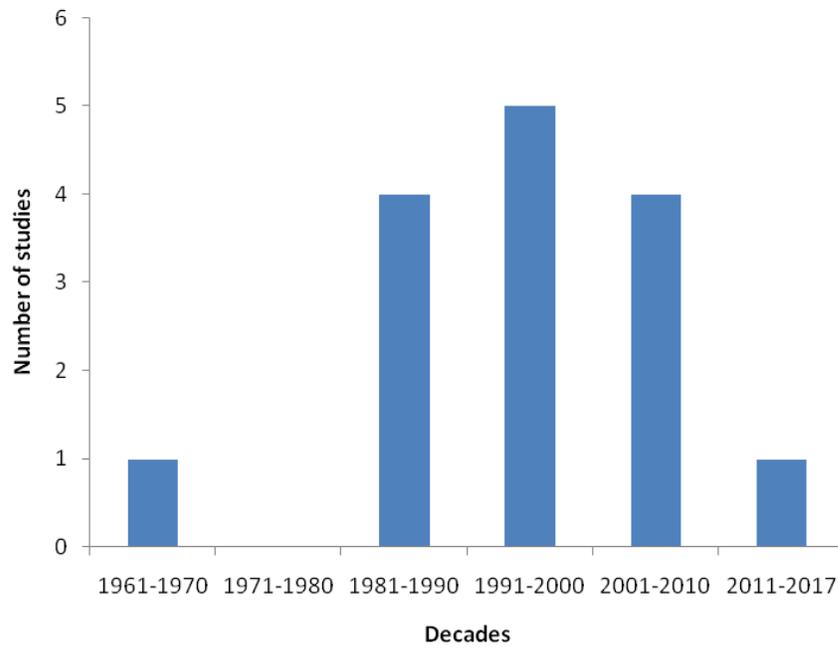
Table 6- Information on the fish order, family, genus and species, natural climate occurrence, place where the fish were obtained, experimental temperature range and if the study found that temperature influenced excretion or not. The reference for each study can be found in the Appendix.

Study number	Fish obtained from	Order	Family	Genus and species	Climate occurrence	Range of temperature tested (^{o}C)	Tempera influenc excretio
1	Lake	Perciformes	Centrarchidae	*Lepomis macrochirus*	Subtropical: 1- 36°C	7.2 - 32.2	Yes
2	Aquarium	Perciformes	Cichlidae	*Tilapia mossambica*	Tropical: 17 - 35°C	30 - 35	No
3	Lake	Cypriniformes	Cyprinidae	*Abramis brama L.*	Temperate: 10- 24°C	10 - 20	No
4	Lake	Cypriniformes	Cyprinidae	*Phoxinus phoximus*	Temperate: 2 - 20°C	5 - 15	Yes
5	Aquarium	Perciformes	Channidae	*Channa punctatus*	Tropical: 22 - 28°C	16 - 32	Yes
6	Lake	Perciformes	Percidae	*Stizostedion vitreum*	Temperate: ? - 29°C	20 - 25	Yes
7	Aquarium	Perciformes	Cichlidae	*Oreochromis nilotica*	Tropical: 14 - 33°C	16 - 33	Yes
8	Aquarium	Salmoniformes	Salmonidae	*Oncorhynchus mykiss*	Subtropical: 10- 24°C	13 - 26	Yes
9	Lake	Perciformes	Terapontidae	*Bidyanus bidyanus*	Subtropical: 10-30°C	25 - 30	Yes
10	Lake	Salmoniformes	Salmonidae	*Salvelinus alpinus*	Temperate: 4 - 16°C	11 - 17	Yes
11	Aquarium	Esociformes	Esocidae	*Esox lucius*	Subtropical: 10 - 28°C	20 - 24	No
12	Aquarium	Perciformes	Moronidae	*Palmetto bass* (*Morone saxatilis x M. chrysops*)	Temperate: ?	18 - 28	Yes

13	Lake	Esociformes	Umbridae	*Umbra limi*	Temperate: 17- 22°C	13 - 31	Yes
14	Lake	Cypriniformes	Cyprinidae	*Carassius auratus*	Subtropical: ?- 41°C	7 - 25	No
15	Lake	Perciformes	Percidae	*Sander lucioperca*	Temperate: 6 - 22°C	13 - 25	No

Temperature effect on freshwater fish excretion

We found that the overall effect size increased with temperature, but it presented high variability between studies (SMD=0.90, I^2=89%) and none of the moderators tested (delta temperature, body size, acclimatization time and feeding status) explained the heterogeneity (Table 7).

Table 7- Overall effect size as Standardized Mean Difference (SMD), which is the difference in excretion rate between the lowest and highest temperature. The Total refers to the sample size, the Mean is the mean excretion rate (μmol N g^{-1}h^{-1}), Standard Deviation as SD, 95% Confidence Interval, and Weight as the weight of each study.

Study	Experimental			Control			Standardised Mean Difference	SMD	95%-CI	Weight
	Total	Mean	SD	Total	Mean	SD				
McKenzie 1996	8	0.12	0.0498	8	0.09	0.0382		0.64	[-0.37; 1.65]	2.6%
McKenzie 1996	8	0.26	0.1114	8	0.12	0.0498		1.53	[0.38; 2.69]	2.3%
Savitz 1969	40	0.34	0.2155	20	0.17	0.1482		0.86	[0.30; 1.42]	3.6%
Savitz 1969	40	0.34	0.2155	14	0.14	0.3164		0.81	[0.18; 1.43]	3.4%
Savitz 1969	15	0.85	0.3766	40	0.34	0.2155		1.88	[1.18; 2.57]	3.3%
Savitz 1969	40	0.36	0.3231	20	0.18	0.1793		0.63	[0.08; 1.17]	3.6%
Savitz 1969	40	0.36	0.3231	14	0.17	0.4973		0.50	[-0.12; 1.12]	3.5%
Savitz 1969	15	0.87	0.2007	40	0.36	0.3231		1.70	[1.02; 2.38]	3.3%
Lyytikaeinen and Jobling 1999	590	0.59	0.7224	590	0.42	0.7224		0.24	[0.12; 0.35]	4.3%
Lyytikaeinen and Jobling 1999	590	0.62	0.7224	590	0.42	0.7224		0.28	[0.16; 0.39]	4.3%
Tatrai 1986	12	0.03	0.0341	10	0.02	0.0049		0.38	[-0.47; 1.23]	2.9%
Tatrai 1986	16	0.04	0.0394	12	0.03	0.0341		0.26	[-0.49; 1.01]	3.1%
Ramaballav and Baran 1990	6	0.52	0.1810	6	0.41	0.1917		0.54	[-0.62; 1.71]	2.3%
Ramaballav and Baran 1990	6	0.88	0.1769	6	0.52	0.1810		1.86	[0.41; 3.31]	1.8%
Ramaballav and Baran 1990	6	0.59	0.1873	6	0.22	0.1777		1.87	[0.42; 3.32]	1.8%
Ramaballav and Baran 1990	6	1.01	0.1962	6	0.59	0.1873		2.02	[0.52; 3.52]	1.7%
Cai and Summerfelt 1992	90	2.07	2.7086	90	1.57	2.7086		0.18	[-0.11; 0.48]	4.1%
Cai and Summerfelt 1992	15	1.78	0.5529	15	1.36	0.5529		0.74	[0.00; 1.48]	3.2%
Cai and Summerfelt 1992	13	0.86	0.2574	13	1.00	0.2574		-0.53	[-1.31; 0.26]	3.1%
Cai and Summerfelt 1992	9	1.36	0.2141	9	1.21	0.2141		0.67	[-0.29; 1.62]	2.7%
Mohamed 1982	16	599.57	77.0878	15	463.95	16.5867		2.33	[1.39; 3.27]	2.7%
Cui and Wooton 1988	5	1.13	0.2128	5	0.15	0.0086		5.88	[2.34; 9.41]	0.5%
Cui and Wooton 1988	3	0.21	0.0489	5	1.13	0.2128		-4.54	[-7.98; -1.11]	0.5%
Cui and Wooton 1988	5	0.24	0.0818	5	1.13	0.2128		-4.99	[-8.06; -1.92]	0.6%
Dockray 1996	142	1.01	7.9840	152	1.35	5.9178		-0.05	[-0.28; 0.18]	4.2%
Dockray 1996	142	1.06	1.1916	152	1.35	5.9178		-0.07	[-0.30; 0.16]	4.2%
Dockray 1996	142	1.62	2.5024	152	1.35	5.9178		0.06	[-0.17; 0.29]	4.2%
Kibria 1998	24	0.73	0.1960	24	0.14	0.0490		4.06	[3.04; 5.08]	2.6%
Zakes 2007	3	0.49	0.0001	3	0.41	0.0742		1.22	[-0.77; 3.21]	1.2%
Zakes 2007	3	0.28	0.0493	3	0.25	0.0029		0.69	[-1.05; 2.42]	1.5%
Zakes 2007	3	0.12	0.0143	3	0.11	0.0014		0.79	[-0.98; 2.56]	1.4%
Liu 2009	20	1.16	0.0157	20	0.75	0.0186		23.35	[17.92; 28.77]	0.2%
Liu 2009	20	1.68	0.0186	20	1.16	0.0157		29.61	[22.75; 36.48]	0.1%
Currie 2010	8	0.46	0.1131	8	0.33	0.1414		0.96	[-0.09; 2.01]	2.5%
Currie 2010	8	0.46	0.1131	8	0.25	0.0849		1.99	[0.73; 3.25]	2.1%
Currie 2010	7	0.65	0.3439	8	0.46	0.1131		0.72	[-0.34; 1.78]	2.5%
Currie 2010	7	0.85	0.3175	8	0.46	0.1131		1.59	[0.38; 2.80]	2.2%
Perry 2010	6	0.29	0.0735	6	0.19	0.0490		1.48	[0.14; 2.82]	2.0%
Frisk 2013	6	0.13	0.0245	6	0.06	0.0490		1.67	[0.27; 3.06]	1.9%
Frisk 2013	5	0.14	0.1118	6	0.13	0.0245		0.12	[-1.07; 1.31]	2.2%
Random effects model	2140			2126				0.90	[0.65; 1.16]	100.0%

Heterogeneity: I^2 = 89%, τ^2 = 0.3966, $p < 0.01$

-20 -10 0 10 20 30 40
Standardized mean diference (g)

However, after we performed a sensitivity test by excluding two studies whose effect sizes were standing out (Liu et al. 2009, Cui and Wooton 1988), we found that acclimatization time (Figure 6, Table 8) and feeding status (Table 9) influenced the magnitude of temperature change on fish excretion, although both factors explained little of the variance. For analyzing

feeding status, we could not consider the study from Cai and Summerfelt (1992) because they do not specify the animal's feeding status.

Acclimatization time was negatively correlated with the effect of temperature increase in excretion rates (Figure 6, Table 8), with longer acclimatization periods resulting in lower differences in excretion with temperature change (I^2=81%, T^2=0.21). Feeding status also influenced how temperature affected excretion rates, with fish that were starved prior to excretion measurements presenting higher excretion rates (SMD=1.33, 95% CI=0.97; 1.70) than fish that were fed (SMD=0.63, 95% CI=0.39; 0.87). Moreover, the starved fish group presented lower heterogeneity (I^2= 24%) compared to the fed fish group (I^2=87%), but the variance between studies were similar (T^2=0.15 for the starved group, T^2=0.11 for the fed fish group). Delta temperature and body size did not have a significant influence on the effect size (p>0.05).

Figure 6- Negative correlation between the effect size and acclimatization time.
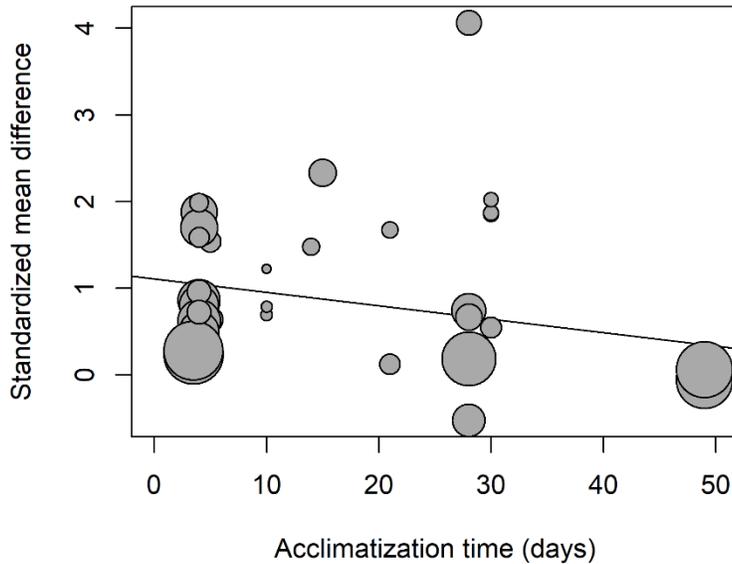
Legend: Graph showing the Standardized Mean Difference in relation to the acclimatization time. Each circle represents a study and the size of the circle is proportional to the study weight in the overall effect size.

Table 8- Acclimatization time model results.

	Estimate	SE	Z value	P value	Lower 95% CI	Upper 95% CI
Intercept	1.1063	0.1640	6.7469	<.0001	0.7849	1.4277
Acclimatization time	-0.0155	0.0066	-2.3606	0.0182	-0.0284	-0.0026

Table 9- Effect size as Standardized Mean Difference (SMD), which is the difference in excretion rate between the lowest and highest temperature, separated between studies that measured excretion of starved individuals and studies that used fed individuals. The Total refers to the sample size, the Mean is the mean excretion rate (µmol N g^{-1}h^{-1}), Standard Deviation as SD, 95% Confidence Interval, and Weight as the weight of each study.

Study	Experimental			Control			Standardised Mean Difference	SMD	95%-CI	Weight
	Total	Mean	SD	Total	Mean	SD				
Fed										
Savitz 1969	40	0.34	0.2155	20	0.17	0.1482		0.86	[0.30; 1.42]	4.5%
Savitz 1969	40	0.34	0.2155	14	0.14	0.3164		0.81	[0.18; 1.43]	4.2%
Savitz 1969	15	0.85	0.3766	40	0.34	0.2155		1.88	[1.18; 2.57]	3.9%
Savitz 1969	40	0.36	0.3231	20	0.18	0.1793		0.63	[0.08; 1.17]	4.6%
Savitz 1969	40	0.36	0.3231	14	0.17	0.4973		0.50	[−0.12; 1.12]	4.3%
Savitz 1969	15	0.87	0.2007	40	0.36	0.3231		1.70	[1.02; 2.38]	4.0%
Lyytikaeinen and Jobling 1999	590	0.59	0.7224	590	0.42	0.7224		0.24	[0.12; 0.35]	6.2%
Lyytikaeinen and Jobling 1999	590	0.62	0.7224	590	0.42	0.7224		0.28	[0.16; 0.39]	6.2%
Tatrai 1986	12	0.03	0.0341	10	0.02	0.0049		0.38	[−0.47; 1.23]	3.3%
Tatrai 1986	16	0.04	0.0394	12	0.03	0.0341		0.26	[−0.49; 1.01]	3.7%
Dockray 1996	142	1.01	7.9840	152	1.35	5.9178		−0.05	[−0.28; 0.18]	5.9%
Dockray 1996	142	1.06	1.1916	152	1.35	5.9178		−0.07	[−0.30; 0.16]	5.9%
Dockray 1996	142	1.62	2.5024	152	1.35	5.9178		0.06	[−0.17; 0.29]	5.9%
Kibria 1998	24	0.73	0.1960	24	0.14	0.0490		4.06	[3.04; 5.08]	2.7%
Zakes 2007	3	0.49	0.0001	3	0.41	0.0742		1.22	[−0.77; 3.21]	1.1%
Zakes 2007	3	0.28	0.0493	3	0.25	0.0029		0.69	[−1.05; 2.42]	1.3%
Zakes 2007	3	0.12	0.0143	3	0.11	0.0014		0.79	[−0.98; 2.56]	1.3%
Random effects model	**1857**			**1839**				**0.63**	**[0.39; 0.87]**	**68.9%**
Heterogeneity: $I^2 = 87\%$, $\tau^2 = 0.1553$, $p < 0.01$										
Starved										
McKenzie 1996	8	0.12	0.0498	8	0.09	0.0382		0.64	[−0.37; 1.65]	2.8%
McKenzie 1996	8	0.26	0.1114	8	0.12	0.0498		1.53	[0.38; 2.69]	2.4%
Ramaballav and Baran 1990	6	0.52	0.1810	6	0.41	0.1917		0.54	[−0.62; 1.71]	2.3%
Ramaballav and Baran 1990	6	0.88	0.1769	6	0.52	0.1810		1.86	[0.41; 3.31]	1.7%
Ramaballav and Baran 1990	6	0.59	0.1873	6	0.22	0.1777		1.87	[0.42; 3.32]	1.7%
Ramaballav and Baran 1990	6	1.01	0.1962	6	0.59	0.1873		2.02	[0.52; 3.52]	1.6%
Mohamed 1982	16	599.57	77.0878	15	463.95	16.5867		2.33	[1.39; 3.27]	3.0%
Currie 2010	8	0.46	0.1131	8	0.33	0.1414		0.96	[−0.09; 2.01]	2.6%
Currie 2010	8	0.46	0.1131	8	0.25	0.0849		1.99	[0.73; 3.25]	2.1%
Currie 2010	7	0.65	0.3439	8	0.46	0.1131		0.72	[−0.34; 1.78]	2.6%
Currie 2010	7	0.85	0.3175	8	0.46	0.1131		1.59	[0.38; 2.80]	2.2%
Perry 2010	6	0.29	0.0735	6	0.19	0.0490		1.48	[0.14; 2.82]	1.9%
Frisk 2013	6	0.13	0.0245	6	0.06	0.0490		1.67	[0.27; 3.06]	1.8%
Frisk 2013	5	0.14	0.1118	6	0.13	0.0245		0.12	[−1.07; 1.31]	2.3%
Random effects model	**103**			**105**				**1.33**	**[0.97; 1.70]**	**31.1%**
Heterogeneity: $I^2 = 24\%$, $\tau^2 = 0.1174$, $p = 0.19$										
Random effects model	**1960**			**1944**				**0.87**	**[0.65; 1.10]**	**100.0%**

Heterogeneity: $I^2 = 83\%$, $\tau^2 = 0.2073$, $p < 0.01$
Test for subgroup differences: $\chi_1^2 = 9.77$, df = 1 ($p < 0.01$)

−4   −2   0   2   4   6
Standardized mean diference (g)

We tested all moderators together in the same model and only feeding status was statistically significant (Table 10). After taking Delta temperature and Body size from the model, feeding status and acclimatization time were statistically significant. The model with all four moderators explained 9% of the heterogeneity (Figure 7a), but when we consider only the

statistically significant variables (acclimatization time and feeding status), the explained heterogeneity rose to 22% (Figure 7b).

Table 10- Model with all four moderators tested together. The moderators are acclimatization time (days), feeding status (starved or fed), delta temperature (difference between the control and experimental temperature) and body size (g).

	Estimate	SE	Z value	P value	Lower 95% CI	Upper 95% CI
Intercept	0.9912	0.3323	2.9829	0.0029	0.3399	1.6425
Acclimatization time	-0.017	0.0066	-1.7575	0.0788	-0.0247	0.0013
Feeding status	0.6762	0.2681	2.5225	0.0117*	0.1508	1.2015
Delta temperature	-0.0085	0.0326	-0.2610	0.7941	-0.0724	0.0554
Body size	-0.0005	0.0006	-0.8363	0.4030	-0.0018	0.0007

Figure 7- Within and between studies variability and percentage of variance explained by the model a) with all four moderators; and b) with the statistically significant moderators.
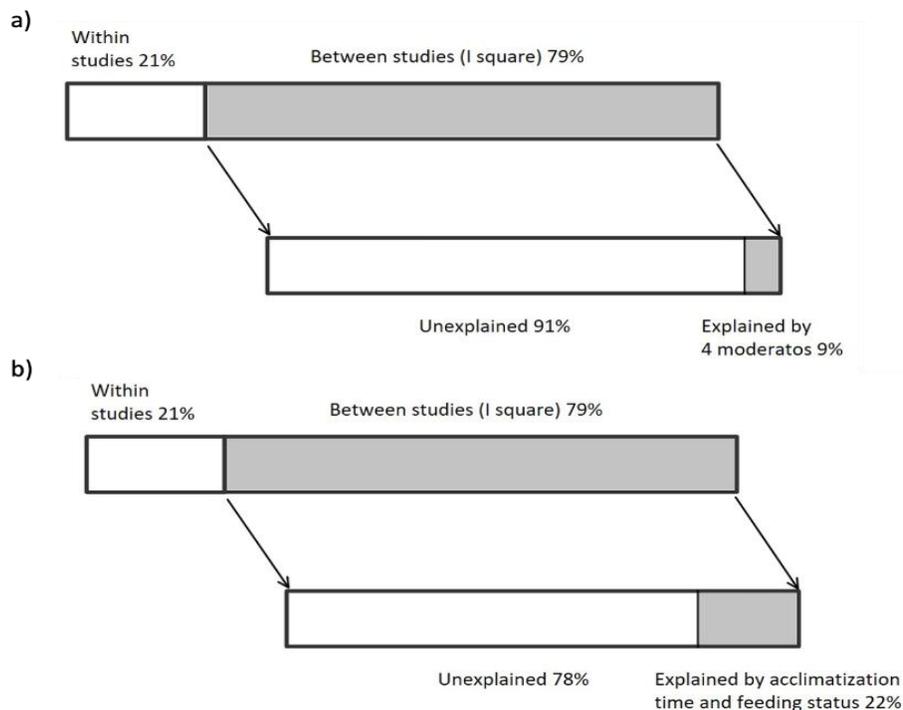
Discussion

There is a common sense that increases in temperature lead to increases in biological rates since the Arrhenius equation was established in 1889. However, when it comes to excretion rates, many studies do not see this pattern for reasons that are not well understood. In our meta-analysis, we found that freshwater fish nutrient excretion rates increased with increasing temperature. Yet, the extent to which temperature can influence fish excretion rates presents high variability.We have presented two sets of results, one with our complete dataset for which none of the moderators tested explained the substantial heterogeneity, and the other without two studies (Liu et al. 2009; Cui and Wooton 1988), as a sensitivity test, where feeding status and acclimatization time together answered for 22% of heterogeneity.In both cases, our study shows that the mechanisms controlling an animal's response to temperature increase are not well understood, evidencing the need for more studies on the subject with standardized methodology, across species and ecosystems.

After excluding the two studies that were standing out in our dataset (Liu et al. 2009; Cui and Wooton 1988), we found that the heterogeneity was partially explained by methodological differences between studies, such as different acclimatization periods of time and feeding status (fish being starved or fed during the experiment).The acclimatization time used in the studies we analyzed ranged from three days to seven weeks. Studies that applied longer periods of acclimatization presented lower differences in fish excretion with temperature increase (Figure 2). This result shows that fish are able to acclimate over time, which means adjusting their standard metabolic rate to the new temperature and, consequently, changes in nutrient excretion rates can be modest. In face of global warming, this fact could represent good news regarding the nutrient cycling process, since changes in temperature will occur gradually, allowing fish to acclimate and maintain their excretion at a rather similar rate. However, this acclimatization probably comes with a physiological cost that could affect the animal's fitness (growth, reproduction, survival), especially for tropical fish that are already living closer to their thermal limits compared to temperate fish.

Feeding status was also a factor influencing how fish excretion rates responded to temperature increase. Contrary to what we would expect, we saw that fish starved before experiments showed higher excretion rates (Table 4). This is surprising because studies that were not included in this meta-analysis, such as Oliveira-Cunha et al. (2018), Capps and Flecker (2013) and Whiles et al. (2009), have found that fish that are fasting usually show

lower excretion rates compared to those that have their stomachs full. However, our meta-analysis includes many more papers and show a different pattern. It could be that the combination of fasting and temperature increase caused the starved fish to be more stressed than the fish that were fed previously, and that this resulted in higher excretion rates for the starved fish group. Several studies show that stress from the risk of predation affects the nutrient excretion of animals, generally increasing it (Guariento et al. 2018). Animals' physiological response to stress leads to higher energy expenditure, which means that the prey use energy, as C, from their tissues, and to maintain their stoichiometric balance, the prey increase their nutrient excretion (Guariento et al. 2018). Therefore, it could be that the stress from fasting leads to a similar physiological response as in the stress from predation risk.We also saw higher variability in excretion among fish fed before the experiments (I^2=87%) than among starved fish (I^2=24%). This result is expected considering that you can offer a controlled diet, but consumption rate and food intake is difficult to estimate and to control. So, when you starve fish for hours, it is certain that they will empty their stomach with time. But, when you offer fish food, you cannot be sure they are all eating the same amount at the same rate. Therefore, it is more likely to see higher variability in excretion within fish from the fed group.

Moreover, we expected studies with the highest delta temperature to show the highest differences in excretion rates, but we saw no effect of the delta temperature on the effect size. It is possible that this was due to the studies testing gradual changesin temperature, allowing fish to acclimate, instead of abrupt changes. This way, the delta temperature effect could have been masked by the effect of acclimatization time. It is also known that body size can influence fish nutrient excretion rates, yet we saw that body size did not explain the differences in effect size, probably because we are using the mass-specific excretion rate to calculate the effect size of each study.

Our study shows that there are unknown factors influencing how temperature change affects nutrient excretion, which could be related to species identity and phylogeny. Uliano et al. (2010) tested the effect of changes in temperature for two phylogenetically distant fish species, *Gambusia affinis* and *Danio rerio,* under the same laboratory conditions and found opposing outcomes. For *Gambusia affinis*, declines in temperature showed no effect over total N excretion, demonstrating the species high tolerance to temperature stress. For *Danio rerio*, however, declines in temperature were followed by declines in NH_3 excretion and increases in urea excretion (Uliano et al 2010). The study suggests that the difference in the species

response to temperature increase could be related to the gills' histology, since *Gambusia affinis* presents a hypertrophy and hyperplasia of mitochondria-rich cells that are related to the regulation of urea transport, and such effect is not observed for *Danio rerio.*This study highlights the importance of species identity, showing that temperature may or may not influence nutrient excretion rates in some cases, for reasons that are not clear, but most likely related to physiological characteristics of each speciesTherefore, it is likely that the extent to which temperature can affect fish nutrient excretion may be contingent to the species.

Additionally, in our literature search, few studies measured P excretion and fewer extrapolated the effects of temperature on nutrient excretion of fish to the whole ecosystem. One study did both and found that gizzard shad's (*Dorosoma cepedianum*) P excretion was much more affected by temperature than N excretion, which led to low N:P excretion during the summer, due to higher temperatures (Schaus et al. 2010). It also found that, on a seasonal basis, P excretion by gizzard shad will probably have the biggest ecosystem impact during spring and autumn, due to reduced external loading (Schaus et al. 2010). These results stress the need for more comparative studies among excreted elements, to determine whether some elements are more affected by temperature than others, and more studies on the impact temperature can have for ecosystem functioning.

Conclusions and recommendations for future studies

Our studyhighlights the effect of temperature on nutrient excretion rates using freshwater fish as model organisms, but the magnitude of its effect is variable. Although there is a common sense that increases in temperature lead to increases in nutrient excretion rates, few studies have tested this relationship and our data show that the number of studies has decreased during recent years. The existing studies, present strong bias for fish populations originated from lake ecosystems and for N excretion. For untangling the role of temperature on nutrient excretion rates, more comparative and integrative studies are needed.

Future research should test the current assumptions of MTE on the effects of temperature on nutrient excretion rates of fish, by comparing different species and excreted elements, conducting experiments with species captured from distinct ecosystems. Fasting and handling stress can influence nutrient excretion rates of fish and must be taken into consideration when deciding the experimental design (Whiles et al. 2009, Oliveira-Cunha et al. 2018). For fish, fasting starts having an effect at approximately 40 minutes of incubation and handling stress can affect about the first 20 minutes (Whiles et al. 2009). Starving the fish

prior to excretion trials can underestimate their nutrient excretion rates. On the other hand, our data show that the heterogeneity is higher among fish that were fed prior to excretion measurements. Therefore, for the purpose of evaluating the effect of temperature increase in nutrient excretion, we recommend starving the fish for at least an hour before excretion measurements so that the effect of temperature is clearer. Also, we recommend that incubation trials last approximately 1 hour, which allows time for fish to recover from handling stress and it is also the incubation period that the majority of studies that measure fish excretion use. However, in excretion trials, there is not an optimal incubation period that applies to all living organisms and this should be a taxon specific decision. Also, laboratory experiments that test the effect of abrupt changes in temperature are not as interesting since, under natural conditions, abrupt changes in temperature are unlikely to occur and thus not very realistic. Therefore, we recommend that studies make gradual changes in temperature to investigate its effects on nutrient excretion.

Furthermore, future research should aim to extrapolate the effects of temperature on fish nutrient excretion to the whole ecosystem, coupling those effects with other abiotic factors, such as ambient nutrient concentrations, nutrient external loading, water flow and light availability. Therefore, to increase our understanding on when, where and how temperature influences fish excretion, more comparative and experimental studies among species, elements and across freshwater ecosystems are needed.

I. Appendix

Appendix 1: Publications selected from our literature search and used in our meta-analysis on the effects of temperature on nutrient excretion rates of freshwater fish.

Study number	Year	Authors	Title	Journal, volume, page numbers
1	1969	Savitz, J.	Effects of temperature and body weight on endogenous nitrogen excretion in the bluegill sunfish (*Lepomismacrochirus*).	Journal of the Fisheries Research Board of Canada **26**, 1813-1821
2	1982	Mohamed, M. P.	Metabolic rates and quotients in the cichlid fish, *Tilapia mossambica* (Peters) in relation to random activity.	Proceedings of the Indian Academy of Science (Animal Science) 91, 217-223.
3	1986	Tátrai, I.	Influence of temperature, rate of feeding and body weight on nitrogen metabolism of bream, *Abramis brama L.*	Comparative Biochemistry and Physiology 83A, 543-547.
4	1988	Cui, Y. and Wooton, R. J.	Bioenergetics of growth of a cyprinid, *Phoxinus phoxinus:* The effect of ration, temperature and body size on food consumption, faecal production and nitrogenous excretion.	Journal of Fish Biology 33, 431-443.
5	1990	Ramaballav, R. and Baran, D. A.	The effect of thermal adaptation on the level of nitrogenous excretion of *Channa punctatus* (Bloch).	Journal of Thermal Biology 15, 251-254.
6	1992	Cai, Y. and Summerfelt, R. C.	Effects of temperature and size on oxygen consumption and ammonia excretion by walleye.	Aquaculture, 104 127-138.
7	1996	McKenzie, D. J., Serrini, G., Piraccini, G., Bronzi, P. and Bolis, C.L.	Effects of diet on responses to exhaustive exercise in Nile tilapia (*Oreochromis nilotica*) acclimated to three different temperatures.	Comparative Biochemistry and Physiology 114A, No. I, 43-50.
8	1996	Dockray, J. J., Reid, S. D. and Wood, C. M.	Effects of elevated summer temperatures and reduced pH on metabolism and growth of juvenile rainbow trout (*Oncorhynchus mykiss*) on unlimited ration.	Canadian Journal of Fisheries and Aquatic Sciences 53, 2752–2763

9	1998	Kibria, G., Nugegoda, D., Fairclough, R. and Lam, P.	Effect of temperature on losses and retention of nitrogen by silver perch, *Bidyanus bidyanus* (Mitchell 1838) (Teraponidae) fed on artificial diets.	Journal of Aquaculture in the Tropics 13(3) 203-214.
10	1999	Lyytikaeinen, T. and Jobling, M.	Effects of thermal regime on energy and nitrogen budgets of an early juvenile Arctic charr, *Salvelinus alpinus*, from Lake Inari.	Environmental Biology of Fishes 54, 219-227.
11	2007	Zakés, Z., Szczepkowski, M., Demska-Zakés, K. and Jesiotowski, M.	Oxygen consumption and ammonia excretion by juvenile pike, *Esox lucius L.*	Archives of Polish Fisheries 15, 79-92.
12	2009	Liu, F., Yang, S. and Chen, H.	Effect of temperature, stocking density and fish size on the ammonia excretion in palmetto bass (*Morone saxatilis* x *M. chrysops*).	Aquaculture Research 40, 450-455.
13	2010	Currie, S., Bagatto, B., DeMille, M., Learner, A., LeBlanc, D., Marka, C., Ong, K., Parker, J., Templeman, N., Tuftd, B. L. and Wright, P. A.	Metabolism, nitrogen excretion, and heat shock proteins in the central mudminnow (*Umbra limi*), a facultative air-breathing fish living in a variable environment.	Canadian Jounal of Zoology 88, 43–58.
14	2010	Perry, S. F., Schwaiger, T., Kumai, Y., Tzaneva, V. and Braun, M. H.	The consequences of reversible gill remodelling on ammonia excretion in goldfish (*Carassius auratus*).	The Journal of Experimental Biology 213, 3656-3665.
15	2013	Frisk, M., Steffensen, J. F. and Skov, P. V.	The effects of temperature on specific dynamic actionand ammonia excretion in pikeperch (*Sanderlucioperca*).	Aquaculture 404-405, 65-70.

3 TAXONOMIC AFFILIATIONS AND ENVIRONMENT INLUENCE FISH BODY STOICHIOMETRY OF EIGHT ELEMENTS

Abstract

There are several biologically-relevant chemical elements, however most ecological stoichiometry studies are focused on C:N:P. This fact represents a knowledge gap, pressing the need for better understanding stoichiometric patterns across differentelements. In our study, nineteen species of tropical and temperate freshwater fish had their whole-body calcium (Ca), phosphorus (P), sodium (Na), potassium (K), sulfur (S), magnesium (Mg), manganese (Mn) and zinc (Zn) concentrations determined. Patterns in body stoichiometry were examined in relation to the species habitat, trophic guild and taxonomic order, and each pair of nutrients were tested for correlations. Overall, elements with similar functions were correlated with each other, specially calcium and phosphorus that showed the highest correlation of any pair in our dataset, due to their role in bone formation.Stoichiometric variation was greater among tropical fish, which could be due to a more specialized stoichiometric niche as a consequence of higher biodiversity in the tropics or to a bigger sample size (12 tropical species versus 7 temperate species), and was mostly driven by differences in Ca and P content. Fish body composition differed between taxonomic orders, highlighting the influence of phylogeny. We also found differences according to the species feeding groups, where omnivorous fish showed an overall higher variation in body composition, but then again, this result seems to be mostly driven by morphological characteristics related to phylogeny. Overall, our study suggests that environment and elemental investments in morphological characteristics affect body stoichiometry. Moreover, this work provides information on fish body stoichiometry for elements other than C:N:P, across distinct ecosystems, taxonomic orders and trophic guilds.

Key words: ecological stoichiometry, phylogeny, bioelements, freshwater.

Introduction

Bioelements are the basis of organismal biology since their atoms are used in the construction of molecules, tissues, organisms and in various ecological interactions. We typically study these interactions and the importance of elements through the stoichiometric framework. Ecological stoichiometry (ES) is based on two general laws: Law of Mass Conservation and Law of Definite Proportions. The first, states that mass cannot be created or destroyed, it can only change form. The second posits that a chemical compound always contains the same elements in the exact same proportion by mass. Through the stoichiometric framework, we look for the acquisition of the element, its assimilation, allocation and release back to the environment (Sterner and Elser 2002). Also, we can investigate what makes up a living organism and in what concentrations certain elements are needed for proper organism function, which can be called the organism's ionome (Salt et al. 2008).

The most abundant bioelements in nature are hydrogen (H) 59%, oxygen (O) 24%, carbon (C) 11% – used in primary structural molecules, such as CO_2, H_2O, O_2 - and nitrogen (N) 4%, phosphorus (P) 1% and sulfur (S) 0.1-1% – components of several molecular structures, such as chlorophyll, RuBisCo, ATP, NAD, DNA, and RNA (percentages of the total amount of atoms in organisms, Slade 2006, Penuelas et. al 2019). Other important bioelements present in lower concentrations are calcium (Ca), magnesium (Mg), sodium (Na), potassium (K), manganese (Mn) and zinc (Zn) (Slade 2006). These are all biologically relevant elements (see Table 1), however, ecological stoichiometry is largely based on C:N:P. Such fact represents a gap in our knowledge concerning the stoichiometry of other elements and their influence on biochemical cycling. Future studies should investigate stoichiometric patterns and constraints of different bioelements, across different species and ecosystems.

Choosing which elements are worth assessing is a case-by-case decision, and revolves around the interaction between the environment's ionome and the ionome of its living organisms. Here, we will focus on freshwater ecosystems, where fish are often the most abundant consumers and nutrient rich taxa. Fish participate in the ecosystem nutrient cycling as sinks by stocking nutrients in their body tissues, sources by releasing nutrients through excretion, egestion and as their bodies decompose, and as transporters by moving nutrients between habitats (Vanni 2002, Glaholt and Vanni 2005, Boros et al. 2015, Nobre et al. 2019). Fish present high demand for phosphorus to build their bone skeleton, especially fish species that are popularly known as armored fish, with boney plate structures (Hood et al. 2005,

McIntyre and Flecker 2010). Because the association of phosphorus and calcium forms hydroxyapatite ($Ca_5(PO_4)_3(OH)$), the major mineral component of bone (Sugira et al. 2004), fish present a high demand for calcium as well. Besides their role in bone formation, phosphorus and calcium participate in many important functions. For instance, phosphorus is a component of nucleic acids and cell membranes and calcium is essential to muscle contraction and blood clotting (NRC 1993). As vertebrates, it is likely that fish present high demands for other elements present in bone, such as magnesium and manganese. Magnesium is mostly stored in bone (around 50-70% of body Mg is in fish bone), although its main functions are related to the soft tissue, such as participating in osmoregulation and neuromuscular transmission, and acting as an essential cofactor in many enzymatic reactions (NRC 1993, Bijvelds et al. 1998). Manganese is considered a trace element, but no less important since it is a key component to the metabolism of carbohydrates, lipids and proteins (Ashner and Ashner 2005) and to the immune system and growth (Kehl-Fie and Skaar 2010). Zinc is also considered a trace element in fish and an essential nutrient for its role in catalytic functions (NRC 1993). Sulfur is a component of two aminoacids, methionine and cysteine, and their dietary deficiency is associated with fish showing early stages of cataract (Candebat et al. 2021). Also, cysteine is present in fish scales (Harikrishna et al. 2017). Sodium and potassium are essential ions to vertebrates (Sh 2013), especially to animals in freshwater environments with hyperosmotic bodies to their surroundings, due to their role in osmoregulation. Besides Na^+ and K^+, the electrolytes Mg^{2+} and Ca^{2+} also participate in the extra- and intracellular osmotic and ionic regulation of fluids in fish (NRC 1993).

Table 11- Elements, their biological importance and how they are obtained by fish.

Element	Biological importance	Source to fish
Calcium, Ca	Important constituent of bone. Present in scales as well. Participates in muscle contraction, blood clot formation, regulation of enzymatic processes, acts as secondary messenger for endocrine signals, and helps maintain cell integrity and acid-base equilibrium (NRC 1993).	Absorption from surrounding water through the gills and skin, but mostly through diet (NRC 1993, Hossain and Yoshimatsu 2014).
Phosphorus, P	Important constituent of bone. Component of cell membranes, adenosine triphosphate (ATP), part of ribossomal RNA and other roles in catalytic	Mostly obtained through diet (Sugira et al. 2004, Sterner and George

	functions(NRC 1993).	2000).
Sodium, Na	Used in cellular osmotic regulation(NRC 1993).	Mainly absorbed by the gills through ionic transfer (Heisler 1984).
Potassium, K	Used in cellular osmotic regulation(NRC 1993).	Mainly absorbed in water via gills, but can be obtained through diet (Haswell et al 1980, Eddy 1985, NRC 1993).
Sulfur, S	Component of some amino acids, also serves as a structural brace for stabilizing protein function (Maret 2004).	Taken up primarily via diet (Carr et al. 2017).
Magnesium, Mg	Participates in skeletal tissue metabolism. It is an essential cofactor in many enzymatic reactions and also plays a role in osmoregulation and neuromuscular transmission (NRC 1993).Stored in bone, but primarily used in soft tissue (Bijvelds et al 1990).	Obtained through diet or surrounding water (NRC 1993).
Manganese, Mn	Needed in minute amounts, stored in bone, a key component for the metabolism of carbohydrates, proteins and lipids, and is also needed for good immune function, development and growth (Kehl-Fie and Skaar 2010, NRC 1993).	Absorbed from water (Srivastava and Agrawal 1983), but primary obtained through diet (Wantanable et al. 1997).
Zinc, Z	Integral part of more than 70 enzymes (NRC 1993). Needed in minute amounts for catalytic functions and the immune system.	Can be absorbed through water, as well as through diet (Wantanable et al. 1997)

Animals body elemental composition is determined by a combination of ecological, environmental and evolutionary factors (El-Sabaawi et al. 2012, Allgeier et al. 2020). One of the premises of ES is that animals are homeostatic, which means that they do not alter their body composition with their diet composition (Sterner and Elser 2002). However, ES also states that, over evolutionary time, predator body tissues becomes more similar to their prey, in order to maximize their nutrient uptake (Sterner and Elser 2002). Despite animals showing a rather constant body composition regardless of their diet, there is evidence that some species are not strictly homeostatic and present some degree of stoichiometric plasticity (Cross et al., 2003; Small and Pringle, 2009; McManamay et al., 2011, El- Sabaawi et al. 2012). This means that diet may drive stoichiometric patterns in animals body stoichiometry over an evolutionary time scale, but also over a short time scale. El-Sabaawi et al. (2012) found that

environment and diet can alter freshwater fish body stoichiometry in a study conducted with *Rivulus hartii* across 6 streams that showed wide differences in environmental variables. This study found that streams explained up to 18% of fish body stoichiometry overall variance, due to the high variability in basal resources and diet, showing a stronger predictive power than organismal traits (El-Sabaawi et al. 2012).

On the other hand, morphological characteristics, such as P-rich bone skeleton, can answer for most of an organism's nutrient demand or body nutrient content. Some studies did observe a strong influence of phylogeny (Hendrixon et al. 2007, Allgeier et al. 2020). A study conducted with 20 species of freshwater fish found that their body P was almost entirely determined by skeleton investment, showing a strong influence of phylogeny (Hendrixon et al. 2007). McIntyre and Flecker (2010) even found that stream fish body composition differed between trophic guilds, howeverthese differences were mainly driven by the presence of loricariid catfishes, indicating phylogeny overpowered the diet effect. But then again, all studies above focus on C:N:P. Therefore, in order to better understand the relation between body stoichiometry, phylogeny and environment, and to what degree certain elements are linked to each other, we must investigate elemental composition of elements aside from the C:N:P spectrum.

Here, we test the influence of phylogeny, environment and diet on the elemental composition of 12 fish species from a lowland tropical stream in Rio de Janeiro, Brazil, and 7 fish species from post-glacial temperate lakes in New York, USA. We measured 8 bioelements - Ca, P, Na, K, S, Mg, Mn and Z – to see in which concentrations they occur in fish body tissues and compare them in relation to diet (trophic guilds as proxy), environment (tropical or temperate) and phylogenetic groups (at the order taxonomic level).

The tropical region harbors the highest species richness on the planet, which also translates into phylogenetic diversity (Willig et al. 2003, Lomolino et al. 2010). It is only intuitive to assume that this biodiversity will be reflected in a diverse body stoichiometry for its organisms, including fish. Moreover, in the tropics, species niches are more specialized, as a result of ecological interactions and coevolutionary adaptations (Brown 2014), which could lead to lower stoichiometric niche overlap. Therefore, we hypothesize that body stoichiometry among fish from the tropical site will present higher variability compared to fish from the temperate site. We expect fish from the Siluriformes order to show more variation in their body stoichiometry, especially concerning bone related elements, since this order comprises three species of armored fish in our dataset. Also, we expect to see differences in body

stoichiometry according to feeding groups, mostly due to morphological demands for nutrient allocation, since two omnivore and one detritivore fish are armored fish in our dataset. Therefore, we expect that armored fish will drive differences in body stoichiometry among trophic guilds, concerning bone related elements. This way, we believe that both environment (tropical or temperate) and taxonomic affiliation have an effect in the elemental composition of fish. And, finally, we expect elements with similar function to covary, such as Ca, P, Mg and Mn for bone formation, and Na and K used in osmoregulation. This would mean that if elements are stoichiometrically linked, by measuring the concentrations of one of them, we would be able to predict the concentrations of the other, as stated by ES.

Materials and Methods

Study site, species and capture method

The fish from this study were collected in two distinct ecosystems: a Brazilian tropical stream and four American temperate lakes. The tropical stream is called Rio Guapiaçu (-42.7595, -22.4355) and its waters run close to REGUA (Reserva Ecológica de Guapiaçu), a private nature reserve in the state of Rio de Janeiro. The 12 species of fish from this site are: *Scleromistax barbatus, Pimelodella lateristriga, Rhamdia quelen, Acentronichthys leptos, Ancistrus multyinis, Rineloricaria sp, Bryconamericus ornaticeps, Mimagoniates microlepis, Characidium vidali, Phalloceros harpagos, Synbranchus marmoratus.* They were collected through electrofishing (Smith Root, LR-24 Backpack Electrofisher). The temperate lakes are located inside the Adirondack Park in the state of New York. The fish samples were collected from four lakes that are routinely surveyed by the Andirondack Fisheries Research Program: First Bisby Lake (-74.9323, 43.6061), Little Moose Lake (-74.9237, 43.6925), East Lake (-74.8959, 43.6946) and Rock Lake (-74.8691, 43.9672). The fish from Little Moose Lake and First Bisby Lakeare: *Lepomis gibbosus*, *Micropterus dolomieu*, *Luxilus cornutus*, *Semotilus atromaculatus*, *Umbra limi* and *Catostomus commersoni*. They were collected through boat electrofishing. The only species collected in East Lake and Rock Lake was *Salvelinus fontinalis*. In East Lake, the fish were sampled with gill nets, which are placed perpendicular out from shore and left for approximately 30 minutes before being pulled up and collecting the fish trapped in the net. Rock Lake was surveyed using trap nets, which work by leading fish through a large funnel into a pot that consists of a box made out of netting with a funnel opening, so that once the fish swims in it, they are trapped inside.

In total, our dataset comprises 4 trophic guilds, 8 taxonomic orders, 13 families and 19 species of freshwater fish, of which 3 species are known as armored fish: *Ancistrus multyinis, Rineloricaria sp* and *Scleromystax barbatus.* See the list of species and more details in Table 12.

Table 12- Taxonomy, sampling sites, size range, feeding group and whether the fish species is considered an armored fish.

Order	Family	Genus and species	Site	Dry mass (g)	Sample size	Feeding group	Armored fish
Siluriformes	Callichtyidae	*Scleromystaxbarbatus*		1.43 – 2.62	4	Omnivore	Yes
	Heptopteridae	*Pimelodellalateristriga*		0.58 – 2.06	4	Invertivore	No
		Rhamdiaquelen		2.90 – 12.15	3	Piscivore	No
		Acentronichthys leptos		0.25	2	Invertivore	No
	Loricariidae	*Ancistrusmultyinis*		1.07 – 3.49	4	Detritivore	Yes
		Rineloricariasp	Rio Guapiaçu, Brazil	1.33 – 4.33	2	Omnivore	Yes
	Trichomicteridae	*Trichomycterus sp*		0.34 – 3.36	2	Invertivore	No
Characiformes	Characidae	*Bryconamericusornaticeps*		0.20 – 7.99	4	Invertivore	No
		Mimagoniatesmicrolepis		0.13 – 0.33	4	Invertivore	No
	Crenuchidae	*Characidium vidali*		0.62 – 1.06	4	Invertívore	No
Ciprinodontiformes	Poecilidae	*Phallocerosharpagos*		<0.01 – 0.28	4	Omnivore	No
Synbranchiformes	Synbranchidae	*Synbranchusmarmoratus*		0.85 – 8.22	2	Piscivore	No
Cypriniformes	Catostomidae	*Catostomuscommersonii*		2.36 – 2.86	5	Detritivore	No
	Cyprinidae	*Luxiluscornutus*		3.31 – 4.10	2	Omnivore	No
		Semotilusatromaculatus		1.55 – 3.83	11	Invertivore	No
Perciformes	Centrachidae	*Lepomisgibbosus*	Andirondack Lakes, USA	1.95 – 8.46	5	Invertivore	No
		Micropterusdolomieu		1.04 – 2.58	6	Piscivore	No
Salmoniformes	Salmonidae	*Salvelinusfontinalis*		2.81 – 8.60	2	Invertivore	No
Esociformes	Umbridae	*Umbralimi*		0.66 – 1.81	8	Invertivore	No

*Some dry mass data were estimated from the fish measured wet mass.

Sample preparation and nutrient analysis

To determine fish elemental composition for Ca, P, Na, K, Mg, Mn, S and Z, we used Inductively Coupled Plasma Optical Emissions Spectroscopy (ICP-OES). For this method, fish tissue samples need to be transformed into a liquid solution. The first step consists of drying and grinding the whole fish (fins included), followed by the digestion process that will liquefy the samples. The fish from Brazil were dried in the drying oven at 60°C and the fish from USA were dried in the freeze dryer set to -10°Cat the shelf temperature and -60°C at the coil temperature. All fish were dried until they reached a stable minimum mass. The Brazilian fish samples were ground using a mortar and pestle until a fine homogeneous powder was obtained. The Adirondack fish were ground using a freezer mill with liquid nitrogen bath after an initial rough grind using a spice blender. The grinding chambers were cleaned between fish using isopropyl alcohol as to not contaminate the tissue samples.

The digestion of the samples started by weighing 0.15 $\pm$0.005 g of finely ground tissue into a 50 ml Falcon tube. Afterwards, 5 ml of 69% nitric acid is added to each tube. The tubes were placed in a hot bath for 30 minutes, of which in the first 10 minutes the samples were heated to 83°C and then they stayed at 83°C for the next 20 minutes. The samples are given time to cool to 50°C and, once that happens,0.5 ml of 30% hydrogen peroxide is added to each tube. For the second time, the samples were placedinside the hot bath at 83°C for 20 minutes. After that, the samples were given time to cool to 50°C and, once that happened,0.5 ml of 30% hydrogen peroxide was added to each tube one more time. The hot bath was activated for a third time to 83°C for 20 minutes. The tubes were then allowed to cool to room temperature and 20 ml of deionized water were added to dilute the samples. After 8 hours, the solutions were transferred to ICP tubes and ran through the spectrometer. The fish were analyzed using an optical emissions spectrometer (Spectro Analytical). Quality control was tested using digested NIST Tomato Leaves and 3 blanks per sample. Three tissue samples of one fish from each Adirondack species were also run to quantify analytical precision.

Statistical analysis

The data were analyzed in two forms: as a percentage of body mass comprising each element, and as the molar ratio of calcium to each element. Calcium was chosen as the reference

because,among the elements we measured in this study, it is the element that makes up the largest portion of fish dry mass due to its role in bone.

Prior to other analysis, we performed a Pearson Correlation to determine significant relationships between nutrient percentages. To discern overall patterns across all fish, we used a Principal Components Analysis (PCA) to represent groups of individuals according to their body stoichiometry (elements as percent of fish mass) into a multivariate space. To test for multivariate differences in the body stoichiometry between groups of "Sites", "Trophic guilds", and "Orders" (grouping fixed factors), we performed a PERMANOVA (999 permutations) based on the body stoichiometry similarity matrix using a Euclidian index. Also, to account for centroid differences made by inter-group variances, we used a PERMDISP (999 permutations) to evaluate dispersion differences in ordinate space between the same grouping fixed factors used before.

To evaluate what causes overall differences among species, we used multivariate analysis of variance (MANOVA) of molar ratios to test the influence of 3 factors: taxonomic order, site and trophic guild. We proceeded to run analysis of variance (ANOVA) tests for each molar ratio looking at the significant factors indicated by the MANOVA (order, site, trophic guild), to further analyze the data. When all factors were statistically significant in the MANOVA, we performed a three-way ANOVA model which nests order within site (referred to as "Nested Model" in the tables), which accounts for all the factors, giving results for sites, trophic guild, and orders within sites.

All analyses were performed in statistical program R 4.1.0 (R Core Team 2021). To perform the Pearson Correlationwe used the function ggpairs from the GGally package (Schloerke et al. 2021). To run the PCA, we used the function prcomp and to plot the data we used function fviz_pca_biplotfrom the factoextra package (Kassambara and Mundt 2020).We performed the PERMANOVA and PERMDISP using the functions adonis2 and betadisper from vegan package (Oksanen et al. 2020). The MANOVA and ANOVA tests were performed with the functions manova and aov, respectively.

Results

General patterns in fish elemental composition show that Ca was the most abundant element, followed by P, K, S, Na, and lastly Mg. Mn and Zn, both were trace elements within the fish. Tropical fish presented noticeably higher Ca and P concentrations (Figure 8a). The order

Ciprinodontiformes showed higher body Ca and P, closely followed by the Siluriformes. Curiously, the Esociformes presented much higher body Na than all the other orders (Figure 8b). As for differences between trophic guilds, omnivorous fish show higher body Ca, P, Mg, Mn and Na, and piscivores show higher body S and K (Figure 8c).

Figure 8- Element percent body mass according to a) site, b) order and c) trophic guild. Manganese and zinc data are shown in the right-side graphs for better visualization.
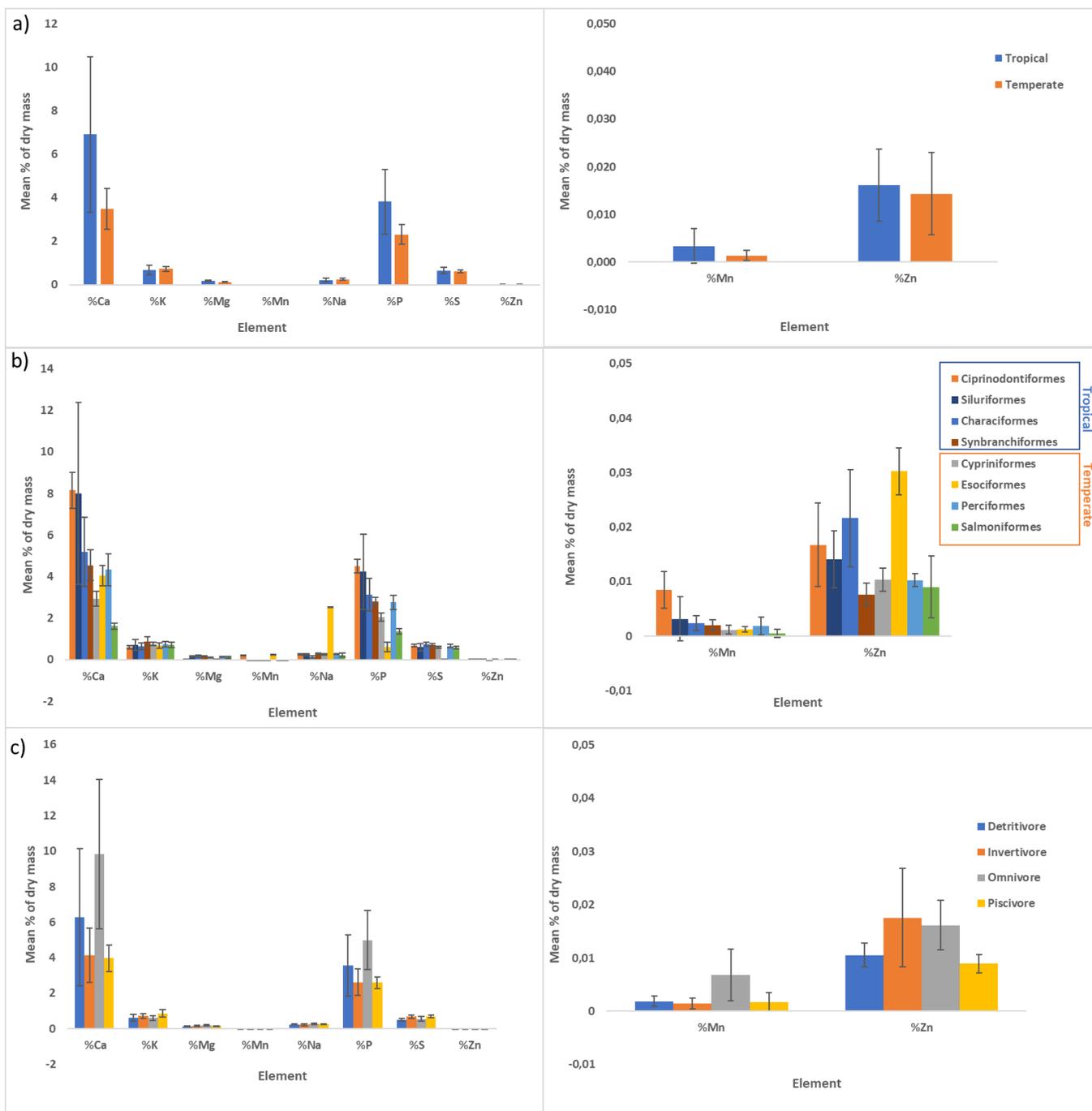
Legend: Means ±SD of the percent mass of the fish for each element sampled, separated by a) site, b) order, and c) trophic guild. Manganese and zinc data are shown at a different scale by the side of each graph for better visualization.

Overall, there is a high positive correlation between elements present in bone – Ca, P, Mg and Mn (0.99 for Ca and P, 0.75 for P and Mg, 0.71 for Ca and Mg, 0.69 for P and Mn, 0.69 Ca and Mn and 0.60 for Mg and Mn) and a more loose correlation between elements involved in osmotic and ionic regulation of extra - and intracellular materials (0.24 for Na and K, 0.22 for Na and Ca, -0.45 for K and Ca) (Figure 9).

Figure 9- Correlation plots for all elements as percentages of body dry mass.
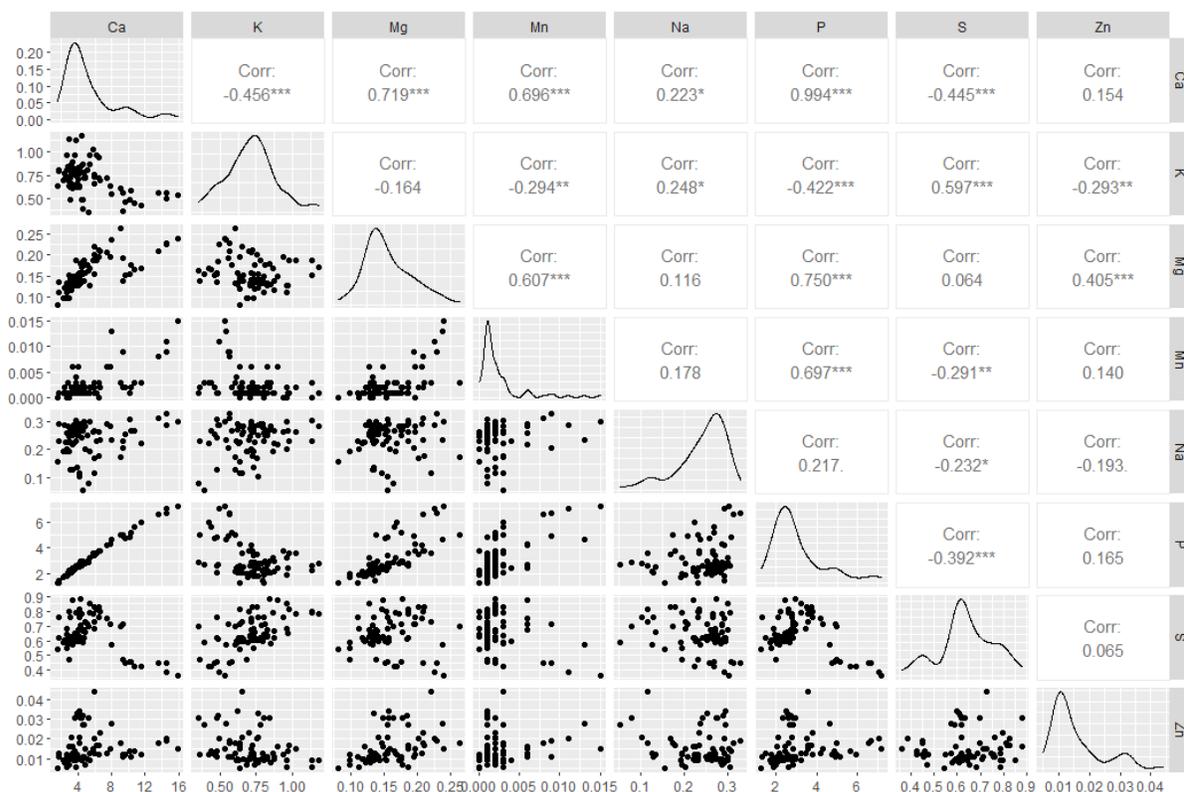
Legend: Each circle in the plots indicates an individual fish and the line graphs illustrate the overall element distribution across all fish.

The PCA model axes show that PC1 explains 46.7%, PC2 explains 17.9% and PC3 explains 17.5% of the variation in the data. There is a tight grouping of Ca, P and Mn across all plots (Figure 10). On the PC1 + PC2 plots, Mg and Zn were grouped with Ca, P and Mn on the PC1 axis, while S and K were closely grouped on the PC2 axis. Sodium had clear alignment with the PC3 axis (Figure 10). When the data is grouped by site, tropical fish were more variable than temperate fish on all plots (Figure 10a, 10b and 10c). When grouped by order, the Siluriformes

group had the most variability on both axes, while most other groups had the majority of their variation on the PC2 axis (Figure 10d, 10e and 10f). When grouped by trophic guild, omnivorous fishes varied along all three axes, detritivores varied on both the PC1 and PC3 axes, invertivores varied on both PC2 and PC3, and piscivores had most of their variation along the PC3 axis (Figure 10g, 10h and 10i). The PERMANOVA tests showed that there are significant differences among fish from different sites, taxonomic orders and trophic guilds (Table 13).

Figure 10- Biplots of the Principal Component Analysis (PCA), created usingthe percent mass of elements in the fish. The PCA's are grouped by site (a, b, c), by order (d, e, f) and by trophic guild (g, h, i).

Table 13- PERMANOVA results showing significant differences across all the elements percentages of body mass for site, order and trophic guild. Significant p-values are defined as being<0.05 and are signaled with an *.

	DF	F value	P value
Site	1, 77	9.0753	0.001*
Order nested in Site	7, 71	36.344	0.001*
Trophic Guild	3, 75	16.195	0.001*

The PERMADISP shows that there are significant differences in the data dispersal relative to the centroid for species between site, order and trophic guilds (Table 14). Species from the tropical site present higher variation in body stoichiometry in comparison to species from the temperate site (Figure 11a). The Siluriformes order stands out with a much higher variation than all the other orders (Figure 11b). Omnivorous fish present higher variation compared with the other trophic guilds (Figure 11c).

Figure 11- Data dispersion relative to the centroid for a) site, b) order and c) trophic guild.
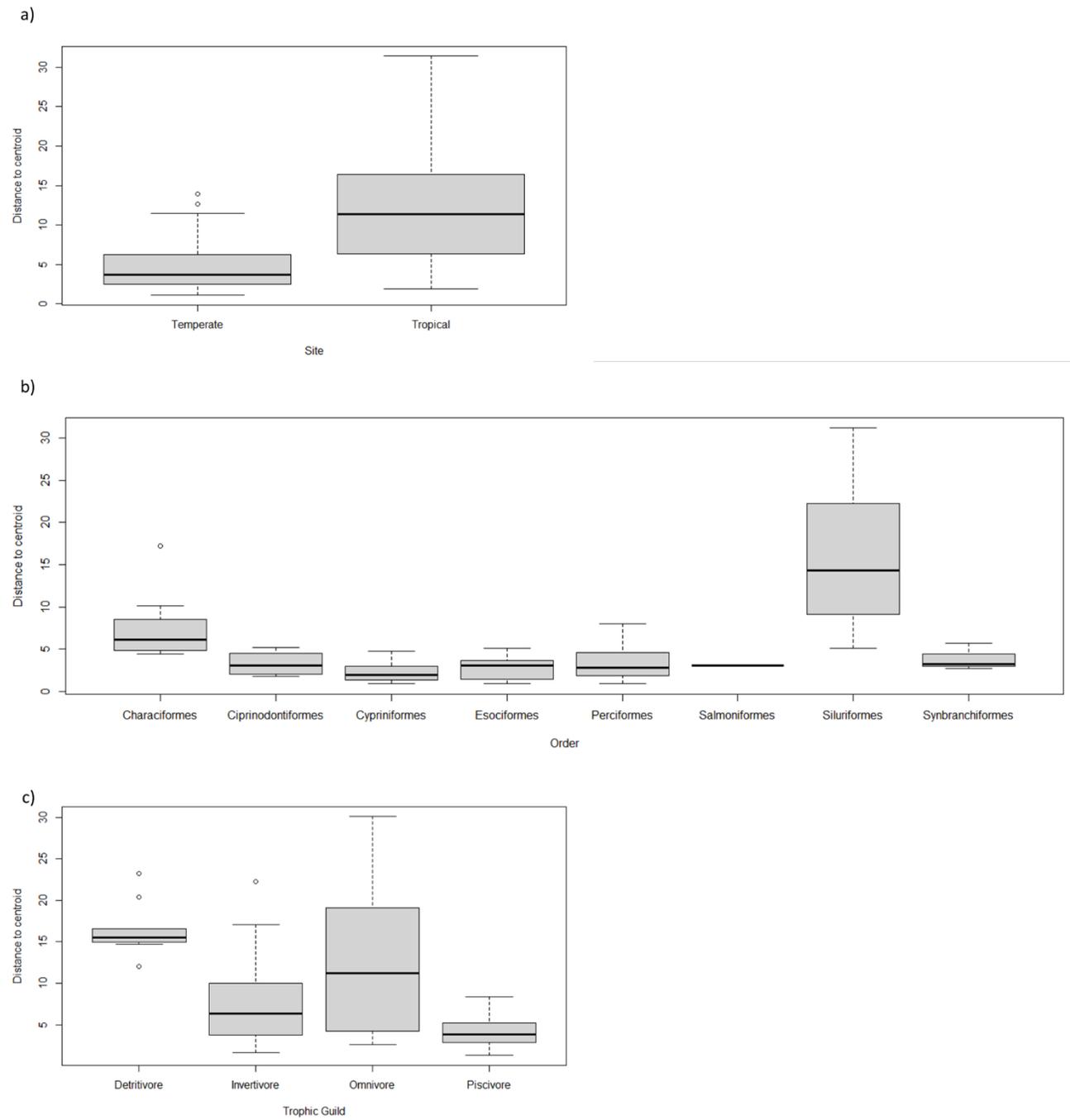
Table 14- PERMDISP results showing significant differences in the data dispersion (distance from centroid) across all the elements percentages. Significant p-values are defined as being<0.05 and are indicated with an *.

	DF	F value	P value
Site	1, 77	34.079	0.001*
Order	7, 71	16.928	0.001*
Trophic Guild	3, 75	13.668	0.001*

For understanding which trophic guilds differed from one another, we ran a post-hoc test (Tukey HSD), that showed that there is a statistically significant difference in body stoichiometry between: detritivores and invertivores (p<0.001), detritivores and piscivores (p<0.001), omnivores and invertivores (p=0.006) and omnivores and piscivores (p<0.001).

The MANOVA analysis showed that there are significant differences in all the elemental molar ratios according to site, order and trophic guild (Table 15 and Figure 12). The ANOVA analysis for each molar ratio of element to calcium demonstrates that all elemental ratios differ according to site, order and trophic guild (Table 16).

Table 15- MANOVA results showing significant differences across all the elemental molar ratios for site, orderand trophic guild. Significant p-values are defined as being<0.05 and are signaled with an *.

	DF	Approximate F value	P value
Site	1, 77	11.222	<0.001*
Order	7, 71	5.266	<0.001*
Trophic Guild	3, 75	4.88	<0.001*

Table 16- ANOVA models for each molar ratio of element to Ca. Significant p-values are defined as being<0.05 and are signaled with an *.

	F- value	P- value
Ca:P		
Nested Model		
.Site	77.51	<0.001*
.Trophic Guild	15.20	<0.001*
.Order nested in Site	11.69	<0.001*
Ca:Mg		
Nested Model		
.Site	46.75	<0.001*
.Trophic Guild	21.88	<0.001*

.Order nested in Site	10.44	<0.001*
Ca:Mn		
Nested Model		
.Site	0.003	0.9569
.Trophic Guild	1.687	0.1780
.Order nested in Site	2.592	0.0254*
Ca:K		
Nested Model		
.Site	65.543	<0.001*
.Trophic Guild	18.494	<0.001*
.Order nested in Site	8.664	<0.001*
Ca:Na		
Nested Model		
.Site	77.51	<0.001*
.Trophic Guild	15.20	<0.001*
.Order nested in Site	11.69	<0.001*
Ca:S		
Nested Model		
.Site	52.137	<0.001*
.Trophic Guild	27.029	<0.001*
.Order nested in Site	8.191	<0.001*
Ca:Zn		
Nested Model		
.Site	35.94	<0.001*
.Trophic Guild	26.12	<0.001*
.Order nested in Site	9.75	<0.001*

Figure 12- Elemental ratios in relation to calcium grouped by a) site, b) order and c) trophic guild.
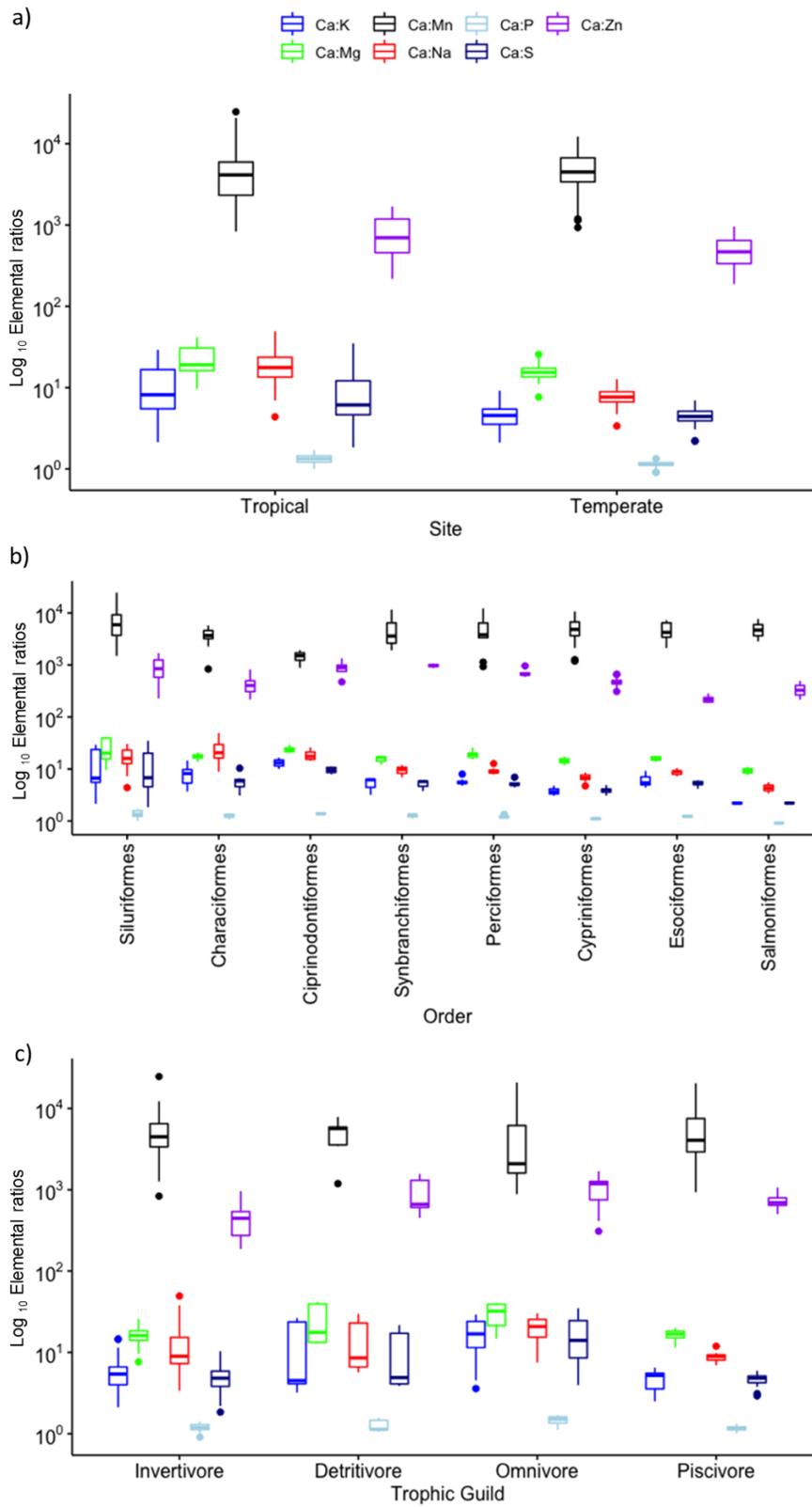
In Figure 13, Ca:P molar ratios are zoomed in for better visualization.

Figure 13- Graph showing the Ca:P molar ratios across all orders.
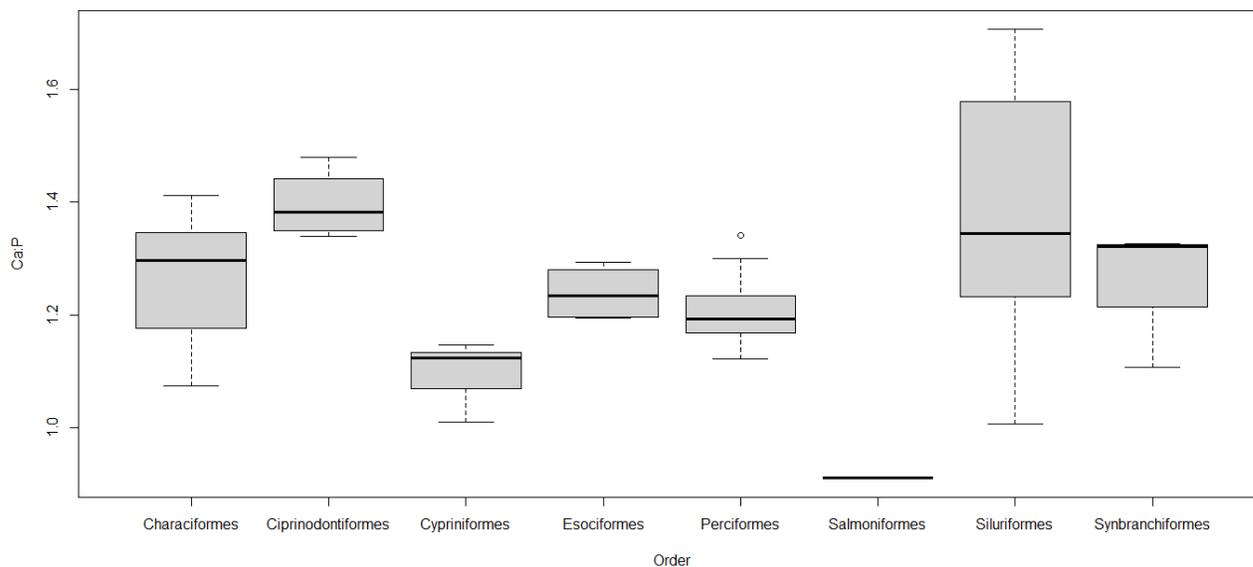
The relative abundances of Ca to P differ a lot between orders, and are mostly driven by the armored fish species: *Scleromistax barbatus*, *Ancistrus multispinis* and *Rineloricaria sp* (Figure 14).

Differences in bone related elements between armored and non-armored fish are represented in terms of percentages of dry mass (Figure 15a) and ratios to calcium (Figure 15b), stressing the differences between these groups.

Figure 14- Relationship between whole fish %Ca and %P for all species. The tendency line was fitted by y= 2.32x - 1.93(R² = 0.98).
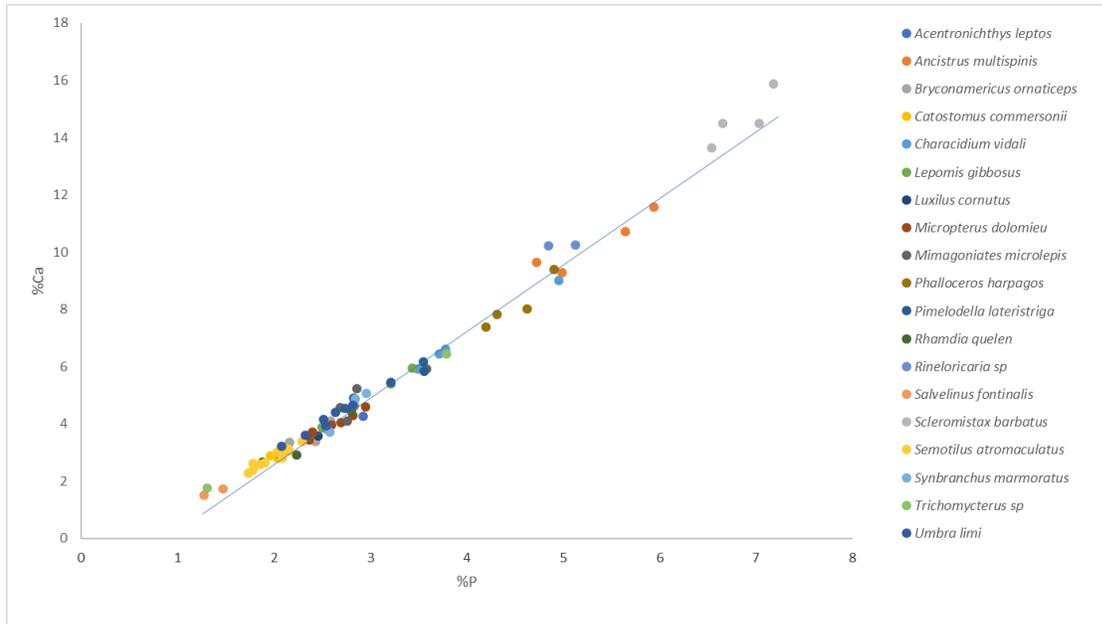
Figure 15- Differences in bone related elements between armored and non-armored fish a) as %P, %Ca and %Mg, and b) as Ca:P and Ca:Mg molar ratios.
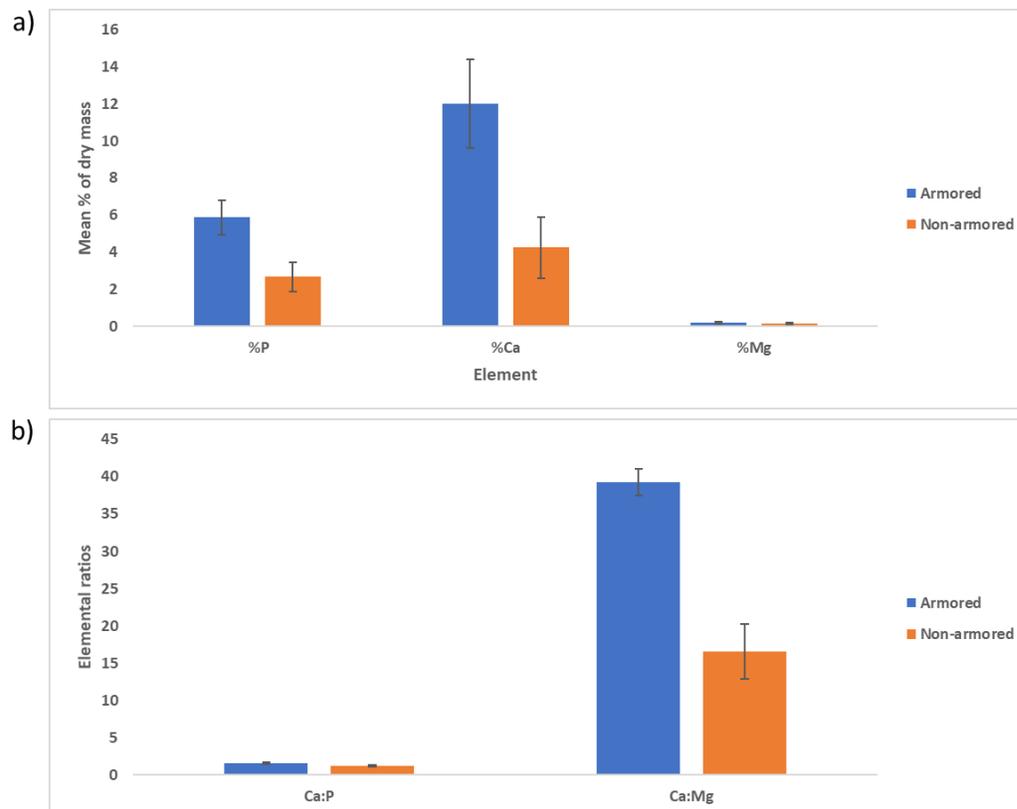
Legend: Means ±SD of the a) percent mass of the fish for P, Ca and Mg, and b) Ca:P and Ca:Mg ratios, separated by armored and non-armored fish.

Discussion

Our study shows that environmental and phylogenetic factors influence fish body stoichiometry and that elements with similar functions are correlated to each other. Specifically, we found results supporting our expectations that bone related elements are tightly correlated, especially Ca and P, and that taxonomic affiliation has a strong influence in fish elemental content, determining nutrients allocation to morphological traits, such as boniness in skeleton.

The correlation between calcium and phosphorus was the highest observed of any pair in our dataset (0.99 Pearson correlation - Figure 9, and R^2=0.98 – Figure 14). This result is similar to what Hendrixon et al. (2007) found in their study and it indicates that the tight correlation between Ca and P has a structural basis related to boniness, as both nutrients are the main components of hydroxyapatite ($Ca_5(PO4)_3(OH)$, molar ratio:1.67, mass ratio:2.15) the major mineral present in bone. However, we can see great differences in the relative abundances of Ca and P (Figure 14) and in the average Ca:P molar ratio across different orders (Figure 13), evidencing that P serves other roles in fish physiology, aside from being a bone component.Similarly, Sterner and George (2000) found that closely related species varied in their P content. Despite being a trace element, Mn is also important for bone formation (Viegas et al. 2021, Gatlin and Wilson 1984), which explains its correlation to Ca and P (0.69 Pearson correlation for both elements), consistent with the chemical signature of bone. And, finally, as Mg is mostly stored in bone, it showed a tight correlation with Ca, P and Mn as well (0.71, 0.75 and 0.60 Pearson correlation, respectively). The correlation between Ca and Mg can also be due to their role in osmoregulation. The electrolytes responsible for extra and intracellular ionic and osmotic regulation of fish fluids are Mg^{2+}, Ca^{2+}, Na^+ and K^+ (NRC 1993). We observed a more loosely correlation between Na and K, Ca and K, Ca and Na (0.24, -0.45, 0.22 Pearson correlation, respectively). It is hard to determine the reasons for the correlations found between Ca and S (-0.44), K and Zn (-0.29), K and S (0.59), K and P (-0.42), K and Mn (-0.29), Mg and Zn (0.40), Mn and S (-0.29), Na and S (-0.23) and P and S (-0.39), since these nutrients serve different functions in fish. It is likely that these elements functions differ between species and are

phylogenetically conserved, which is why their correlations are not as strong as the correlations between bone related elements.

As expected, we found more variation in body composition for all elements in fish from the tropical site (Figures 8a, 3a, 10b, 10c, 11a and 12a). Brown (2014) states that biodiversity is higher in the tropics fundamentally because the high temperatures accelerates ecological and evolutionary rates, generating and maintaining biodiversity. As a consequence of this higher biodiversity of living forms, species may show a narrower stoichiometric niche, as a way to maximizing their survival. However, to fully understand how body stoichiometry changes between tropical and temperate ecosystems, more comparative studies are needed. Especially, fish from the tropics present much higher proportions of body Ca and P (Figure 9a), driven by the presence of armored fish species *Ancistrus multispinis* (Loricariidae), *Rineloricaria sp* (Loricariidae) and *Scleromistax barbatus* (Callicthyidae) (Figure 14). Fish from the Loricariidae and Callicthyidae family present bony plates covering their bodies (Vanni et al. 2002, Sire 1993), which explains their high body P and Ca and highlight the strong role of phylogeny in fish body stoichiometry. This is also an example of how environment and phylogeny overlap and can both simultaneously influence body stoichiometry. One limitation in our study is that we do not have the same taxonomic orders in both tropical and temperate sites. Although we have corrected for this in our nested models (order nested in site), it would have been ideal to compare the same orders across distinct ecosystems.

Sodium is primarily obtained through the surrounding water, therefore we expected to find differences in fish body Na between sites, since our tropical stream and temperate lakes are under very different weathering patterns and landscapes and, consequently, present different nutrient dynamics. Fish body Na is slightly higher in the temperate site (Figure 9a) and the Ca:Na ratios are higher for tropical fish (Figure 12a), which indicates lower levels of Na in the water in the tropical site. In fact, we know our tropical stream has very low conductivity (average conductivity= 16.06 μS/cm), which means low levels of Na in the water. The relatively low variation of Ca:Na ratios within orders (Figure 12b) and the much higher abundance of body Na in Esociformes fish (Figure 8b) also indicates that homeostasis levels of Na can be influenced by phylogeny.

Sodium, potassium and magnesium can be absorbed by the gills from the surrounding water and obtained through diet (NRC 1993, Eddy 1985, Haswell et al. 1980). Body K and body Mg present higher concentrations and variability in fish from the tropical site, which could be due to higher availability of these nutrients in the water (Figure 1a). But there also seems to be a phylogenetic influence on these nutrient ratios to calcium (Ca:K, Ca:Mg), with relatively little variation within orders (Figure 12b). Also, since the fish in this study are from freshwater environments, K levels in water can be low (Shearer 1988) and its primary source may be dietary. This means that trophic guild can also influence the relative abundance of K in fish. Fish body Mg can also be influenced by trophic guild, as shown by a study conducted with *Cyprinus carpio*, where dietary Mg influenced Ca:Mg ratios in fish body (Ogino and Chiou 1976). Armored fish show much higher body P and Ca compared to non-armored fish, and similar body Mg (Figure 8a), consequently, armored and non-armored fish show very different Ca:Mg ratios (Figure 15b). This fact also demonstrates the role Mg plays in organism function aside from being present in bone.

We found that fish trophic guildshowed a significant effect on fish body stoichiometry, however it seems that this result has little to do with their diet and is mostly driven by taxonomic identity. Pairwise comparisons show that detritivores differ from invertivores and piscivores, and that omnivores differ from invertivores and piscivores. Our armored fish species belong to the detritivores and omnivores trophic guilds, which are precisely the trophic guilds differing from invertivores and piscivores, which suggests that phylogeny is driving this pattern. However, piscivores, followed by invertivores, showed higher body S and body K (Figure 8c and 12c). Sulfur is obtained primarily through diet (Carr et al. 2017) and it is a component of two important amino acids, methionine and cystine (NRC 1993), which could explain why fish with a protein rich diet, showed higher body S.

Moreover, our data shows differences in body S and Ca:S ratio across orders of fish (Figure 9b and 12b), meaning that body S is also determined by phylogeny. This result is similar to a study with *Channa sp* fish, that showed that species from this genus differed in their amino acid composition (Zuraini et al. 2006). Therefore, differences in amino acid composition between species could mean differences in body S.

Manganese and zincoccur in minute amounts in fish body, nonetheless they are essential to metabolic functions (NRC 1993). Both can be absorbed through water and obtained via diet, but Mn is primarily obtained through diet (Wantanable et al. 1997, Srivastava and Agrawal 1983). Studies suggest that increases in dietary Mn lead to increases in bone Mn (Viegas et al. 1997, Wantanable et al. 1997). Manganese is also part of the photosynthetic complex in plants (Yachandra et al. 1996), which can explain why omnivores showed the highest Mn body content (Figure 2c). However, regardless of trophic guild, studies demonstrate that species differ in their dietary Mn requirements. For example, *Oncorhynchus mykiss* needs 15 mg Mn kg $^{-1}$for proper organism function (Wantanable et al. 1997), whereas *Ictalurus punctatus* needs 2.4 mg Mn kg $^{-1}$ (Gatlin and Wilson 1984), indicating the role of phylogeny in determining fish body Mn. Zinc can be toxic to fish in relatively low levels in the water (Wantanable et al. 1997) and to some species of fish in high dietary levels (Clearwater et al. 2002). Therefore, fish body Zn is maintained at homeostasis through limited gastrointestinal absorption and excretion (Wantanable et al. 1997). Phylogenetic traits may determine Zn homeostasis levels, which would explain the high variability of body Zn between fish orders (Figure 9b).

To comprehend which factors can shape the stoichiometry of individual organisms, the relationship between elemental composition, phylogeny and environment must be investigated for multiple elements. Our data show that elemental investments in morphological characteristics combined with element availability in the water and prey affect body stoichiometry. Moreover, this work provides information on which nutrients other than C:N:P occur in fish body tissues, across distinct ecosystems, taxonomic orders and trophic guilds. To our knowledge, there are no other studies on freshwater fish stoichiometry that have quantified P, Ca, Mg, Mn, S, K, Na and Zn together for the same species. Hence, patterns in fish body content warrant further investigation.

DISCUSSÃO

Este estudo buscou compreender como características dos consumidores e do ambiente afetam sua excreção de nutrientes e estequiometria corporal usando peixes de água doce como modelo de estudo. Nossos resultados apontam para o enorme poder preditivo que o tamanho corporal tem sobre as taxas de excreção de nitrogênio e fósforo, sendo esta a variável mais importante em comparação a dieta, estequiometria corporal e temperatura. Através de uma meta-análise, vimos que, em geral, um aumento da temperatura do ambiente leva a um aumento das taxas de excreção de nutrientes dos peixes, porém nem sempre este é o caso (como observado no Rio Guapiaçu e descrito no capítulo 1) e as causas para esta variabilidade de resultados precisam ser esclarecidas. Por fim, vimos que a estequiometria corporal dos peixes é fortemente influenciada pela morfologia das espécies, especialmente no que se refere ao seu esqueleto ósseo.

Ao testar a influência da dieta (grupo alimentar como proxy), da estequiometria corporal (%N, %P, N:P), do tamanho corporal dos indivíduos e da temperatura nas taxas de excreção de nitrogênio e fósforo de peixes verificamos que a Teoria Metabólica da Ecologia, através da influência do tamanho do corpo, tem um maior poder preditivo sobre a excreção destes animais, comparada a Teoria de Estequiometria Ecológica. A partir destes dados, podemos concluir que o tamanho corporal, através de sua relação com o metabolismo, é o principal fator a determinar as taxas de excreção de peixes. No entanto, é preciso ressaltar que a ausência de dados de ingestão e egestão podem afetar o poder preditivo das variáveis relacionadas a Teoria de Estequiometria Ecológica e estudos futuros devem buscar medir e incluir esses dados em suas investigações.

Além disso, o efeito da temperatura nas taxas de excreção de nutrientes de peixes se mostrou bastante variado, com alguns estudos indicando uma forte influência da temperatura sobre a excreção de nutrientes, enquanto outros não.Dentre os fatores metodológicos que poderiam interferir nos resultados dos estudos, apenas o período de aclimatação e o fato do animal ter sido alimentado previamente ou não, apresentaram alguma interferência. No entanto, estas variáveis explicam pouco dos resultados obtidos, indicando que fatores desconhecidos interferem no efeito que a temperatura tem sobre as taxas de excreção. É bastante provável que a identidade das espécies seja um fator determinante, onde cada espécie irá apresentar um nível de tolerância específico às mudanças na temperatura.

Por fim, testamos como a taxonomia e o ambiente afetam a composição corporal de

peixes de água doce de um córrego tropical e lagos temperados, em relação a 8 elementos. Os 8 elementos estudados foram: cálcio, fósforo, magnésio, manganês, sódio, potássio, enxofre e zinco, e até onde sabemos, esses elementos foram medidos juntos em espécies de peixes pela primeira vez no nosso estudo. Vimos que as características morfológicas tem forte influência sobre os padrões de estequiometria corporal, especialmente em relação a elementos presentes no esqueleto ósseo, como cálcio, fósforo, magnésio e manganês. A estequiometria corporal dos peixes se mostrou mais diversificada no ambiente tropical, o que pode indicar que o nicho estequiométrico das espécies é mais estreito nesses ambientes, como uma consequência da maior biodiversidade de formas de vida nesses locais. No entanto, são necessários mais estudos comparativos entre ambientes tropicais e temperados para verificar se este é um padrão de fato.

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