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Variation in bulk and amino acid-specific nitrogen isotope enrichment factors in fishes

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Abstract of the thesis presented by **María Teresa Nuche Pascual** as a partial requirement to obtain the Doctor of Science degree in Marine Ecology.

Variation in bulk and amino acid-specific nitrogen isotope enrichment factors in fishes

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Compound-specific isotope analysis (CSIA) of amino acids (AAs) in consumer tissues is a developing technique with wide-ranging application for identifying nitrogen (N) sources and estimating animal trophic level. Controlled experiments are essential for determining which dietary conditions influence variability in N stable isotopes (δ15N) trophic enrichment factors in bulk tissue (TEFbulk) and AAs (TEFAA). In this study, address the effect of potential sources of variation on trophic enrichment factors at the species-specific and taxonomic group-specific levels. At the species level evaluated independently the effect of the quantity and quality (digestibility) of the dietary protein on the trophic enrichment factors in two tissues differing in isotope turnover rates through controlled feeding experiments performed on juveniles of a carnivorous fish (Seriola lalandi) as a model. At the taxonomic group level, evaluated the relationship between nutritional and ecological factors on trophic enrichment factors of teleost fishes using a meta-analysis of studies reporting TEFs derived from controlled feeding experiments. Results suggest that within a single species, dietary protein quantity and quality relative to protein requirements can contribute to variability in TEFAA; the effect varied with tissue type. TEFbulk values in liver tissue showed a limited but significant relationship with protein quality, but in muscle bulk TEFs did not differ with protein quantity. At the taxonomic level, the TEF values of phenylalanine remained relatively constant in relation to nutritional and ecological factors, while those of lysine, glycine and serine varied in relation to the type of diet or life stage, which confirms the glycine and serine should not be considered source AA. Among trophic AAs, there is a relationship between TEFs and feeding regime, diet type and the aquatic habitat typically inhabited by a given species. It should be noted that glutamic acid TEFs were similar when estimated for singe teleost species, groups of teleosts, or global values calculated for many taxa. Further controlled species-specific feeding experiments are needed to elucidate the relationship between TEFs and factors that potentially have an influence on AA-specific δ15N values of fishes in their natural environment.

Keywords: carnivorous fish, isotopic fractionation, liver, muscle, nitrogen, nutrient requirement

Resumen de la tesis que presenta **María Teresa Nuche Pascual** como requisito parcial para la obtención del grado de Doctor en Ciencias en Ecología Marina.

Variación en los isótopos estables de nitrógeno y en los factores de enriquecimiento trófico en tejidos completes y aminoácidos

Resumen aprobado por:

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Dra. Sharon Z Herzka Llona
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El análisis isotópico de compuestos específicos de aminoácidos (CSIA-AA por sus siglas en inglés) en tejidos de consumidores es una técnica en desarrollo con una amplia aplicación para identificar fuentes de nitrógeno (N) y estimar el nivel trófico de animales. Los experimentos controlados son esenciales para determinar qué características de la dieta influyen sobre la variabilidad en los factores de enriquecimiento trófico (TEF) de N en tejidos completos (TEFbulk) y en AA específicos (TEFAA). En este estudio, se examinó la relación entre factores de enriquecimiento trófico en tejidos de peces en función de aspectos nutricionales y ecológicos. Se evaluó la relación entre la cantidad y calidad (digestibilidad) de la proteína de la dieta de manera independiente y los TEF de 11 AA en dos tejidos que difieren en sus tasas de recambio metabólico: hígado y músculo. Los TEFs se calcularon a través de un experimento de alimentación controlado con juveniles de un pez carnívoro (Seriola lalandi) como especie modelo. También se evaluó la relación entre factores nutricionales y ecológicos y los TEFAA mediante un meta-análisis de estudios en teleósteos que han llevado a cabo experimentos de alimentación bajo condiciones controladas. Los resultados sugieren que en nivel especie, la cantidad y calidad de proteína en la dieta pueden contribuir a la variabilidad en los valores de TEFAA para ciertos AA (fenilalanina, lisina, isoleucina y leucina), y que esta relación varía según el tipo de tejido. Los valores de TEFbulk en el hígado mostraron una relación limitada pero significativa con la cantidad de proteína, mientras que en el músculo no hubo relación. Los resultados del meta-análisis indicaron que los TEF de fenilalanina permanecieron relativamente constantes en relación a los diferentes factores nutricionales y ecológicos, mientras que los de lisina, glicina y serina variaron en relación a factores tales como tipo de dieta o estadio de vida, lo que confirma que la glicina y serina no deben ser considerados como AAs fuente. Entre los AAs tróficos, hubo una relación entre los TEFs y factores como el tipo de dieta, el régimen de alimentación y el hábitat acuático típico de cada especie. Hay que destacar que los valores de ácido glutámico, considero el AA trófico canónico, fueron similares entre los TEF reportados en estudios particulares sobre una sola especie, y los TEF calculados a partir de datos de varios grupos taxonómicos. Es necesario realizar más estudios controlados de alimentación para elucidar la relación entre los TEFs y factores que potencialmente ejerzan una influencia en los animales en su ambiente natural, con el fin de obtener estimaciones de posición trófica precisas y robustas por medio de mediciones de la composición isotópica de AA.

Palabras clave: Pez carnívoro, fraccionamiento isotópico, hígado, músculo, nitrógeno, requerimiento nutricional, teleósteos

Dedication

"El hecho más sorprendente es el conocimiento de que los átomos que constituyen la vida en la tierra, los átomos que componen el cuerpo humano, son rastreables a los crisoles que cocinaron los elementos ligeros en elementos más pesados en su núcleo, bajo temperaturas y presiones extremas. Estas estrellas, las de mayor masa entre ellas colapsaron y explotaron, diseminando sus entrañas enriquecidas a través de la galaxia, entrañas hechas de carbono, nitrógeno, oxígeno, y todos los ingredientes fundamentales para la vida. Estos ingredientes se hicieron parte de nubes de gas que se condensaron, colapsaron, formaron la siguiente generación de sistemas solares estrellas con planetas orbitándolas, y esos planetas ahora tienen los ingredientes para la vida misma.

Nosotros formamos parte de este universo, estamos dentro del universo.

Y el universo está en nosotros. Mis átomos vinieron de esas estrellas."

(Neil deGrass Tyson)

A mi madre

Fuente de inspiración y fortaleza en los momentos de alegría y de debilidad.

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CHAPTER 1. General introduction.

The study of trophic food webs provide ecological information about the role of organisms and the fluxes of organic matter and energy within and between ecosystems. Trophic food webs are formed by a large number of organisms and functional groups that can exhibit numerous complex trophic relationships. Properly sampling and representing the organisms within an ecosystems in trophic studies is this challenging, but crucial for tracing the fate of nutrients and understanding the contribution of dietary resources to consumers and characterizing food web structure. Various approaches, including gut content analysis, stable isotopes analysis (SIA), compound-specific isotopic analysis of amino acids (CSIA-AA), fatty acid analyses and more recently molecular techniques, have been used to reconstruct food web structure, characterize animal dietary composition and estimate trophic position (TP).

Traditionally, gut or stomach content analysis has been used to identify prey items from consumer´s stomach and estimate trophic level (Hyslop, 1980). This technique has inherent limitations because it provide a short-term dietary consumption of the recently consumed prey, and requires a large sample size. Also, the samples may not be fully representative of ingested items in the case of small prey, those with no solid structures (i.e. spines, vertebrates or scales) such as jellyfish, and the identification of partially or completely digested items is challenging (Hyslop, 1980), which may yield bias when identifying prey composition and in TP estimations.

Stable isotope analysis (SIA) has long used for studies in trophic ecology. Nitrogen stable isotope ratios ($\delta^{15}N$) of bulk consumer tissues have the potential for tracing dietary nutrients and provide spatial and time-integrated information of the assimilation of energy or mass flow within ecosystems in field studies, and have been particularly useful for estimating trophic position (Peterson and Fry, 1987). However, the difference in the bulk tissue $\delta^{15}N$ values of consumer relative to its diet, also known as trophic enrichment factors (TEFs), may vary due to the spatial and temporal availability of nutrient sources at the base of the food web and physiological processes of consumers (Vanderklift and Ponsard, 2003; Martínez del Río et al., 2009). TEFs have been reported to vary with diet type (Vander Zanden and Rasmussen, 2001), protein quality (e.g. Florin et al., 2011) and quantity (Kelly and Martínez del Río, 2010), tissue type (e.g. Malpica-Cruz et al., 2012), taxa and the mode of nitrogen excretion (McCutchan et al., 2003). Trophic enrichment factors are applied in trophic position estimation formulas and incorporated into mixing models to calculate the contribution of potential food sources in consumer tissues (Post, 2002). Therefore, erroneous estimations of TEFs would lead to inaccurate TP estimates and the contribution of food sources to tissues production (Post, 2002; Phillips, 2012).

In addition, the use of SIA to estimate TP requires the characterization of the isotopic baseline, which is a function of local or regional geochemical processes that influence the isotopic composition of inorganic nitrogen, as well assimilation by primary producers. Characterizing the isotopic baseline implies a big effort in human and economic resources to collect samples from primary producers and potential prey of consumers in a manner that adequately reflects spatial and temporal variability in isotopic composition.

Trophic position estimates have been also estimated using a complementary approach, which relies in measuring the compound-specific stable isotope ratios of amino acids (CSIA-AA). The CSIA-AA approach has the potential for reducing the limitations of SIA on bulk tissue for estimating TP (McClelland and Montoya, 2002; Popp et al., 2007; Chikaraishi et al., 2007), but also requires empirical estimates of TEFs, which is also calculated as the difference in $\delta^{15}N$ values of AAs between a consumer´s tissues and those of its diet. The approach is based on the premise that $\delta^{15}N$ values of some AAs reflect the isotopic baseline of primary producers (known as "source" AAs), whereas "trophic" AAs reflect trophic position. Using CSIA-AA has been reported to have more precision and accuracy than bulk SIA for estimating TP (McClelland and Montoya, 2002). One of the main advantages of CSIA-AA is that a single tissue sample holds isotopic information about the primary producer (isotopic baseline) and the consumer, which reduces the need of collecting samples from the primary producers and potential prey, and a greater resolution can be reached regarding energy sources and food web structure (Chikaraishi et al., 2009). However, the biochemical and physiological mechanisms causing isotopic variation in $\delta^{15}N$ values of amino acids and thus TEFs values are not fully understood (McMahon and McCarthy, 2016). Also, differences in samples preparation methodology, derivatization techniques and chromatography and combustion procedures may contribute to isotopic variation. The reproducibility of each sample is still a challenge within and between laboratories (Yarnes and Herszage, 2017).

Source and trophic AAs have been grouped based on the nitrogen isotopic discrimination, respectively (Popp et al., 2007), where phenylalanine (Phe), lysine (Lys) and methionine (Met) are currently classified as source AAs due to their small isotopic discrimination with each trophic step, and hence they reflect the isotopic baseline (Popp et al., 2007). Aspartic acid (Asp), glutamic acid (Glu), isoleucine (Ile), proline (Pro), leucine (leu), valine (Val) and alanine (Ala) are classified as trophic AAs given their large isotopic discrimination with each trophic step, and thus they can be used to estimate consumer trophic level (Popp et al., 2007). Serine (Ser), threonine (Thr) and glycine (Gly) were initially considered source AAs, but are currently classified as "metabolic" AAs due to their high variability in isotopic discrimination in high trophic level consumers (Germain et al., 2013; McCarthy et al., 2007).

The source and trophic AA classification has been related to whether transamination involves cleavage of the C-

N bond, which is observed in trophic AA (Chikaraishi et al., 2009). Source AA do not exhibit cleavage of the C-N bond, which explains the low level of isotope discrimination that is observed. Hence, source AA supposedly have small ^{15}N discrimination because they are not able to freely exchange nitrogen. Phenylalanine, which is considered the canonical source AA, has two potential catabolic pathways, one involves hydroxylation to tyrosine through tyrosine aminotransferase to form glutamate and p-hydroxyphenylpyruvate. This route is dominant and irreversible. The other pathway involves transamination with pyruvate to form Ala and phenylpiruvate (Mathews and van Holde, 1996). It has been suggested that variation in nitrogen isotope discrimination of AA is related to level of transamination and deamination during anabolic and catabolic processes (Macko et al., 1986; Hare et al., 1991; McClelland and Montoya, 2002; O´Connell, 2017). In a recent article, O´Connell (2017) suggested that in addition to transamination and deamination, the source and trophic AA classification is also associated with the availability of AAs, their role as energy substrates, protein synthesis and nitrogen cycling within a heterotroph. Further, nitrogen from source AAs is directly excreted in the form of ammonia by irreversible deamination (Gly, Met, Ser), or it is transferred to the metabolic pool incorporated into glutamate by direct irreversible transamination with α-keto-glutarate (Lys) or with pyruvate (Ser). Hence, the N in the so-called source AA can be subject to isotopic discrimination in specific biochemical and physiological processes.

In contrast to source AA, trophic AAs are able to interchange their amino nitrogen via glutamic acid, which is the central transamination route of many amino acids (Cammarata and Cohen, 1950). Asp, Ile, Val, Leu and Ala form glutamic acid and their respective keto-acids via transamination with α-keto-glutarate (Cammarata and Cohen, 1950), whereas Pro does not transaminate because its amino nitrogen is part of the ring structure since it is synthesized via ring closure from glutamic acid (O´Connell, 2017). The amino-nitrogen of proline is derived from the same AAs pool as the glutamate from which it was originated that had previously suffered deamination.

The use of CSIA-AA as a tool for ecosystems studies and TP estimation remains challenging and requires a better understanding of the mechanisms and factors that underlie the variation in the $\delta^{15}N$ values of AAs within and between species (Nielsen et al., 2015). Laboratory experiments can help to understand the variability in $\delta^{15}N$ values of AA and TEFs by controlling some factors that may have an effect on isotopic composition and isotopic discrimination. Recent literature surveys have reported high variability in empirical estimates of TEFs of source and trophic AAs. The level of variation reported for each AA challenges the application of universal values for TEFs across taxa and trophic levels in ecological studies.

In animals, nutritional components of the diet (AAs, fatty acids and glucose) constitute the energy and organic matter sources (NRC, 2011) and thus it is logical to think that nutritional factors may have an effect on nitrogen

isotopic discrimination. The source and trophic AA classification derived from the isotopic literature does not correspond with the nutritional classification of essential (EAA) and non-essential amino acids (NEAA). Essential AA can be synthesized by some bacteria and autotrophs, but heterotrophs must assimilate them from their diet, whereas NEAA can be synthesized by heterotrophs.

Ecological and physiological factors also influence AA isotopic fractionation. Two of the main factors influencing the variability in TEFs bulk and AA are quantity and quality of dietary protein (Martínez del Río, Wolf, Carleton, & Gannes, 2009; McMahon, Thorrold, Elsdon, & McCarthy, 2015; Nielsen et al., 2015). Protein is a primary body constituent and an energy substrate. Protein requirements, that is, the minimum amount of protein needed to maximize growth (Dacosta- Calheiros, Arnason, & Bjornsdottir, 2003), are determined by the EAA requirements of a given species. Protein accretion is a determinant of biomass gain and utilization of AAs, and varies due to endogenous (e.g., life stage) and exogenous (e.g., diet) factors.

Therefore, it is necessary to run controlled feeding experiments to evaluate the effect of nutritional factors on the variation of isotopic discrimination from an ecological stand point, as Florin et al. (2011) and Kelly and Martínez del Río (2010) did for protein content in bulk tissues. The main achievement of this work is the inclusion of nutrition as a theoretical framework and tool as well as ecological factors that may contribute to the understanding of physiological and biochemical processes that lead to variation in the isotopic nitrogen composition through controlled experiments in the laboratory which may contribute to a correct interpretation in an ecological context.

1.2. Objetives

Evaluate the relationship between protein quantity and quality on the nitrogen isotope discrimination in bulk tissues and amino acids in liver and muscle tissues of a carnivorous fish, *Seriola lalandi* based on controlled laboratory experiments (Chapter 2).

Evaluate the relationship between dietary components (protein and lipid content and nutritional requirements, feeding regime) and ecological factors (prey type, habitat type and life stage) on the trophic enrichment factors of amino acids of teleosts based on a meta-analysis of existing literature (Chapter 3).

CHAPTER 2. Amino acid-specific $\delta^{15}N$ trophic enrichment factor in fish fed with formulated diets varying in protein quantity and quality

2.1. Introduction

Tracing organic material and energy fluxes through food webs is important for determining the functional role of species within an ecosystem. The nitrogen stable isotope ratios ($\delta^{15}N$) of bulk consumer tissues have served as powerful natural tracer to infer nutrient sources, characterize animal dietary composition, estimate trophic level, and reconstruct food web structure (Peterson & Fry, 1987). The differences in $\delta^{15}N$ values between a consumer and its diet, also known as the trophic enrichment factor (TEF), were believed to be relatively constant across food webs and are essential for estimating trophic position (TP) (DeNiro & Epstein, 1981; Minigawa & Wada, 1984). The TEF in bulk tissue (TEF_{bulk}) ranges from 2.5 to 5‰ for most soft tissues (reviewed by Vanderklift & Ponsard, 2003; McCutchan, Lewis, Kendall, & McGrath, 2003) and varies depending on diet type (Vander Zanden & Rasmussen, 2001), protein quality (Florin, Felicetti, & Robbins, 2011; Robbins, Felicetti, & Sponheimer, 2005), tissue type (Hobson & Clark, 1992; Malpica-Cruz, Herzka, Sosa-Nishizaki, & Lazo, 2012), taxa, and the mode of nitrogen excretion (McCutchan et al., 2003). Because TEF_{bulk} values are incorporated into isotope mixing models to elucidate trophic relationships and food web structure, the use of imprecise TEF_{bulk} values would lead to inaccurate estimates of both TP and the contribution of food sources to tissue production (Phillips, 2012; Post, 2002). Estimating TP requires characterization of the isotopic baseline by measuring the isotopic composition of primary producers (or primary consumers as their proxy) (Cabana & Rasmussen, 1996; Post, 2002). Determination of the $\delta^{15}N_{baseline}$ is difficult due to high temporal and spatial variability in primary producer isotopic ratios, as well as the temporal uncoupling between source isotope ratios and those integrated by higher level consumers (McMahon, Hamady, & Thorrold, 2013; Popp et al., 2007; Post, 2002).

Compound-specific isotope analysis (CSIA) of amino acids (AAs) is a developing complementary technique with the potential for reducing the limitations of N stable isotope analysis (SIA) on bulk tissue for estimating TP (e.g., Chikaraishi et al., 2009; McClelland & Montoya, 2002; Ohkouchi et al., 2017; Popp et al., 2007). Some AA $\delta^{15}N$ values quantified from animal tissues reflect baseline isotope ratios and others consumer trophic level. Currently, source AAs include phenylalanine (Phe), methionine (Met), and lysine (Lys). These AAs presumably reflect primary producer values due to low isotopic discrimination with each trophic step (Popp et al., 2007). In contrast, trophic AAs such as glutamic acid (Glu), aspartic acid (Asp), alanine (Ala), isoleucine (Ile), leucine (Leu), proline (Pro), valine (Val) show large isotopic discrimination with each trophic step. Serine (Ser), threonine (Thr), and glycine (Gly) were initially considered source AAs, but they can exhibit variable and high isotopic fractionation in high trophic level consumers,

and do not fit strictly into the source category (Germain, Koch, Harvey, & McCarthy, 2013; McCarthy, Benner, Lee, & Fogel, 2007; McMahon & McCarthy, 2016). N isotope discrimination associated with source AAs (minimal) and trophic AAs (large) has been attributed to whether transamination involves cleavage of a C–N bond (Chikaraishi, Kashiyama, Ogawa, Kitazato, & Ohkouchi, 2007; Chikaraishi et al., 2009). However, isotopic discrimination can also occur during deamination, and both essential AAs (EAA; those that cannot be synthesized de novo by a heterotroph) and nonessential AAs (NEAA) can serve as energy sources producing substrates involved in enzymatic chemical reactions (O'Connell, 2017). A more integrative understanding of the biochemical conditions and processes that discriminate nitrogen isotopes is required. O'Connell (2017) specifies that N isotope discrimination should be considered as the result of an AA transamination, deamination, and the exchange of amino groups within the active N pool.

The difference in TEF_{AA} between a trophic and a source AAs is used to estimate TP, and this difference (e.g., $TEF_{Glu} - TEF_{Phe}$ = 7.6‰ for the canonical AAs) was initially assumed to be constant across species, tissues, and trophic levels from all ecosystems (e.g., Chikaraishi et al., 2009; Popp et al., 2007). Meta-analyses of AA isotopic fractionation indicate that trophic AAs TEFs exhibit high variability between taxa due to differences in diet composition, taxa, and mode of nitrogen excretion (McMahon & McCarthy, 2016; Nielsen, Popp, & Winder, 2015). Source AAs TEFs can also vary substantially (Steffan et al., 2013; McMahon & McCarthy, 2016 and references therein, O'Connell, 2017). For example, Nakashita et al. (2011) measured blood $\delta^{15}N$ values of Phe and Glu of long-term captive black bears (*Ursus thibatanus*) and wild black bears fed with known diets, and found differences of up to 4.6 and 8.5‰ in TEF estimates, respectively. Taxon-specific empirical estimates of TEFs that evaluate the role of specific dietary protein attributes are necessary. Furthermore, the TPs of marine mammals and other high trophic level predators have been underestimated (e.g., McMahon & McCarthy, 2016; Nielsen et al., 2015) when using CSIA-AA $\delta^{15}N$ values and applying the "universal" TEF proposed by Chikaraishi et al. (2009); these results highlighting the need for taxon and TP-specific TEF estimates.

Two of the main factors influencing the variability in TEFs bulk and AA are quantity and quality of dietary protein (Martínez del Río, Wolf, Carleton, & Gannes, 2009; McMahon, Thorrold, Elsdon, & McCarthy, 2015; Nielsen et al., 2015). Protein is a primary body constituent and an energy substrate. Protein requirements, that is, the minimum amount of protein needed to maximize growth (Dacosta-Calheiros, Arnason, & Bjornsdottir, 2003), are determined by the EAA requirements of a given species. Protein accretion is a determinant of biomass gain and utilization of AAs, and varies due to endogenous (e.g., life stage) and exogenous (e.g., diet) factors. Martínez del Río and Wolf (2005) made three predictions regarding the relationship between food protein and bulk tissue isotope discrimination: (a) TEF_{bulk} should increase with dietary protein content given that excess dietary protein is catabolized and used as an energy substrate and hence excreted in urine depleted in ^{15}N, (b) TEF_{bulk} should decrease with higher protein quality due to the increase in protein intake to meet energy and protein requirements and thus higher AA catabolism, and (c) TEF_{bulk}

should decrease with the efficiency of N deposition due to reduced protein catabolism. Experimental studies on fish and other taxa are inconsistent or contradictory regarding the relationship between TEF_{bulk} or TEF_{AA} and protein quality (see review by Martínez del Río et al., 2009; McMahon & McCarthy, 2016). Early studies on CSIA-AA analyzed the effect of protein quantity on TEF_{AA} dynamics using both wild-caught and captive specimens of various taxa (e.g., Bradley, Madigan, Block, & Popp, 2014; Chikaraishi et al., 2007, 2009; McClelland & Montoya, 2002; McMahon, Polito, Abel, McCarthy, & Thorrold, 2015; McMahon, Thorrold, et al., 2015). As it has been recognized for SIA in bulk tissues (McCutchan et al., 2003; Vanderklift & Ponsard, 2003), recent studies using CSIA-AA indicate that diet quality can account for the reported variation in TEF_{AA} between taxonomic groups and trophic levels (Chikaraishi, Steffan, Takano, & Ohkouchi, 2015; Ohkouchi et al., 2017). Feeds with the same protein quantity that overlook variability in protein sources can show pronounced differences in protein quality (McGoogan & Reigh, 1996) due to variations in protein digestibility and AA profile (Masumoto, Ruchimat, Ito, Hosokawa, & Shimeno, 1996). Digestibility is the term used to assess the availability of nutrients to the fish. The term refers to the process of digestion and absorption of nutrients in the digestive system of the organism. Digestion refers to the process of solubilization and hydrolization of nutrient polymers (proteins) into their monomers (amino acids) for latter absorption. Not all proteins are easily digested by fishes; in particular plant proteins have typically low digestibility (see NRC, 2011). For these reasons, independently elucidating the effect of protein quantity and quality within specific taxa will provide the foundation for robust comparisons with other groups with different physiological characteristics.

In fishes, some studies have shown that protein quantity is positively related to TEF_{bulk} (Focken, 2001; Kelly & Martínez del Río, 2010), while others indicate a negative significant relationship (Barnes, Sweeting, Jennings, Barry, & Polunin, 2007; Martín-Pérez et al., 2013). Regarding CSIA-AA, an omnivorous fish fed with a low-protein plant-based diets resulted in very high $\delta^{15}N$ TEFs of trophic AAs in comparison with those fed with diets containing animal protein and higher content (McMahon, Thorrold, et al., 2015). Therefore, carnivorous and omnivorous fish fed with vegetable-based diets with very-low-protein content may yield ecologically unrealistic TEFs that should not be applied to wild fish that feed at high trophic levels.

To date, the number of studies investigating the underlying variability in TEF_{AA} is lower than that conducted for TEF_{bulk}. Early studies on CSIA-AA analyzed the effect of protein quantity on TEF_{AA} dynamics using both wild-caught and captive specimens of various taxa (e.g., Bradley et al., 2014; Chikaraishi et al., 2007, 2009; McClelland & Montoya, 2002; McMahon, Polito, et al., 2015; McMahon, Thorrold, et al., 2015), and only the most recent studies indicate that diet quality influences TEF_{AA} (Chikaraishi et al., 2015; McMahon, Thorrold, et al., 2015). However, studies that report TEF_{AA} estimates based on multiple food sources covaried protein quantity and quality (Table 1), making it impossible to separate the effect of protein quality from protein quantity on TEF variability.

Table 1. Summary of studies that examined the effect of dietary protein quantity and quality on TEF_{bulk} and TEF_{AA} in fish. Experiments in which fish were fed a single diet are included for comparative purposes.

TEF	Species	Tissue	Protein source in diet	Co-vary protein quantity and quality	Co-vary AA profile and digestibility	WR min-max	Reached equilibrium*	Reference
TEF_{bulk}	*Oreochromis niloticus* (Nile tilapia)	muscle	Fish meal, wheat gluten and soybean concentrate	No: only vary protein quantity	No	1.3-2.1	No	Focken (2001)
TEF_{bulk}	*Oreochromis niloticus* (Nile tilapia)	whole body	Two diets: 1) wheat gluten+EAA 2) fish meal+wheat meal	Yes	Yes	1.0-3.0	Probably only in fish with highest biomass gain	Gaye-Siessegger, Focken & Abel (2003)
TEF_{bulk}	*Ciprinus carpio* (Carp)	whole body	Fish meal+wheat meal-based commercial diet	No: only varied protein quantity	No	1.0-5.4	Probably only in fish with highest biomass gain	Gaye-Siessegger, Focken & Muetzel (2004)
TEF_{bulk}	*Oreochromis niloticus* (Nile tilapia)	whole body	Wheat gluten+synthetic AA	No: only varied protein quantity	ND	1.3	No	Gaye-Siessegger, Focken & Abel (2004)
TEF_{bulk}	Dicentrarchus labrax (European sea bass)	muscle	Sandeels (non-formulated diet)	No: only varied protein quantity	N/A	5.8-7.7	Yes	Barnes et al. (2007)
TEF_{bulk}	*Oreochromis niloticus* (Nile tilapia)	whole body	Three diets: 1) EAA+NEAA 2) EAA+AA precursor 3) EAA+glutamate	No: only varied protein quantity	Yes	0.8-1.1	No	Gaye-Siessegger, Focken & Abel (2007)
TEF_{bulk}	*Oncorhynchus mykiss* (Rainbow trout) *Sparus aurata* (Gilthead sea bream)	liver, muscle, intestine + perivisceral fat	Fish meal, corn gluten meal, wheat gluten, extruded peas, rapeseed meal soybean meal, extruded whole wheat	Yes	Yes	5.8-7.4	Yes	Beltrán et al., (2009)

Table 1.(continued)

TEF	Species	Tissue	Protein source in diet	Co-vary protein quantity and quality	Co-vary AA profile and digestibility	WR min-max	Reached equilibrium*	Reference
TEF_{bulk}	*Oreochromis niloticus* (Nile tilapia)	muscle	Casein	No: only varied protein quantity	No	3.0	Probably	Kelly & Martínez del Río (2010)
TEF_{bulk}	*Sparus aurata* (Gilthead sea bream)	muscle	Fish meal, wheat gluten and soybean concentrate	No: only varied protein quantity	ND	2.5-3.0	Probably only in fish with highest biomass gain	Martín-Pérez et al. (2013)
TEF_{bulk}	*Micropogonias undulatus* (Atlantic croaker)	liver, muscle	1) Low quality: terrestrial sources (fish meal+plant-based) 2) Medium quality: terrestrial (fish meal + plant-based)+marine sources (fish meal) 3) Control feed: marine sources (fish meal)	No	Yes	2-4	Yes, only in fish with highest biomass gain	Mohan et al. (2016)
TEF_{AA}	*Acanthopagrus butcheri* (Black bream)	muscle	1) Fish meal feed 2) Vegetable feed	Yes	Yes	0.9-1.2	No	Bloomfield et al. (2011)
TEF_{AA}	*Thunnus orientalis* (Pacific bluefin tuna)	muscle	70% sardine+ 21% squid+ 9% gelatin (N=non-formulated diets)	No comparison, only one treatment	N/A	93.5	Yes**	Bradley et al. (2014)

Table 1.(continued)

TEF	Species	Tissue	Protein source in diet	Co-vary protein quantity and quality	Co-vary AA profile and digestibility	WR min-max	Reached equilibrium*	Reference
TEF_{AA}	*Carcarias taurus* (Tiger shark), *Negaprion brevirostris* (Lemon shark), *Triakis semifasciata* (Leopard shark), *Pristipomoides filamentosus* (Opakapaka)	muscle	Non-formulated diets: Anchovy, haddock, trevally, saithe, mackerel, whiting, mullet, octopus, krill, squid	No comparison, only one treatment	N/A	ND	ND	Hoen et al. (2014)
TEF_{AA}	*Fundulus heteroclitus* (Mummichug)	muscle	1) Plant-based commercial fish pellet: wheat meal, soy meal, corn meal 2) Omnivorous commercial fish pellet: fish meal, krill meal, wheat gluten, whey protein 3) Clam 4) Squid	Yes	Yes	2	ND	McMahon, Thorrold & Elsdon (2015)
TEF_{AA}	*Seriola lalandi* (Pacific yellowtail)	liver, muscle	Fish meal	No comparison, only one treatment	N/A	4	Yes	Barreto-Curiel et al. (2017)

* A WR = 3 (≈67% change in isotope turnover when assuming simple dilution conditions) was considered as a threshold for isotopic equilibrium.

** According to Madigan et al. (2012), during the experiment sardines and squid were caught several times from the wild and may have varied in isotopic composition. Although fish increased in weight substantially, small variations in the isotopic composition of prey may have led to small biases in TEFs.

ND=no data.

N/A= Not applicable.

Furthermore, the use of artificial formulated fish feeds that do not consider nutrient requirements or that are not representative of the nutritional characteristics of natural diets consumed in the wild (such as the use of vegetable-based diets to feed carnivorous fish) limits our ability to understand the sources of variability in TEF_{AA}. Fish increase consumption rates to compensate for diets with low-protein quality, and to meet both energy and essential nutrient demands for AAs, fatty acids and vitamins (e.g., Saravanan et al., 2012). This adjustment leads to an increase in the amount of dietary protein intake and catabolic activity that can ultimately increase isotope discrimination. From a nutritional perspective, the criteria for formulating or selecting diets and feeding regimes are key in feeding experiments designed to evaluate which dietary factors drive variability in TEFs.

Most studies on CSIA-AA $\delta^{15}N$ focusing on fish have analyzed a single tissue (mainly muscle) (e.g., Blanke et al., 2017; Bradley et al., 2015). Consequently, it is relatively unknown whether AA isotopic discrimination varies between different tissues for fish fed under the same dietary regime. Given that fish tissues can vary substantially in isotope turnover rates and reflect information for different feeding periods (Bradley et al., 2014; Herzka, 2005; Hesslein, Hallard, & Ramlal, 1993), analyzing more than one tissue from the same individuals can yield insights into switches in trophic level and feeding habits over different time scales (e.g., Kurle, 2009; Malpica-Cruz, Herzka, Sosa-Nishizaki, & Escobedo-Olvera, 2013; McNeil, Drouillard, & Fisk, 2006). Muscle and liver metabolism are innately different and play specific functional roles. Muscle tissue is responsible for movement, while the liver is involved in assimilation processes, storage of glycogen and lipids, and excretion, as well as the metabolism of proteins and AA, carbohydrates, and lipids. The metabolism of the fish liver can adapt to variations in AA availability to meet energy and metabolic requirements (Kaushik & Seiliez, 2010); the same AA pool serves for both catabolic and anabolic processes (Cowey, 1975). Moreover, liver serves a regulatory function, adapting to nutrient fluxes in response to tissue and whole-body requirements and the availability of dietary AAs (Enes, Panserat, Kaushik, & Oliva-Teles, 2009). Isotope discrimination in AAs in muscle and liver tissues may therefore differ substantially, rendering the empirical determination of tissue-specific TEFs necessary.

Fish fed high-quality diets (with an adequate amino acid profile and high digestibility) assimilate and accrete as protein between 25% and 55% of the total AA in their diets (Cowey & Walton, 1989; Halver & Hardy, 2002; National Research Council, 2011). The rest of the dietary AA pool (45%–75%) is used to sustain metabolic processes, including maintenance AA requirements and inevitable AA catabolism. The former refers to the AA required to maintain the protein pools in equilibrium and has been estimated to comprise a small proportion of total AA requirements (5%–20%). The latter refers to AA catabolism that occurs even when enough energy for protein synthesis is provided (National Research Council, 2011). Thus, fish have inevitable catabolic processes that cannot be shut down. This inevitable AA catabolism is estimated to be between 20% and 40% of the digestible AAs consumed by the fish above maintenance

requirement (National Research Council, 2011). While source and trophic AAs have been broadly characterized based on whether transamination (and the resulting isotope discrimination) occurs (e.g., Chikaraishi et al., 2009), deamination resulting from AA catabolism will also lead to isotope discrimination (see review by O'Connell, 2017). All AAs are subject to catabolic processes, and hence, the observed variation in both source and trophic TEF_{AA} can be at least partially attributed to AA catabolism.

Considering these facts, we evaluated independently the effect of protein quantity and quality on nitrogen TEF_{bulk} and TEF_{AA} for both liver and muscle tissues of the Pacific yellowtail (*Seriola lalandi*), a model carnivorous species. We assessed the relationship between TEF_{bulk} and TEF_{AA} and protein quantity and quality as a function of fish performance (growth rates, feed conversion ratios, protein efficiency rate, and protein productive value). We hypothesized that TEFs of source AAs would not differ among fish tissues equilibrated with diets differing in protein quantity and quality. For bulk tissue and trophic AAs, we hypothesized that TEFs would increase with increasing protein quantity, because fish should catabolize excess dietary protein resulting in higher excretion of ^{15}N-depleted nitrogen and decrease with increasing protein digestibility (quality) due to direct routing and assimilation of available protein into fish tissues, which involves limited catabolic processes.

2.2. Methods

2.2.1. Experimental diets

We formulated five experimental diets to contain increasing levels of digestible protein (DP) by changing the quantity and quality of a single batch of high-quality fish meal (that contain highly digestible protein and with an AA profile that meets nutritional requirements; Table 2 and Supporting Information Table S1). The main protein source was a high-quality 60% crude protein (CP) content fishmeal (Special Select, Omega Protein, Texas, USA) made from menhaden that containing a reported 60% crude protein, 6% crude fat, 2% crude fiber, 4.3%–5.3% calcium, and 2.5% phosphorus. A review of the AA content reported in the Special Select fish meal relative to the AA-specific dietary requirements of *S. lalandi* indicated that the diets had sufficient AA content to meet the species requirements (data not shown).

Table 2. Experimental diet design. Diet codes reflect the percentage of digestible plus non-digestible crude protein in each diet.

Diet code	Digestible crude protein (%)	Non-digestible crude protein (%)	Total protein (%)
40+0	40	0	40
50+0	50	0	50
60+0	60	0	60
40+10	40	10	50
50+10	50	10	60
Commercial	57	0	57

Seriola lalandi was used as a model for a carnivorous marine teleost species because it is easy to raise in captivity, its nutritional requirements are well characterized, and it exhibits very fast growth rates. Diets were formulated based on the known protein and AA requirements for *S. lalandi* (Masumoto, 2002; NRC, 2011). One had the optimal required protein level as described in those two references that are based on nutritional studies (50% CP), another one with lower protein level (40% CP) and a third one with higher protein level (60% CP; hereafter referred to as diets 40 + 0, 50 + 0, and 60 + 0, respectively). Two additional experimental diets were formulated to contain 50% and 60% total crude protein but with 40% and 50% estimated digestible protein, respectively. This was achieved by combining 10% non-digestible protein with the 40% and 50% digestible protein for a total of 50% and 60% crude protein (hereafter 40 + 10 and 50 + 10 diets, respectively). The nondigestible protein was prepared using the fish meal treated with formaldehyde to reduce the digestibility of the protein source using the well-known protocol described by Antoniewicz, van Vuuren, van der Koelen, and Kosmala (1992). This technique is commonly used in terrestrial animal (ruminants) nutrition studies to reduce protein digestibility (Wulf & Südekum, 2005), and has been successfully applied to fish nutrition studies (Durazo et al., 2010). Formaldehyde (FA) treatment of dietary protein sources is not harmful to experimental fish as indicated by high growth rates, and allows for the formulation of diets with the same protein source and amino acid profile but different digestible protein content.

Feed ingredients (Table 3) were ground to pass through a 1.02 mm diameter sieve. The ingredients were blended with the fish oil using a food mixer for 15 min, cold-extruded with a meat grinder using a 3 mm die and air-dried to a moisture content <10%. A commercially formulated diet for marine fish (Skretting, UK; ≥55% crude protein, ≥15% crude fat, ≥1% crude fiber, ≥11.4% ash) was used as reference to evaluate fish growth and nutritional performance (here after referred to as commercial diet).

Table 3 Formulation of the experimental diets (g ingredient/100 g diet) on dry weight basis and proximate analysis of the prepared diets and commercial reference diets. FA: formaldehyde

Ingredient (g/100 g diet)	Diet (40+0)	Diet (50+0)	Diet (60+0)	Diet (40+10)	Diet (50+10)	
Casein	5	6.4	7.7	5	6.4	
Fish meal[1]	50	64	77	50	64	
Fish meal treated with FA	0	0	0	15.4	14.7	
Jelly	3	3	3	3	3	
Fish oil	17	12	8	14	8	
Gelatinized starch	15	8	0.8	9.1	0.4	
Cellulose	6.5	3.1	0	0	0	
Vitamins	2	2	2	2	2	
Mineral mix	1	1	1	1	1	
Vitamin C	0.5	0.5	0.5	0.5	0.5	
Total	100	100	100	100	100	
Proximate composition	**Diet (40+0)**	**Diet (50+0)**	**Diet (60+0)**	**Diet (40+10)**	**Diet (50+10)**	**Commercial Diet**
Total crude Protein (%)	42.1 ± 0.2	51.9 ± 2.7	61.3 ± 1.6	49.5 ± 3.2	60.0 ± 0.2	56.9 ± 0.2
Lipids (%)	20.4 ± 0.5	16.1 ± 0.3	12.1 ± 1.0	10.1 ± 0.2	8.9 ± 1.7	9.0 ± 0.6
NFE[2] **(%)**	19.6	14.2	8.6	14.3	7.2	
Ash (%)	16.8 ± 0.1	18.7 ± 0.1	21.5 ± 0.1	18.9 ± 0.5	21.5 ± 0.2	12.5 ± 0.2
Energy (kJ/g)	21.3	21.3	21.4	19.3	18.6	20.5
P:E (mg/kJ)	18.8	23.5	28.0	25.9	32.2	27.6

1 Omega Protein high digestibility fish meal: 60% crude protein, 6% crude fat, 2% crude fiber, 4.3-5.3% Calcium, 2.5% Phosphorus, <0.015% ethoxyquin
2 NFE, Nitrogen-free extract

Efficiency of the FA treatment was evaluated using a simple multienzyme pH-STAT in vitro digestibility protein assay (Lazo, Holt, & Arnold, 2002). We consider the non-FA-treated fish meal as the digestible crude protein source and the FA-treated fish meal as the non-digestible crude protein (Table 2). Protein hydrolysis by commercial digestive enzymes was reduced by 91% in FA-treated fish meal compared to non-FA-treated fish meal.

2.2.2. Animal culture and feeding

Juveniles were produced from eggs at a commercial Pacific yellowtail hatchery (Baja Seas, Baja California, Mexico). Early juveniles were brought to the Marine Fish Laboratory at the Center for Scientific Research and Higher Education of Ensenada (CICESE) and acclimated for 40 days in two 3 m^3 raceways connected to a recirculating system. Juveniles were maintained at 20 ± 2°C, and salinity at 35 ± 1. Dissolved oxygen (DO) concentrations were kept above 6mg/L and total ammonia [NH^{3+} NH^{4+}] was lower than ≤1.0 mg/L. Raceways were cleaned twice a day and >70% of the water exchanged daily. Fish were hand-fed four times a day using a feeding rate of 6% body weight per day (Nakada, 2000) with commercial diet containing: ≥57% crude protein, ≥15% crude fat, ≤0.2% crude fiber. Individual mortality was recorded daily.

Immediately before the experimental phase, juveniles *S. lalandi* were weighed to the nearest 0.1 g. We observed a bimodal size distribution, and therefore, fish were separated into two groups to minimize the initial variation in size and obtain precise relative weight gain estimates (Carleton & Martínez del Río, 2005). Fishes with an initial weight of 26 to 30 g (mean ± SD: 28 ± 2 g) were assigned to treatments 40+0, 50+0, and 60+0, and fishes with initial weights of 19 to 24 g (22±2g) to treatments 40+10, 50+10, and the commercial diet. Treatments were randomly allocated to tanks (n=12 fish per tank, and n=3 tanks per treatment), for a total of 216 individuals.

Each experimental tank had a recirculating water system coupled to a biological filter and a UV light lamp. Temperature, DO, food consumed, and mortality were recorded daily for each experimental tank. Juveniles were held near the optimal temperature for this species (22 ± 2°C) (Pirozzi & Booth, 2009). Other environmental conditions were maintained as described above.

Fish were fed a fixed amount based on the feeding rates suggested by Nakada (2000) for Pacific yellowtail. Feeding regimes were adjusted weekly based on the mean weight of the fish of each tank (range 5.5% body wt/day at the beginning to 2.4% body wt/day at the end of the trial). Feedings were fed three times a day for the first 26 days and twice a day thereafter. Weight (g) and standard length (SL; mm) of 5 individuals (randomly selected per tank) were measured weekly.

2.2.3. Sample collection

Ten fish were collected on day 0 for isotope and proximate analyses. Fish fed with treatments 40+0, 50+0, 60+0, and commercial diets were sampled four to five times throughout the experiment depending on the average

relative increase in biomass (WR = $weight_t$/$weight_{initial}$) for each treatment. Fish in the 40 + 10 and 50 + 10 treatments were only sampled at the beginning and end of the experiment. WR was used to monitor growth because weight gain is a conservative estimate of the percent of isotopic turnover in juvenile fishes; isotopic equilibrium (a steady state between a consumer's isotope composition and its diet) to a new food source can be approached after a four fold to six fold increase in fish biomass (Herzka, 2005). Two fish were collected at ca. WR = 2, WR = 3, WR = 5, WR = 7 for isotope analysis of bulk tissue and individual amino acids during the experiment, and three fishes were collected at the end of the experiment. Fish were euthanized by placing them on ice, weighted and standard length (SL) measured before dorsal muscle and liver tissues were dissected. An additional individual from each tank was sacrificed for proximate analysis. Diet, muscle, and liver samples were frozen at −20°C pending isotope and proximate analyses.

2.2.4. Proximate analysis

Fish feeds, fish muscle, and liver tissues were analyzed for protein, lipid, ash, and nitrogen-free extract. Liver was only analyzed for crude protein at the start of the experiment due to their small size. Crude protein content was estimated based on the percent nitrogen determined during bulk isotope analysis (see below) and calculated as % N × 6.25 (Jones, 1941). Lipid content and ash content were analyzed using the Folch method (Folch, Lees, & Stanley, 1956) and by incineration (Association of Official Analytical Chemists, A.O.A.C., 1990), respectively. Carbohydrate (including fiber) content was estimated as nitrogen-free extract, or NFE (%) = 100 − % protein − % lipids − % ash. Dietary energy was estimated assuming 1 g protein = 5.6 kcal, 1 g lipid = 9.4 kcal, 1 g carbohydrate = 4.1 kcal (Webster & Lim, 2002). The P:E ratio was calculated for each diet.

2.2.5. Sample preparation for bulk isotope analysis

Liver and muscle, diets, and the fish meal were thawed and dried at 60°C and ground into a powder. Lipids were not extracted from any of the samples to avoid bias associated with lipid extractions because several studies have documented shift in $\delta^{15}N$ values after lipid extractions in bulk tissues (Hesslein et al., 1993; Ingram et al., 2007; Pinnegar & Polunin, 1999; Ruiz-Cooley, Garcia, & Hetherington, 2011). Lipid extraction may remove not only lipids but also lipoprotein compounds that have low $\delta^{15}N$ values (Bodin, Le Loc'h, & Hily, 2007; Sotiropoulos, Tonn, & Wassenaar, 2004). Moreover, the variability of $\delta^{15}N$ values may depend on the amount of fat, fatty acids, and

lipoproteins of individuals that vary between tissues, and C:N ratios may not be a good predictor of lipid content (Ruiz-Cooley et al., 2011).

For bulk isotope analysis, 0.8–1.2 mg of homogenized samples were weighed into tin capsules and sent to the Stable Isotope Facility of UC Davis. Fish feeds and samples were analyzed using an Elementar CUBE elemental analyzer (Elementar Analysensysteme GmbH, Langenselbold, Hessen, Germany) interfaced to a VisION isotope ratio mass spectrometer (IsoPrime, Stockport, U.K.). The standard deviations (SD) of the laboratory's quality assurance materials, bovine liver, nylon 5, and glutamic acid, were 0.1‰, 0.3‰, and 0.2‰ for $\delta^{15}N$, respectively. For CSIA-AA, sample preparation involved acid hydrolysis of the fish feeds, fish muscle, and liver samples to liberate amino acids from proteins and subsequent derivatization by methyl chloroformate before sample injection into gas chromatograph (GC, protocol detailed in (Yarnes & Herszage, 2017) before analysis by gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS). The $\delta^{15}N$ values were determined by gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS). CSIA of AAs was performed on a Thermo Trace Gas Chromatograph coupled to a Delta V Advantage IRMS via a GC IsoLink combustion interface (Thermo Electron, Bremen, Germany). During each measurement, provisional values were calculated by adjusting measured values to a coinjected internal reference material, D-norleucine. Subsequently, an external reference mixture was used to calibrate each individual amino acid, such that the known $\delta^{15}N$ value was obtained (Yarnes & Herszage, 2017). Each experimental sample was analyzed in duplicate. The use of alkyl chloroformates in the measurement of $\delta^{15}N$ is relatively new, however, a recent comparison of $\delta^{15}N$-AA measurements as methoxycarbonyl methyl esters (MOC; Walsh, He, & Yarnes, 2014) and N-acetyl isopropyl esters (NAIP; Styring, Knowles, Fraser, Bogaard, & Evershed, 2012), a more traditional esterification-acylation technique, yielded comparable $\delta^{15}N$-AA results across a range of sample types (Yarnes & Herszage, 2017). The following amino acids were reproducibly quantified in all analyzed samples: Ala, Val, Gly, Ile, Leu, Pro, Asp, Phe, Glu, Lys, and Met. The SD was calculated from duplicate measurements on each liver and muscle sample and values are reported in the Supporting Information Table S1 (overall mean SD: 0.5‰ for liver and 0.4‰ for muscle; range SD: 0.2%–0.7‰ for liver and 0.2–0.6‰ for muscle). The SD of individual AAs from duplicates was generally below 0.8‰ for all AAs, except for Asp, Glu, and Lys in the diet samples only (1.0, 1.5, and 1.0‰, respectively). Accuracy of calibration and quality assurance mixtures was high, and the standard deviations of all AA standards were ≤1.2 ‰ (mean SD: 0.8‰; Supporting Information Table S2). Stable isotope values are expressed in standard delta notation (δ) with respect to atmospheric nitrogen: $\delta^{15}N\,(‰) = ([R_{sample}/R_{standard}] - 1) \times 10^3$, where R is the isotope ratio $^{15}N:^{14}N$.

2.2.6. Growth performance and survival

Growth performance was assessed by calculating final body weight, absolute weight gain, specific growth rate (SGR; Halver & Hardy, 2002) and WR as a function of time. Nutritional response variables were calculated using the following formulas (De Silva & Anderson, 1995), where the initial weight (W_i) and the weight at time t (W_t) are in grams:

$$\text{Feed intake (g fish}^{-1}\text{98 day}^{-1}\text{)} = \text{sum 98-day feed intake per fish} \quad (1)$$

$$\text{Feed Conversion ratio (FCR)} = \text{feed intake (g)/fish weight gain (g)} \quad (2)$$

$$\text{Protein efficiency ratio (PER)} = \text{fish weight gain (g)/protein intake (g)} \quad (3)$$

$$\text{Protein productive value (PPV)} = \text{fish protein gain (g)/protein intake (g)} \quad (4)$$

$$\text{Survival (\%)} = [100 - (\text{number of dead individuals/total individuals per tank})] \times 100 \quad (5)$$

Fish growth performance calculations using fish weight and body composition are expressed as dry weights and feed consumption rates are reported as wet weights.

2.2.7. Evaluation of isotopic equilibrium

To evaluate whether isotopic equilibrium was reached we first evaluated the pattern of isotopic turnover for two source (Phe and Gly) and two trophic (Glu and Ala) AAs. Phe and Glu were selected based on their widespread use and importance described in the literature. An asymptotic pattern is expected in the isotopic composition of liver and muscle tissue as a function of WR if isotopic equilibrium is reached. We also estimated the percent of isotopic turnover achieved in each treatment as a function of weight gain following Herzka (2005). These estimates are based on mass balance considerations that assume simple dilution conditions (i.e., growth is considered the only process driving isotopic turnover), and are thereby conservative. The WR for each treatment was also calculated and expressed relative to absolute weight. Because fish size differed between treatments on d = 0, percent isotopic turnover and WR were calculated separately for treatments with a mean initial weight of 22 and 28 g. The consistency between the final ($\delta^{15}N_{Final}$) and prefinal ($\delta^{15}N_{Final-1}$) isotopic measurements in fish tissues was evaluated using an independent sample Student's t test.

2.2.8. Data and statistical analysis

Final measurements of tissue-specific bulk $\delta^{15}N$ values were calculated as $TEF_{bulk} = \delta^{15}N\text{tissue} - \delta^{15}N\text{diet}$. In the CSIA-AA literature, TEF refers to the ^{15}N enrichment with each AA with trophic level following Chikaraishi et al. (2015) and McMahon, Thorrold, et al. (2015):

$$TEF_{AA} = \delta^{15}N\text{-}AA_{tissue} - \delta^{15}N\text{-}AA_{diet} \quad (6)$$

where $\delta^{15}N\text{-}AA_{tissue}$ and $\delta^{15}N\text{-}AA_{diet}$ represent the nitrogen isotopic value each AA in the consumer's tissue and diet, respectively. Average values ±1SD of TEF_{bulk} and TEF_{AA} for each treatment were calculated based on individual $\delta^{15}N$-AA values (n = 3) measured at the end of the experiment relative to the diets.

Statistical analyses were carried out using SYSTAT V 11. One-way ANOVAs were used to test for differences in proximate composition, growth performance (WR, SGR), nutritional performance (FCR, PER, PPV) and survival between treatments. The effect of protein quantity and quality on final fish weight was tested with an ANCOVA using mean initial size as a covariate. Statistical analyses included the reference diet only when evaluating growth performance and nutritional response.

The absolute difference between TEF_{AA} for liver and muscle tissues were plotted for each amino acid and treatment. The effect of treatments on TEF_{bulk} and TEF_{AA} for liver and muscle were also tested with one-way ANOVA. Assumptions of homogeneity of variances were checked using Levene's equal variance test. Tukey's honestly significantly different (HSD) test with p = 0.05 was applied to identify significant differences between treatment when ANOVA results indicated significant differences between treatments. To determine whether protein quantity influenced TEFs, we focused on post hoc test results comparing the 40 + 0, 50 + 0, and 60 + 0 treatments. To evaluate the effect of protein quality, we compared the 50 + 0 vs. 40 + 10 and the 50 + 10 vs. 60 + 0 treatments. The TEFs estimated for fish fed with the reference commercial diet were excluded from statistical analysis when evaluating the effect of protein quantity and quality because its quality varied in an uncontrolled fashion relative to our formulated experimental diets. Power analyses were run using a one-way ANOVA model to estimate the probability of correctly rejecting the null hypothesis by setting an alpha level of 0.5 and n = 3. Student's t tests were applied to identify differences between liver and muscle tissue TEF_{bulk} and TEF_{AA} (alpha = 0.05).

2.3. Results

2.3.1. Survival, growth, and nutritional response

There were no significant differences in mortality ($p > 0.05$, Table 4) among dietary treatments. Specific growth rates differed significantly among treatments (one-way ANOVA, $df = 5$, $F = 17.3$, $p < 0.001$) and ranged from 1.3 to 2.1%/day. Growth rates differed significantly between protein levels, but did not differ significantly between treatments with same protein level but with different protein quality; 50+0 vs. 40+10 and 60+0 vs. 50+10 (Table 4). Final relative biomass gain (WR) ranged from 3.6 (40+0 diet) to 7.9 (commercial diet). The lowest WR value was found with the diet containing the lowest protein content. Final WR varied significantly between treatments with different protein content, but protein quality did not have a significant effect on final WR (Table 4).

Feed conversion ratios (FCR) ranged from 1.4 (commercial diet) to 2.6 (diet 40 + 0) (Table 4) and differed significantly among treatments ($F=5.3$, $df=5$, $p=0.008$). The lowest (best) FCR value (1.4) was achieved by fish fed the commercial diet, followed by the 60+0 diet (1.7). Significant differences (one-way ANOVA $F=5.3$, $df=5$, $p=0.036$) were found in FCR among fish fed the higher protein quantity (60 + 0) treatment compared to the treatment with the lowest protein quantity (40 + 0). Treatments with different protein quality were not statistically significantly different in FCR. Protein efficiency ratios (PER) differed significantly among treatments ($F=3.3$, $df=5$, $p=0.04$) and were lower in the higher protein and lower digestibility treatment. Protein productive values (PPV) differed significantly among treatments ($F=3.2$, $df=5$, $p=0.046$). However, PPV did not differ between fish fed with diets varying in protein quantity and quality.

Table 4. Growth performance and nutritional parameters of juvenile *Seriola lalandi* fed with diets differing in quantity and quality of digestible protein (DP) during a 98-day feeding experiment ($n = 3$). Parameters: SGR = specific growth rate, WR = relative weight gain ($W_t/W_{initial}$), FCR = feed conversion rate, PER = protein efficiency rate, PPV = protein productive value. Values with different superscripts within a line are significantly different ($p < 0.05$) based on one-way ANOVA followed by Tukey's HSD multiple comparison test. Diet codes indicate the percentage of digestible crude protein + nondigestible crude protein

	40+0 (mean ± SD)	**50+0** (mean ± SD)	**60+0** (mean ± SD)	**40+10** (mean ± SD)	**50+10** (mean ± SD)	**Commercial** (mean ± SD)
Initial body weight (g)	28.0 ± 2	28.0 ± 2	28.0 ± 2	21.5 ± 2	21.5 ± 2	21.5 ± 2
Final body weight (g)	100.1 ± 14.9^{a}	153.4 ± 21.0^{ab}	153.4 ± 11.8^{b}	113.9 ± 2.9^{a}	129.5 ± 15.2^{a}	169.8 ± 6.3^{b}
SGR (% body weight day^{-1})	1.3 ± 0.2^{a}	1.6 ± 0.2^{ab}	1.7 ± 0.1^{b}	1.7 ± 0.0^{b}	1.8 ± 0.1^{bc}	2.1 ± 0.0^{c}
WR	3.6 ± 0.2^{a}	5.5 ± 0.1^{b}	5.5 ± 0.5^{b}	5.3 ± 0.4^{b}	6.0 ± 0.7^{bc}	7.9 ±0.9^{c}
Feed intake (g 98 day^{-1} fish^{-1})	182.0 ± 4.4^{a}	217.2 ± 0.7^{b}	213.7 ± 5.0^{b}	180.2 ± 4.2^{a}	188.2 ± 2.3^{a}	209.0 ± 3.8^{b}
FCR	2.596 ± 0.5^{a}	2.125 ± 0.5^{ab}	1.712 ± 0.1^{b}	1.953 ± 0.1^{ab}	1.765 ± 0.3^{ab}	1.411 ± 0.0^{b}
PER	0.938 ± 0.2^{ab}	0.900 ± 0.2^{a}	0.939 ± 0.1^{ab}	1.037 ± 0.1^{ab}	0.953 ± 0.1^{ab}	1.247 ± 0.0^{b}
PPV	0.506 ±0.1^{ab}	0.465 ± 0.1^{a}	0.488 ± 0.0^{ab}	0.545 ± 0.1^{ab}	0.526 ± 0.1^{ab}	0.733 ± 0.2^{b}
Survival (%)	89 ± 4.8^{a}	75 ± 8.3^{a}	84 ± 8.3^{a}	81 ± 21.0^{a}	81 ± 4.8^{a}	81 ± 4.8^{a}

2.3.2. Proximate analysis

The protein content of initial liver tissue did not differ significantly between fish with initial mean weight of 28 and 22 g: Only lipid content in muscle tissue differed significantly (p = 0.05; Table 5). In liver tissue, the mean protein content of fish at the end of the experiment was variable but did not differ significantly among treatments. Table 5). There were no significant differences in protein, lipid, and ash content of muscle tissue at the end of the experiment among treatments (Table 5).

Table 5. Proximate analysis of liver and muscle tissues (mean ± *SD*; *n* = 3 replicates per treatment) of juvenile *Seriola lalandi*. Fish with a mean weight of 28 and 22 g were fed diets differing in percentage and quality of digestible protein and sampled after a 98-day feeding experiment. Proximate analyses are reported on dry weight basis. Percent ash and lipids could not be determined for liver tissue due to their small size. Diet codes indicate the sum of digestible protein + nondigestible protein. Different superscripts within a line are significantly different ($p < 0.05$) based on one-way ANOVA followed by Tukey's HSD multiple comparison test

	Dietary treatment							
	Initial wt 28 g	**Initial wt 22 g**	**40+0**	**50+0**	**60+0**	**40+10**	**50+10**	**Commercial diet**
Fish liver								
Protein (%)	68.4 ± 7.8[a]	68.9 ± 2.5[a]	64.2 ± 7.3[a]	55.6 ± 12.6[a]	62.3 ± 7.3[a]	59.8 ± 10.3[a]	55.0 ± 5.6[a]	48.2 ± 3.4[a]
Fish muscle								
Protein (%)	83.8 ± 2.6[a]	84.0 ± 0.2[a]	87.1 ± 1.6[a]	84.2 ± 3.1[a]	85.0 ± 0.4[a]	85.7 ± 3.1[a]	88.2 ± 1.5[a]	85.0 ± 3.2[a]
Lipids (%)	7.7 ± 0.2[a]	8.8 ± 0.5[b]	5.4 ± 0.3[a]	3.7 ± 1.6[a]	3.9 ± 0.9[a]	7.9 ± 1.3[a]	9.5 ± 2.3[a]	4.9 ± 1.1[a]
Ashes (%)	12.3 ± 1.8[a]	12.7 ± 3.7[a]	9.8 ± 0.4[a]	8.4 ± 0.9[a]	8.8 ± 0.6[a]	8.8 ± 1.3[a]	10.1 ± 0.7[a]	8.8 ± 1.5[a]

2.3.3. Evaluation of isotopic equilibrium

Isotopic shift patterns from the selected source and trophic AAs exhibited an asymptotic behavior after the switch in diet (Figure 1). Isotopic equilibrium was approached at WR ≈ 3 by the four selected amino acids for all treatments and both tissues as well as the commercial diet. The calculated percent of isotopic turnover as a function of weight ranged from 72% to 87%. Fish with the slowest growth rate achieved a conservative estimate of isotopic turnover of 72% (Figure 2) at final WR = 3.6. The final (day 98) and next to last $\delta^{15}N$ values from fish liver and muscle tissues did not differ significantly for bulk tissue (t-student, $p > 0.05$) and the four selected AAs (t-student, $p > 0.05$; Figure 1).

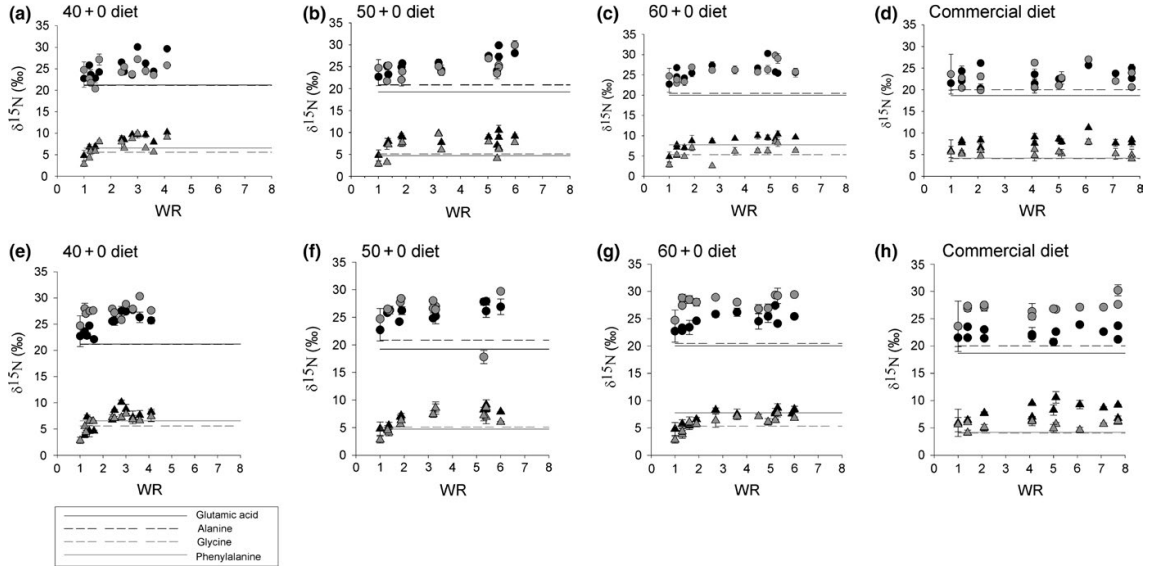

Figure 1. Pattern of nitrogen isotopic turnover of select amino acids in liver and muscle tissue of juvenile Seriola lalandi subjected to an abrupt dietary shift. Changes in isotopic ratios are expressed as a function of relative weight gain (WR = Wt/Winitial). $\delta^{15}N_{AA}$ values are shown for liver (a–d) and muscle (e–h) tissues for two trophic amino acids (glutamic acid (black circles) and alanine (gray circles) and two source amino acids (phenylalanine in black triangles and glycine in gray triangles). Symbols represent individual fish; errors are 1 standard deviation of replicates for each sample. $\delta^{15}N_{AA}$ of the diets are represented by horizontal lines. Diet codes indicate the percentage of digestible + nondigestible crude protein (see Table 2)

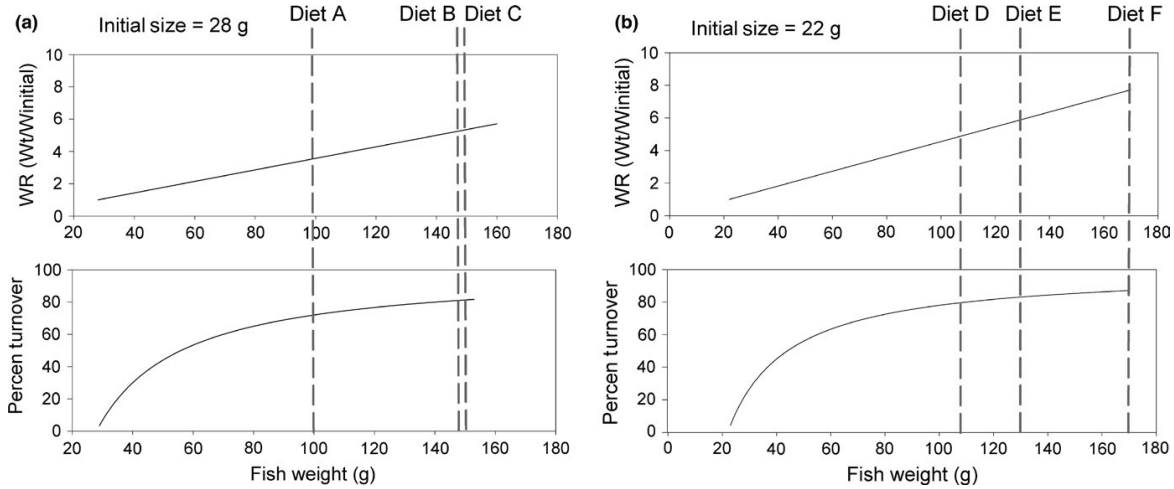

Figure 2. Simple dilution model of the expected isotope turnover pattern for juvenile *Seriola lalandi* subjected to dietary shift at a mean weight of 28 g (a) and 22 g (b). The mean relative weight gain (WR = $W_t/W_{initial}$) achieved by fish fed diets differing in the percentage of digestible + nondigestible crude protein is indicated by vertical lines, (diet A=40+0, diet B=50+0, diet C=60+0, diet D=50+10, diet E=60+10)

Isotopic equilibrium was therefore approached by the end of the experiment for all treatments in both fish tissues as indicated by three criteria: (a) the observed asymptotic isotopic pattern for the selected source and trophic- AAs after an abrupt dietary shift, (b) the estimated high percent of isotopic turnover as a function of weight gain

observed for all diets (>72%), and (c) the absence of statistical differences in the $\delta^{15}N_{AA}$ between the last two sampling times for select AAs. Although we did not measure $\delta^{15}N$ values during the course of the experiment for fish fed diets 40+10 and 50+10, we assume that equilibrium was also approached because fish achieved a greater WR than fish fed the lowest protein diet (i.e., diet 40+0). Also, WRs were similar to those calculated for fish fed diets 50+0 and 60+0. The rigorous confirmation of the approach to isotopic equilibrium is conducive to robust estimates of TEFs.

2.3.4. Isotope values of diets and final fish liver and muscle tissues

There was low variability in bulk $\delta^{15}N$ values among the formulated diets (SD = 0.3‰), and fish liver and muscle tissues at the end of the experiment (Figure 4). Final individual $\delta^{15}N$ values of source amino acids Phe, Lys, Met, and Gly ranged from 6.7 to 12.5‰ for liver, and from 7.6 to 10.9‰ for muscle. Final individual $\delta^{15}N$ values of trophic amino acids Asp, Glu, Ile, Pro, Val, Leu, and Ala ranged from 21.2 to 26.8‰ for liver, and from 17.4 to 26.9‰ for muscle.

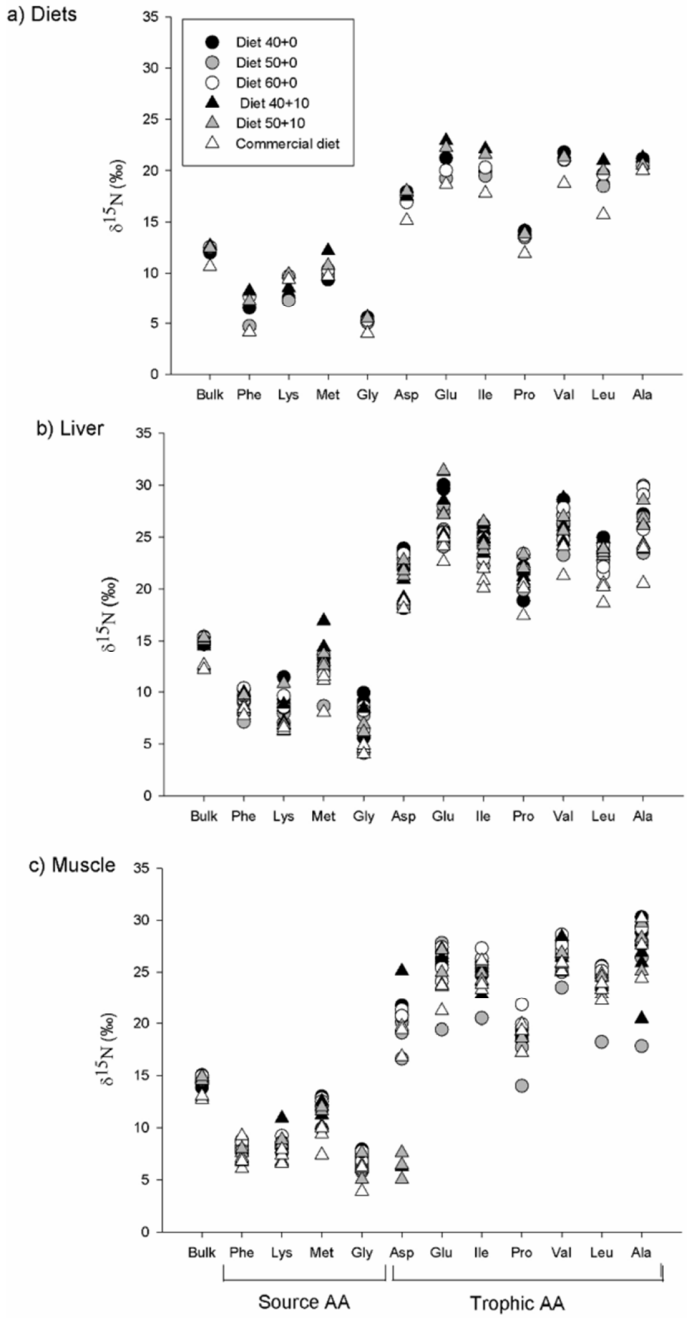

Figure 4. Bulk tissue and CSIA-AA $\delta^{15}N$ values of (a) experimental diets, (b) muscle and (c) liver tissue (n=3) of *S. lalandi* juveniles fed five formulated and one commercial diet for 98 d. Diets varied in the percentageof digestible crude protein (DP) + non-digestible crude protein (NDP) as described in Table 1. Phe = phenylalanine; Lys = lysine; Met = methionine; Gly = glycine; Asp = aspartic acid; Glu = glutamic acid; Ile = isoleucine; Pro = proline; Val = valine; Leu = leucine; Ala = alanine. For simplicity, the error bars corresponding to the two measurements of isotopic composition performed in each sample are omitted.

2.3.5. Bulk tissue TEFs

TEF_{bulk} for both liver and muscle tissues had limited variability among dietary treatments (Figure 5). In liver, TEF_{bulk} ranged from 2.1 ± 0.2‰ for the 40 + 10 diet to 2.8 ± 0.1‰ for the 50 + 10 diet. In fish fed the 40 + 10 diet, TEF_{bulk} was significantly lower compared to estimates for fish fed the other formulated feeds ($p<0.006$, Table 6). In contrast, for muscle tissue, TEFs did not differ significantly ($p = 0.45$, Table 7) as a function of protein content and protein quality, ranging from 2.0 to 2.4‰.

Table 6 Mean±SDof trophic enrichment factors (TEF) in bulk liver tissue and individual aminoacids calculated for fish fed diets differing in protein quantity and quality. When a significant effect of diet was found with a one-way ANOVA, ($p < 0.05$), Tukey's HSD multiple comparison tests were applied. Significant differences are indicated by superscript letters. Overall mean TEFs are reported when ANOVAs did not indicate differences between treatments. TEFs are expressed in ‰

		Treatment-specific TEF (Percent crude protein + non-digestible crude protein)					TEF values (mean±SD)	F ratio	p-value	Power analysis
		40+0	50+0	60+0	40+10	50+10				
	Bulk liver	2.7±0.1^{a}	2.6±0.3^{a}	2.6±0.2^{a}	2.1±0.2^{b}	2.8±0.1^{a}		6.7	0.006	
Source AA	Phe						2.3±1.2	3.1	0.060	0.626
	Lys	2.3±1.4^{a}	0.4±0.6^{ab}	-0.8±0.7^{ab}	-1.0±1.1^{ab}	-1.9±2.5^{b}		3.9	0.037	0.824
	Met						2.5±1.4	0.7	0.580	0.218
	Gly						1.8±1.5	0.5	0.700	0.152
Trophic AA	Asp						4.2±2.0	0.9	0.500	0.243
	Glu						6.3±2.2	0.3	0.850	0.109
	Ile						4.0±1.5	2.0	0.170	0.514
	Pro						8.0±1.3	1.6	0.260	0.42
	Val						4.9±1.5	0.2	0.900	0.087
	Leu	5.4±0.9^{a}	4.8±1.0^{ab}	3.0±1.4^{b}	3.6±0.5^{ab}	3.5±0.3^{ab}		3.6	0.040	0.772
	Ala						5.6±2.4	1.9	0.170	0.457

Table 7. Mean ± SD of trophic enrichment factors (TEFs) for bulk muscle tissue and individual amino acids calculated for fish fed diets differing in protein quantity and quality. When a significant effect of diet was found with a one-way ANOVA, ($p<0.05$), Tukey's HSD multiple comparison tests were applied. Significant differences are indicated by superscript letters. Overall mean TEFs are reported when ANOVAs did not indicate differences between treatments. TEFs are expressed in ‰.

		Treatment-specific TEF (Percent crude protein + non-digestible crude protein)					Overall TDF or TEF values (mean±SD)	F ratio	p-value	Power analysis
		40+0	50+0	60+0	40+10	50+10				
	Bulk muscle						2.3±0.3	1.0	0.450	
Source AA	Phe	1.7±0.6^{a}	3.3±0.3^{c}	0.3±0.5^{ab}	-0.8±0.6^{b}	0.3±0.4^{ab}		20.3	0.000	1.000
	Lys	0.4±0.4^{ac}	1.2±0.1^{a}	-1.0±0.5^{bc}	-0.1±0.2^{abc}	-1.8±0.7^{b}		9.3	0.004	1.000
	Met	2.8±0.8^{a}	1.1±1.6^{ab}	2.0±0.5^{ab}	-0.3±0.9^{b}	0.5±0.9^{ab}		4.5	0.030	0.765
	Gly						1.4±0.8	0.46	0.760	0.125
Trophic AA	Asp						2.9±1.2	1.95	0.190	0.792
	Glu	5.3±0.9^{ab}	8.1±0.6^{a}	5.6±1.7^{ab}	3.9±0.6^{ab}	3.1±1.7^{b}		5.0	0.020	0.981
	Ile	5.5±0.7^{ac}	5.7±0.3^{ac}	6.1±0.8^{a}	2.0±1.8^{b}	3.4±0.8^{bc}		8.4	0.006	0.996
	Pro						5.5±1.1	2.7	0.100	0.621
	Val						5.3±1.1	3.2	0.070	0.668
	Leu	6.6±0.2^{a}	5.6±0.6^{a}	5.4±0.3^{a}	3.1±0.4^{b}	4.1±0.6^{ab}		8.8	0.005	0.996
	Ala						7.2±1.7	2.25	0.150	0.756

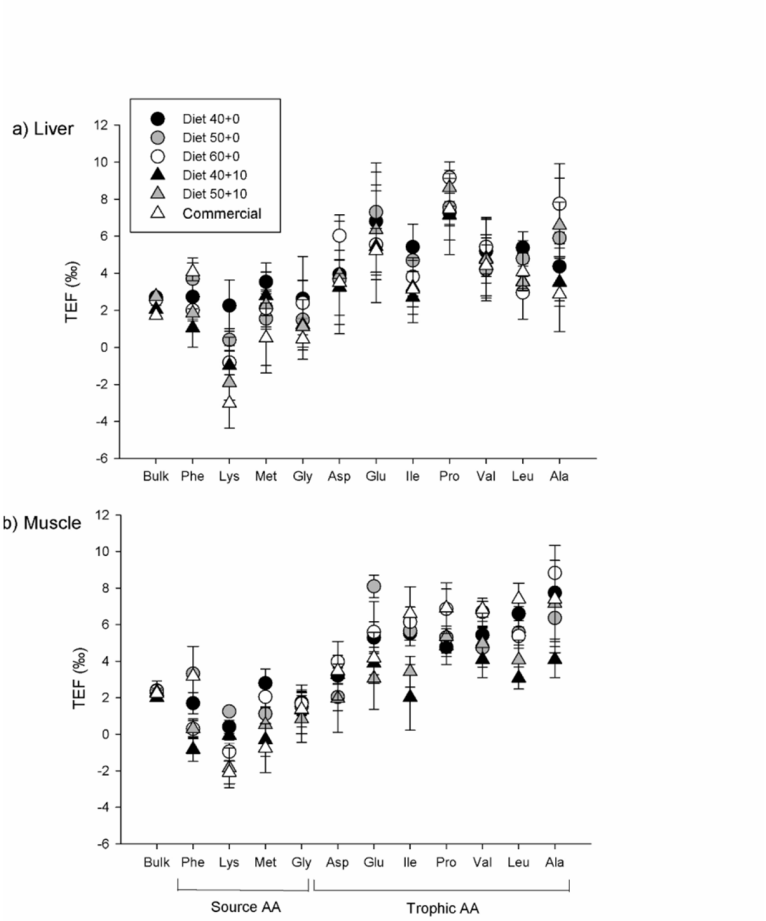

Figure 5. Trophic enrichment factors in bulk tissue (TEF_{bulk}) and individual amino acids (TEF_{AA}) for (a) liver and (b) muscle tissue (n=3) of juvenile *Seriola lalandi* fed with five formulated feeds and one commercial diet differing in protein percentage and quality. Error bars represent the SD of TEFs for each dietary treatment. Diet codes indicate the percentage of digestible protein + non-digestible protein.

2.3.6. Comparison between liver and muscle TEF_{AA}

There was generally a strong positive correlation between AA-specific values between tissues (Supporting Information Figure S1). The strength of the association increased with protein content (r = 0.5 in the 40 + 0 to r = 0.8 in the 60 + 0 treatment). The difference in TEFs between tissues for each AA was inconsistent in magnitude and direction among treatments (Figure 3). In general, source AAs showed a low difference (<1‰) in TEFs between tissues in the optimal protein diet (50 + 0), whereas for the low-protein quality diets (40 + 10 and 50 + 10), there were higher differences (up to 2‰). The difference in TEF_{Phe} was relatively consistent between tissues (1–2‰); Lys and Met had the lowest differences in the optimal and highest protein treatments (<1‰). TEF_{Met} varied little (<1‰) between treatments that did not include formalin-treated fish meal, and showed higher discrimination (2–3.5‰) TEFs in the liver tissue of fish fed diets with decreased digestibility. The difference in TEF_{Gly} was low (<1‰) for all treatments. TEF_{Lys} had the highest difference between tissues in the diets with

lowest protein content (40 + 0; ca. 2‰).

The difference in TEFs between liver and muscle tissues of trophic AAs varied substantially between treatments (Figure 3). Nonetheless, fish fed the optimal protein diet had the lowest difference between tissues for all trophic AAs (less than 2‰). Pro had the highest TEFs in liver tissue, while Ala had the highest TEFs in muscle tissue. TEF_{Glu} had variable difference between tissues (up to 3.5‰) in all treatments except for the high-protein diet. TEF_{Ala} had the lowest difference between tissues in the optimal protein (<1‰) and the highest in the low-protein feed (almost 4‰). Proline was the only trophic AA with consistent and positive differences between liver and muscle tissues; liver tissue was more enriched in ^{15}N. TEF_{Val} differed by <1‰ between tissues in the low-protein treatments (40 + 0 and 50 + 10), and by 1–2‰ for the other treatments, and did not differ in the low-protein digestibility treatment (50 + 10).

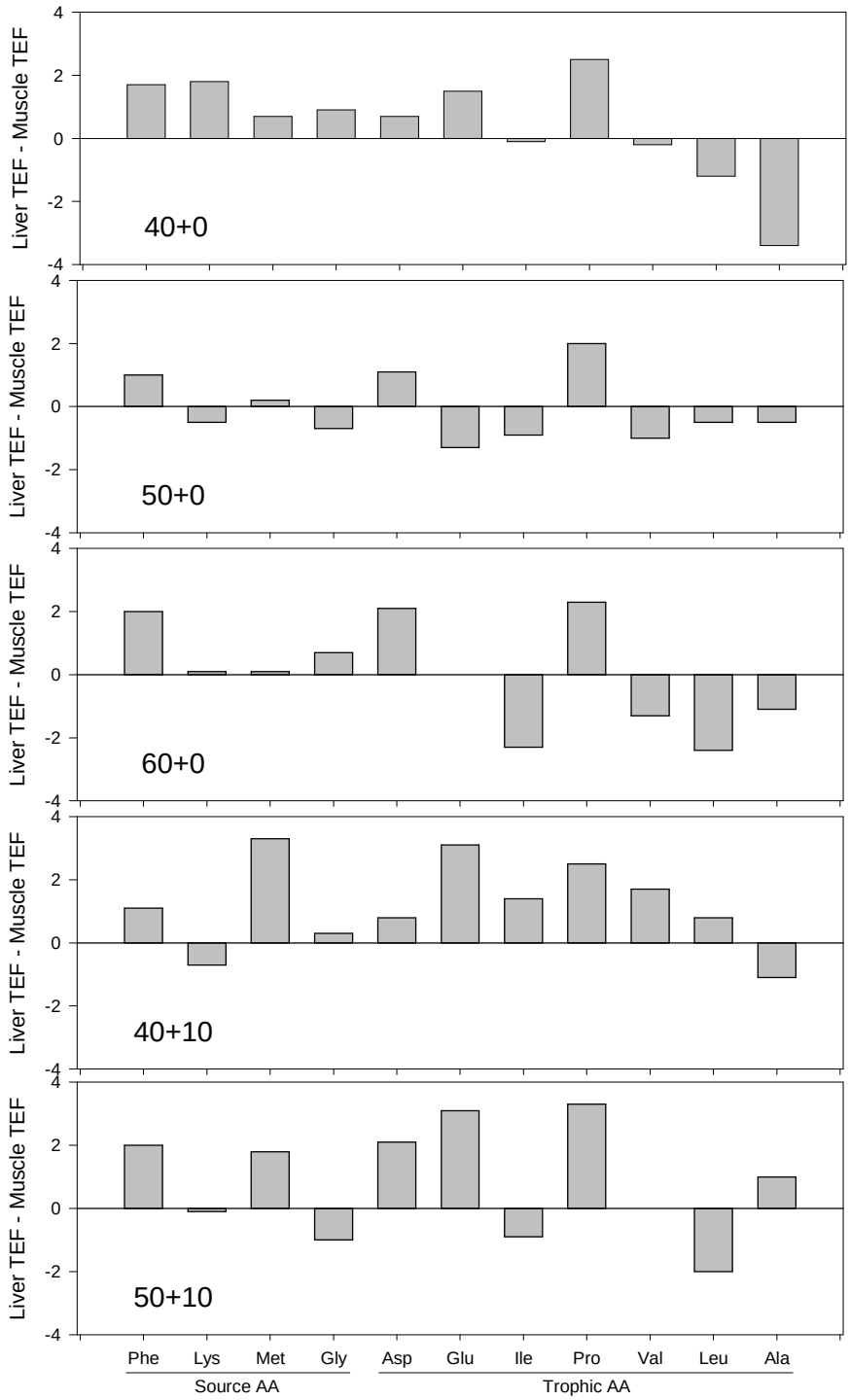

Figure 3. Difference between TEF for liver and muscle for each AA (Phe=phenylalanine, Lys=lysine, Met=methionine, Gly=glycine, Asp=aspartic acid, Glu=glutamic acid, Ile=isoleucine, Pro=proline, Val=valine, Leu=leucine, Ala=alanine) as a function of diets varying in protein quantity and quality. Dietary treatments are described in Table 2.

2.3.7. Amino acids TEF

TEF_{AA} for source and trophic AAs were variable in liver and muscle tissues (Figures 5 and 6). For source AAs in liver, TEF_{Lys} exhibited significant differences among dietary treatments ($p=0.037$, see Table 6), while TEFs for Phe, Met, and Gly did not differ significantly among treatments (Figure 6; Table 6). For muscle, the TEFs for Phe, Lys, and Met differed significantly among treatments ($p<0.001$, $p=0.004$, and $p=0.030$, respectively); only TEF_{Gly} did not differ significantly among all treatments (Table 7).

Regarding TEFs for trophic AAs in liver tissue, TEF_{Leu} was the only one that differed significantly among treatments ($p=0.04$; Table 6). In muscle tissue, TEFs Glu, Ile, and Leu values differ significantly among treatments ($p=0.020$, $p=0.006$, and $p=0.044$, respectively). TEFs Asp, Pro, Val, and Ala did not differ significantly among treatments (Table 7).

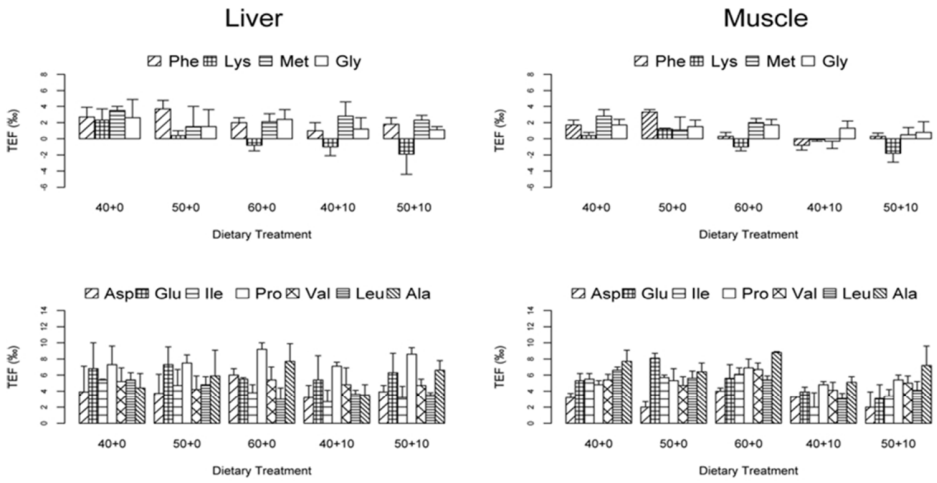

Figure 6. Trophic enrichment factors of individual amino acids (TEF_{AA}) for liver (a) and (b) muscle tissue (n=3) of juvenile Seriola lalandi fed with five formulated feeds differing in protein percentage and quality. Error bars represent the SD of TEF for each dietary treatment. Top panels: source AA. Bottom panels: trophic AA. Diet codes indicate the percentage of digestible protein + non-digestible protein.

2.4. Discussion

The variable TEFs of all trophic AAs, and of some source AAs, indicate that isotopic discrimination varied between tissues depending on the dietary treatment. This may be related to the preferred energy sources used during fish growth, and the degree of transamination and deamination of specific AAs. The latter occurs due to AA catabolism; all AAs can be subject to catabolic processes in fish and other vertebrates (O'Connell, 2017). Below, we briefly discussed results of fish performance in relation to AA isotopic fractionation, and later, we

discussed in detail the N isotopic fractionation for bulk tissues and AAs among and within each tissue.

2.4.1. Survival, growth, nutritional response

Dietary protein content had a significant effect on specific growth rate (SGR), and indicated significantly greater protein accretion in muscle tissue of fish fed the higher protein level diets compared with diet 40+0. Thus, our SGR values reflect adequate growth rates for this species reared under culture conditions irrespective of the presence or absence of treated fish meal. However, we observed a slightly higher SGR in fish fed diets with lower digestibility compared with those with the same crude protein level but higher digestibility. This result can be associated with the small initial fish size assigned to the 40+10 and 50+10 treatments.

Feed conversion rates (FCR) of fish fed experimental diets ranged from 1.7 to 2.6, which is within the range for *S. lalandi* (Moran, Pether, & Lee, 2009; O'Sullivan, 2005). Lower FCR were obtained in fish fed diets with higher protein content, reflecting better feed efficiency (Takakuwa, Fukada, Hosokawa, & Masumoto, 2006). The protein efficiency ratio (PER) and protein productive value (PPV) (that were estimated using total protein in the diets and assuming a decrease in digestibility of 100% in the fish meal treated with formalin), were not significantly different between fish fed diets differing in protein quantity and quality. However, calculating the PPV using the estimated available protein (i.e., the protein in the non treated protein fish meal in the diet) results in a significant negative relationship (data not shown). This suggests that *S. lalandi*, like many other carnivorous fish, may have the ability to utilize dietary protein more efficiently when fed diets with lower protein quantity and/or lower quality (National Research Council, 2011). More efficient protein accretion should lead to lower TEFs, but we did not observe a clear relationship.

Trophic AAs TEFs from the 40+0 and 40+10 diets were the lowest, especially for Asp TEF in both tissues. Fish fed the low-protein diet (40+0) had the lowest growth rates and highest FCR, leading to limited AA catabolism and hence isotope discrimination.

The relationship between protein and energy in diets is important as lipids and carbohydrates can spare protein use as an energy source (i.e., protein sparing effect; National Research Council, 2011). The P:E ratios of our experimental diets ranged from 19.0 to 28.1 mg protein/kJ. The highest growth rates were obtained with a P:E of 23.8 (diet 50+0) and did not increase with higher P:E ratios. These results suggest that protein was in excess for diet 60+0, and the excess protein was probably burnt as energy. The highest trophic AAs TEFs in liver and muscle was found in the 60+0 diet. Fish possibly burned AAs as energy sources and reduced their protein efficiency when protein was in excess,

which explain the high TEF_{AA} because fish likely metabolize more AAs.

2.4.2. Bulk tissue TEF as a function of protein quantity and quality

Despite the range of protein levels included in our formulated feeds, TEF_{bulk} did not vary as a function of protein quantity for either muscle or liver tissue. TEF_{bulk} $\delta^{15}N$ values were consistent with those previously reported for the same tissues in fish (McCutchan et al., 2003; Vanderklift & Ponsard, 2003) and about 1‰ lower than the 3.4‰ value typically used to calculate trophic level from fish muscle SIR.

Our results for TEF_{bulk} are inconsistent with the hypothesis proposed by Martínez del Río and Wolf (2005), and our hypothesis regarding the relationship between protein content and tissue TEFs, at least when considering a limited (albeit ecologically realistic) range of protein contents in the diets. The observed low variability in TEF_{bulk} from *S. lalandi* are also inconsistent with the results from previous studies that varied protein content without simultaneously influencing diet quality (particularly AA profiles) (see Table 1). For instance, Focken (2001) found a positive trend between whole fish TEF_{bulk} and feeding rate in Nile tilapia (*Oreochromis niloticus*) fed diets containing fish and wheat meals. However, there was not clear evidence that tissues reached isotopic equilibrium during the four-week experiment as WRs were low (1.3–2.1). In contrast, the negative relationship between muscle TEF_{bulk} and protein content observed in gilthead sea bream (*Sparus aurata*) (Martín-Pérez et al., 2013) may be a result of the result of an increased feed intake in fish fed the lower protein content diet, because fish were fed to satiation. This may explain the different relationship observed between protein content and TEF_{bulk} between their study and ours.

The preferential assimilation of one of their protein sources may also contributed because different protein sources can drive N isotopic fractionation differently depending upon protein quality (Martín-Pérez et al., 2013). Lastly, another study showed a positive relationship between muscle TEF_{bulk} and protein content in Nile tilapia fed a casein-based diet fed at different levels, including in excess of the estimated maximal intake (Kelly & Martínez del Río, 2010). Higher TEF_{bulk} values were observed in diets with higher protein content, possibly due to high daily ration conducive to protein catabolism. A broader range of protein contents in the diets may therefore yield a positive relationship.

Protein quality (specifically protein digestibility) had a negligible effect on TEF_{bulk} of muscle tissue and a limited effect (0.5‰) in liver tissue. No previous studies report on the effect of protein digestibility on TEFs of fishes or other taxa. Other experiments evaluating the effect of varying the AA profile (another component of dietary quality) on TEF_{bulk} and avoiding a potentially confounding effect of covarying protein digestibility observed

different results. Gaye-Siessegger, Focken, Abel, and Becker (2007) evaluated AAs synthesis from their precursors relative to isotopic discrimination by raising Nile tilapia on three diets using fixed feeding rates. Whole fish TEF_{bulk} values were −0.3, 1.6, and 1.8‰, respectively, which are lower than our TEFs for liver and muscle. Their final WR values (1.1, 0.9, and 0.8, respectively) indicated low growth rates and weight loss; the authors concluded their results were likely due to the lack of absorption of synthetic AAs. Mohan et al. (2016) raised juvenile Atlantic croacker (*Micropogonias undulatus*) on diets considered of low (plant-based, 32% protein) and medium (plant and animal-based, 45% protein) quality in which the AA profiles necessarily differed.

Their muscle TEF_{bulk} values were 6.5 and 4.7‰ for the low and medium protein quality, respectively, which are high relative to the values we obtained (2.3‰), possibly due to an imbalance in some AAs and the consequent metabolism of some NEAA. However, our results for liver TEF_{bulk} values are similar to the range these authors reported for the low and medium protein quality diets (3.0 and 2.1‰, respectively) and their high-protein control diet (48% protein; 1.6‰).

2.4.3. Comparison between liver and muscle TEF_{AA}

We found an increasing level of association between TEFs of liver and muscle in response to higher protein content (Supporting Information Figure S1). Fish fed diets with optimal or higher protein levels had more similar AA-specific isotope enrichment factors. As dietary protein increased, the difference in the amino acid isotopic values between tissues decreased likely due to better feed efficiencies (lower FCE), which implies a lower amount of catabolism and hence lower isotope discrimination.

The differences in TEF_{AA} between liver and muscle support our hypothesis and agree with results from the few studies that estimated TEFs for multiple tissues at the intraspecific level. In harbor seals, Germain et al. (2013) found mean differences between blood serum and muscle of four individuals, ranging from 0.1 and 0.4‰ for Ala and Lys, to 5.9 and 6.7‰ for Gly and Ile. In fish, there is only one study that estimated TEFs for multiple tissues. Barreto-Curiel, Focken, D'Abramo, and Viana (2017) fed *S. lalandi* a single diet with 43% protein content and found a difference of 3.3‰ for TEF_{Met} between liver and muscle, which is comparable to what we found for our low digestibility formulations. However, these authors calculated a difference of 3.4‰ between tissues for TEF_{Phe}, and −0.7 and −0.9‰ for Lys and Gly, respectively, which differed from our results.

Given that our study also used the same species, the differences in tissue-specific TEFs between Barreto-Curiel et

al.'s (2017) and our study are possibly linked to differences in the quality of the protein sources, which includes the AA profiles, and the digestibility of the diets. Future studies should evaluate the effect of varying the dietary availability of specific AA on TEF estimates.

We hypothesized that source TEF_{AA} would have more consistent values between tissues than trophic AAs. Unexpectedly, TEF values of some source AAs varied by up to ca. 4‰ between tissues, and the difference was not consistent among dietary treatments (Figure 3).

TEF_{Met} differed by <1‰ between liver and muscle tissue in treatments varying protein quantity, and by up to 3.5‰ in fish fed diets with lower digestibility. Perhaps, the variable isotopic fractionation between tissues is related to the availability of Met in the diets: The lower availability of Met in the 40+10 diet might not have met the species' dietary requirement, causing catabolism of endogenous Met in the liver.

We hypothesized that the TEFs of trophic AAs would exhibit a greater degree of difference between tissues than source AAs. Our results only partially agree with our hypothesis. The difference in TEF_{Glu} between liver and muscle tissue of fish fed diets of low-protein quality was ca. 3‰, which is consistent with the 2.9‰ estimated by Barreto-Curiel et al. (2017). The observed high differences in the TEFs of Glu between tissues for fish fed with low-protein digestibility diets may be attributed to the dynamic and complex nature of Glu metabolism and its variability between both tissues, which is largely unknown in fishes (Li, Mai, Trushenski, & Wu, 2009). This NEAA plays numerous metabolic roles (Wu, 2009), and it is one of the preferred sources of metabolic energy in fishes. Its use as an energy source can be higher than glucose or fatty acids (Jia, Li, Zheng, & Wu, 2017). Higher isotope discrimination may depend on the degree in which Glu was used as an energy substrate or transaminated. All of these factors may underlie the observed high and variable isotopic discrimination in Glu between tissues and dietary protein attributes (i.e., quality and quantity) during *S. lalandi*'s growth.

In contrast to Glu, TEF_{Pro} showed consistent differences between muscle and liver TEFs for all dietary treatments. A consistent TEF_{Pro} was also detected in fish fed with diets that covaried protein quality and quantity (McMahon, Thorrold, et al., 2015), even in fish fed a plant-based diet that possibly put fish under nutritional stress. Proline is synthesized from arginine (Arg) and glutamate/glutamine and is typically not considered an essential AA. Although ring closure of Glu is a pathway for Pro synthesis, arginine is also a major precursor via arginase; up to 40% of dietary Arg can be metabolized to form Pro, and glutamine and ornithine can be also be used as substrates (Wu et al., 2011). All these factors can lead to the observed differences in Pro and Glu TEFs.

Proline plays many important roles in protein synthesis and structure, metabolism and nutrition, as well as wound

healing, antioxidative reactions, and immune responses (Wu et al., 2011). On a per-gram basis, proline and hydroxyproline are the most abundant AAs in collagen; proline requirements for whole-body protein synthesis are the highest among all AAs in fish (Li & Wu, 2018).

Therefore, physiological needs for proline are particularly high. Although information about the role of proline is limited for fish, a study suggests that the liver probably synthesizes this AA to meet requirements, while muscle tissue may be more dependent upon the amount of proline available in the diet (Li et al., 2009). If true, this difference between tissues may explain the higher TEF_{Pro} in liver than muscle tissue.

A high difference TEF_{Ile} between tissues (>2‰) and higher TEFs in liver than in muscle was also observed by Barreto-Curiel et al. (2017). The difference in TEF_{Ile} was higher in muscle tissue of fish fed the 60+0 diet with highest protein content (>2‰), suggesting higher catabolism in muscle and the consequent higher excretion of ^{15}N-depleted nitrogen. We observed a much higher TEF_{Ala} in muscle than liver tissue, which was also observed by Barreto-Curiel et al. (2017).

In fish, most regulatory effects of nutrient utilization and metabolism initially occur in the liver, and its metabolism generates a cascade of events in other tissues (Enes et al., 2009). Liver tissue has a higher metabolic rate than muscle and it is where most of the NEAA are synthesized (Jürs & Bastrop, 1995), which may explain why the majority of AAs were more ^{15}N-enriched than in muscle tissue. Isotopic routing may also contribute to differences in TEFs between tissues, as nutrients are directed differentially to specific tissues (Tieszen & Fagre, 1993). Our results and the currently available literature to date nevertheless indicate that TEFs are tissue-specific.

2.4.4. AA TEFs as a function of protein quantity

2.4.4.1 Liver tissue

TEFs of Phe, Met, Lys, and Gly did not vary significantly with protein content among treatments, supporting our hypothesis. However, we did observe a marked trend toward a greater depletion in ^{15}N in Lys TEFs with increasing protein content (TEF = 2.3 ± 1.4‰ to −0.8 ± 0.7‰ for diets 40+0 to 60+0, respectively), which is unexpected given its classification as a source AA. Barreto-Curiel et al. (2017) also reported a negative TEFs for Lys (−0.7 ± 0.3‰). This may be related to differences in dietary lipid content, which was lower in the high-protein diets (12.1 vs. 20.4‰ for the 40 +0 vs. 60+0 diet, respectively), and 13.2‰ in the commercial diet of Barreto- Curiel et al. (2017). Lys is used for the

synthesis of carnitine, which is involved in the transport of long-chain fatty acids into cells, and is often a limited AA in commercial fish diets, particularly those formulated with plant-based protein sources (Li et al., 2009). Higher dietary lipid content would require more fatty acids transporters, which would increase Lys catabolism for the synthesis of carnitine, and would cause higher TEF_{Lys} in the low-protein diet. Further studies are required to examine this possibility. Nevertheless, if Lys isotopic composition varies as a function of dietary lipid content, caution should be taken when interpreting its isotopic composition as a source AA in liver tissue.

Our mean TEF_{Phe} and TEF_{Gly} (2.3 ± 1.2‰ and 1.8 ± 1.5‰, respectively) are similar to those reported for the same species (3.2 ± 0.5‰ and 1.0 ± 0.4‰; Barreto-Curiel et al., 2017), despite that Gly is now considered a "metabolic AA" due to its high variability in many taxa (O'Connell, 2017). TEF_{Met}, however, differed by ca. 5‰ between our study (2.5 ± 1.4‰) and Barreto-Curiel et al. (2017) (7.5 ± 1.7‰), possibly due to variations in Met, cysteine (Cys), and taurine (Tau) availability relative to dietary requirements. This is possible because Met is the first AA to be limiting in formulated feeds in fish, and being a sulfur AA, its metabolism is linked with that of Cys and Tau (Li et al., 2009). High TEFs for Met could be indicative of conversion to Cys, which involves the transmethylation–transsulfuration pathway and results in the cleave of the amino group, during which isotope discrimination could occur (O'Connell, 2017). Regardless of the mechanisms underlying the lack of differences in isotope discrimination, Phe, Gly, and Met in liver tissue did not vary with protein content and exhibit limited isotopic enrichment relative to the diets in liver tissue.

Trophic AAs in liver tissue had higher TEFs than those of source AAs, as expected (e.g., Bloomfield, Elsdon, Walther, Gier, & Gillanders, 2011; Chikaraishi et al., 2009; Hoen et al., 2014; McMahon, Thorrold, et al., 2015). In our study, proline exhibited the highest TEF (8.0 ± 1.3‰), followed by Glu (6.3 ± 2.2‰), Ala (5.6 ± 2.4‰), and Val (4.9 ± 1.5‰). This pattern differs from that of Barreto-Curiel et al. (2017), who reported higher TEFs for Glu than Pro (8.4 ± 0.7‰ and 4.9 ± 0.8‰, respectively) and lower values for Ala (4.6 ± 0.88‰) and Val (4.1 ± 0.45‰). The differences in trophic TEFs values between these studies could be attributed to distinct dietary AA profiles and digestibility, and the consequent differential synthesis and catabolism of specific AAs.

We hypothesized an increase in TEF with increasing protein quantity for trophic AAs. However, our results lead us to reject this hypothesis for Asp, Glu, Ile, Pro, Val, and Ala because their TEFs did not differ between treatments. Despite the difference in dietary protein content, and the complexity of the metabolic pathways involved in the metabolism of these AAs (O'Connell, 2017), there were no differences in the level of isotope discrimination. In contrast to the rest of the trophic AAs, TEF_{Leu} showed a negative relationship with protein content, ranging from 5.4 ± 0.9‰ in the 40 + 0 diet to 3.0 ± 1.5‰ in the 60 + 0 diet. Previous studies also reported a negative relationship between dietary protein content and TEF_{Leu} in fish muscle (McMahon, Thorrold, et al., 2015). To our knowledge,

there are no previous studies reporting data for fish liver tissue using a single protein source in experimental diets varying protein content. Although it has not been widely investigated in fish, leucine is considered a functional EAA (it plays a key role in determining the three-dimensional structure of proteins and is thus involved in their functionality), and stimulates muscle protein synthesis in fish and mammals (Nakashima, Yakabe, Ishida, Yamazaki, & Abe, 2007; NRC, 2011). In our study, juvenile Pacific yellowtail grew adequately, but the treatment with the lower protein content exhibited lower growth rates and poorer food conversion efficiency, which could lead to more Leu catabolism (and hence higher isotope discrimination) for energy purposes than in the other treatments. However, it is important to consider that the catabolism of Leu is greater in tissues other than liver, like muscle, kidneys, and the central nervous system (NRC, 2011), and that Leu, Val, and Ile metabolism might be dependent in each other, which render the explanation of the differences in TEF_{Leu} difficult.

2.4.4.2 Muscle tissue

Comparison between our TEF estimates and those of other studies can yield insight into the level of variation in isotope discrimination of AAs in fish muscle tissue. However, these studies covaried protein quantity and quality, and comparisons are necessarily qualitative when attempting to partition the contribution of protein quantity and quality to variation in AA-specific TEFs. Unexpectedly, the TEFs of Phe and Lys showed significant differences among diets differing in protein content that lead us to reject our hypothesis for source AAs because they are not expected to vary as a function of protein content. These results challenge the current paradigm in which the CSIA-AA of Phe and Lys in muscle tissue are assumed to reflect baseline isotope ratios.

TEF_{Phe} was significantly higher in the optimal protein diet (3.3‰), and the overall range of TEFs for Phe was also higher (0.3–3.3‰) than those reported for the omnivorous mummichug (*Fundulus heteroclitus*) fed diets differing in protein sources and quality (0.1–1.0‰; McMahon, Thorrold, et al., 2015). Blanke et al. (2017) also reported a limited range of TEF_{Phe} (−0.3 to 1.0‰) for four fish species fed a range of diets. Phe is an EAA whose metabolism is intimately related to that of Tyr via hydroxylation (Mathews & van Holde, 1996). In turn, Tyr can react with alpha-keto-glutarate, yielding p-hydroxyphenylpyruvate and glutamate, which would imply deamination and consequently isotope discrimination (Mathews, 2007; O'Connell, 2017). Phe transamination with pyruvate can also occur, yielding Ala and phenylpyruvate, although this is thought to be a minor catabolic pathway (O'Connell, 2017). Phe has an important regulatory role in growth performance and Tyr is a precursor of neurotransmitters and hormones (Li et al., 2009). Thus, differences in Phe TEFs in diets differing in protein content and/or AA profile might be related to its specific functional and metabolic roles, and those of Tyr.

Similarly, TEF_{Lys} was the highest TEF (1.2‰) in fish fed the optimal protein diet, and the lowest TEF (−1.0‰) on the 60+0 diet. As Lys in muscle tissue is highly involved in the formation of collagen (Li et al., 2009; NRC, 2011), fish with higher growth rates should need to metabolize more Lys to support collagen production. However, we did not observe differences in growth rates between fish fed the 50 + 0 and 60 + 0 diets. Lys N can be transferred to the nitrogen pool through catabolic processes involving glutamate (O'Connell, 2017). Consequently, differences in the level of Lys catabolism between diets could lead to differences in TEFs.

In contrast, Met and Gly did not show significant differences in muscle tissue between diets differing in protein content, and both TEFs indicated limited discrimination (2.0‰ and 1.4‰, respectively). Barreto-Curiel et al. (2017), however, reported a higher TEF_{Met} (4.5‰) for muscle tissue. As mentioned previously, Met is related to cysteine and taurine synthesis (Li et al., 2009), and as for other nontransaminating AAs, Met can be catabolized through deamination, which would lead to isotope discrimination and enrichment in the residual Met pool. The lack of differences in Met TEFs in muscle tissue therefore suggests a similar level of Met catabolism between diets.

As we mentioned before, the consistency in Gly TEFs was unexpected due to the high variability detected in several taxa of marine and freshwater consumers fed diets differing in protein sources (ca. 4‰; McMahon & McCarthy, 2016 and references therein), and its association with microbial degradation (McCarthy et al., 2007), and transamination. In fish, Gly metabolism is intimately linked with that of Cys; these two NEAA can be interconverted in the liver and kidneys and together they play a complex role in gluconeogenesis, sulfur AAs metabolism and the metabolism of fat (Li et al., 2009). McMahon, Thorrold, et al. (2015) reported Gly TEF values of −0.1 to 1.6‰ for an omnivorous fish, and Barreto-Curiel et al. (2017) reported a value of 1.9‰ for muscle tissue of Pacific yellowtail. Taken together, these data and our results indicate Gly seems not to fractionate isotopically in N in response to changes in dietary protein content in marine fishes.

Despite that we hypothesized increasing TEF_{AA} values for trophic AAs with increasing protein quantity, trophic $TEFs_{AA}$ varied but were not significantly different among 40+0, 50+0, and 60+0 diets and did not exhibit a specific pattern. These results disagree with previous findings in fish (McMahon, Thorrold, et al., 2015) in a study that covaried protein quantity and quality (Table 1). Their highest TEF values for trophic AAs were found in fish fed a plant-based diet with a very-low-protein content. This plant-based diet likely forced fish to catabolize their own body protein to meet energy requirements, leading to high isotope discrimination because, as we mentioned before, fish cannot metabolize carbohydrates efficiently and have high-protein requirements (Booth, Moses, & Allan, 2013; Hemre, Mommsen, & Krogdahl, 2002). In the same study, Ala had the highest mean TEF (11.7‰) followed by Glu (10.8‰), while Pro had a more limited range (6.6–7.3‰) of values and the lowest TEFs among trophic AAs. Nevertheless, their Pro

TEFs were somewhat higher than our mean Pro TEF value of 5.5‰. For *S. lalandi*, Barreto-Curiel et al. (2017) reported higher TEFs for Pro (5.9‰) and than ours (5.5‰; Table 7), while lower TEFs for Ala (6.8‰) than ours (7.2‰) and relatively consistent TEF_{Asp} (3.7‰) with our TEF_{Asp} values (2.9‰). These inconsistencies in the trophic TEF_{AA} between our study and those of McMahon, Thorrold, et al. (2015) and Barreto-Curiel et al. (2017) might be due to differences in protein sources and digestibility, as well as AA profiles.

2.4.5. TEFs as a function of protein quality

2.4.5.1. Liver tissue

In liver tissue, the TEFs of source and trophic AAs did not differ between diets with decreased protein digestibility and hence quality. This is consistent with our hypothesis for source AAs. Liver tissue appears insensitive to variations in protein digestibility, at least within the protein levels and degree of reduced digestibility considered in our study. TEF_{Lys} did not differ significantly between treatments varying in protein quality; however, diets with low-protein quality had negative TEF values, which was also reported by Barreto-Curiel et al. (2017) and as was observed for liver tissue. As mentioned previously, dietary lipid levels may be intimately linked to Lys metabolism and consequently TEF values. Feeding studies with diets that only vary lipid content are required to examine the potential effect of lipid levels on TEF_{Lys}.

2.4.5.2. Muscle tissue

We hypothesized that the TEF_{AA} of source amino acids would not vary as a function of protein quality. However, in muscle tissue Phe exhibited a higher TEF (3.3‰) in the optimal diet (50+0) than in the lowest protein quality diet (−0.8‰ in diet 40 + 10). Notably, the fish fed the low-protein diet that did not contain fish meal treated with formalin (diet 40+0) also had a significantly different TEF (1.7‰) than the 40+10 formulation. Comparison of our results with other studies indicates that TEF_{Phe} in fish muscle is variable. Barreto-Curiel et al. (2017) reported a negative TEF_{Phe} (−0.16‰) for muscle of Pacific yellowtail. Bradley et al. (2014) and Hoen et al. (2014) reported low positive TEF_{Phe} values (1.5‰ in both studies) for Pacific bluefin tuna (*Thunnus orientalis*) and opakapaka, or pink snapper (*Pristipomoides filamentous*), respectively, which is similar to the TEFs of our fish fed the lowest protein content diet. This broad range of TEF_{Phe} values differs from the more limited range reported for fish fed diets

differing in protein quantity that also varied in protein sources, and hence quality (0.1–1.0‰ in McMahon, Thorrold, et al., 2015; −0.3 to 1.0‰ in Blanke et al., 2017). Phe could reflect isotope discrimination when used directly as an energy substrate or when Tyr synthesized from Phe is catabolized, as the reactions involved include deamination (Mathews & van Holde, 1996; O'Connell, 2017). The differences in TEF_{Phe} between diets varying in protein digestibility may be attributed to variations in the extent to which this AA was used as an energy source or channeled for growth. Regardless of the cause, the studies available to date indicate that the isotopic composition of Phe in muscle tissue is sensitive to the nutritional characteristics of a fishes' diet. More specifically, our results strongly indicate that isotope discrimination of Phe is sensitive to protein digestibility.

Although there were no significant differences in TEF_{Lys} between diets differing in protein quality, TEFs were negative in both treatments with decreased protein digestibility (−0.1 and −1.8‰) and TEFs showed a broad range of values for a source AA when considering all formulated feeds (from −1.8 to 1.7‰). Bradley et al. (2014) reported slightly negative TEF_{Lys} value (−0.3‰) for Pacific bluefin tuna and Hoen et al. (2014) reported positive values (ca. 0.5‰) for opakapaka; both studies held the fish in captivity and used wild-caught prey as food sources. Barreto-Curiel et al. (2017) also reported a low TEF_{Lys} in muscle (0.05‰), and McMahon, Polito, et al. (2015) and McMahon, Thorrold, et al. (2015) reported a positive range of TEF_{Lys} values (1.6–3.0‰). Thus, as with Phe, Lys TEFs of muscle do not appear to be consistent.

Similar to Lysine, Met TEFs did not show significant differences between protein quality in the diets, but the overall range of TEF_{Met} was broad for a source amino acid (ca. 3‰), and diets with decreased digestibility had lower TEFs (−0.3 and 0.5‰ for the 40+10 and 50+10 diets). Moreover, Met exhibited a significantly higher TEF (2.8‰) in the lowest protein content diet (40+0) than in the 40+10 diet (−0.3‰), which was formulated to have a similar digestible protein content. Barreto-Curiel et al. (2017) also reported a high TEF_{Met} (4.2‰) for Pacific yellowtail. As we mentioned before, Met is an EAA that can be converted into cysteine and taurine (Li et al., 2009; Wu, 2009), and Met has also an important role as a precursor of other metabolic reactions and participates in the synthesis of glucose and glycogen (NRC, 2011). Differences in TEF_{Met} between dietary treatments may be due to the complexity of Met metabolism and the level of catabolism relative to its dietary availability and nutritional requirements.

Gly had a low mean TEF (1.4‰) in diets differing in protein digestibility. Once again, this consistency in Gly TEFs was unexpected because it has been reported to vary among several taxa of marine consumers that excrete ammonia (McMahon & McCarthy, 2016 and references therein), and may be the result of a limited range of protein levels within our experimental design. Bloomfield et al. (2011) reported TEF_{Gly} of −1.0‰ and 4.0‰ for black bream fish fed diets differing in protein sources. Bradley et al. (2014) reported slightly higher TEF_{Gly} value (3.4‰) than in our study,

whereas Hoen et al. (2014) reported a wide range TEF_{Gly} values (from −7.0 to 5.0‰) for three elasmobranchs and one teleost; the enrichment factor for the teleost was 0.5‰. McMahon and McCarthy (2016) reported a low range TEF_{Gly} values (from −0.1 to 1.6‰) for muscle tissue of fish fed diets differing protein sources and quantity. Gly metabolism is linked to that of threonine (Thr) and Cys, and these three AAs can be catabolized through deamination through several pathways (O'Connell, 2017), which could lead to variation in isotope discrimination. Taken together, the studies available to date indicate that Gly TEFs vary in fish muscle tissue, although the underlying causes remain uncertain. We hypothesized that the TEFs for trophic AAs would decrease with increasing protein digestibility; however, only TEF_{Ile} and TEF_{Leu} showed significant differences between the higher and lower quality diets. In both cases, TEFs were higher in the higher quality diets. The higher TEFs may reflect a greater degree of transamination or deamination in the diets with higher protein quality. Although our range of TEFs for Ile and Leu were similar to those reported for by Barreto- Curiel et al. (2017) (4.9 and 5.1‰, respectively), previous studies have reported some higher TEFs for Ile (range: 5.2–9.4‰) and Leu (range 5.5–10.0‰) (McMahon, Thorrold, et al., 2015). Bloomfield et al. (2011) also reported very high TEF_{Ile} values and TEF_{Leu} values of fish fed fish meal (9.0 and 21.0‰) and vegetable-based (9.5 and 20.1‰); these diets must have differed markedly in their AA profiles, and due to their limited growth, the fish may not have reached isotopic equilibrium (Table 1).

TEFs for Glu differed significantly between diets differing in protein digestibility, despite the relatively large level of variation between replicates in some treatments (maximum *SD* observed among replicates ≈1.7‰). TEFs for Glu spanned a large range of values (3.1– 8.1‰), similarly to what was reported by McMahon, Thorrold et al. (2015) (5.6–10.8‰) and Blanke et al. (2017) (5.9–8.2‰). Bloomfield et al. (2011) reported higher TEF_{Glu} values (11.0 and 20.0‰), but as mentioned previously, fish may not have reached isotopic equilibrium and values may therefore be skewed. The TEFs for Glu reported by Bradley et al. (2014) (7.8‰), Hoen et al. (2014) (range 2.0–3.9‰), and Barreto-Curiel et al. (2017) (5.5‰) also differ. Together, these results indicate that Glu in muscle varies substantially, even within the same taxa.

TEFs of the Asp, Pro, Val, Ala also did not differ significantly between diets differing protein quality, which reject our hypothesis. Among these AAs, Ala had the highest TEF value (7.2‰) and Asp the lowest (2.9‰). Bradley et al. (2014) reported relatively similar TEF_{Ala} (6.8‰), whereas Hoen et al. (2014) reported a wider range but lower TEF_{Ala} (ranged 0.5 to 6.0‰) and TEF_{Asp} (0.2 to 3.0‰). Barreto-Curiel et al. (2017) reported high TEF_{Ala} (6.8‰) and a low TEF_{Asp} (3.7‰) for Pacific yellowtail. The lack of differences in TEFs may indicate that TEFs for Asp, Pro, Val, and Ala reflect the trophic step of a carnivorous fish. These results are unexpected given that diet quality represent one of the main current working hypothesis to explain the variability in many trophic AAs across trophic levels (TLs) including for the canonical trophic AA, Glu TEF, and $TDF_{Glu\text{-}Phe}$ (e.g., McMahon & McCarthy, 2016). For a high trophic level growing fish such as the carnivorous *S. lalandi*, the results of our study indicate that diet quality influence Glu TEFs, but does not

have a significant effect on Asp, Pro, Val, and Ala TEFs. In particular, Asp TEF exhibited overall a relatively low isotope discrimination in muscle in response to diet quality but also quantity in comparison with other trophic AAs. These results suggest that Asp responds slightly to changes in dietary protein attributes.

2.5. Summary and recommendations

In liver tissue, the TEFs of Phe, Met, Lys, and Gly did not vary with protein content and showed limited isotope fractionation relative to the diets. Only TEF_{Lys} decreased with protein content possibly in relation to higher dietary lipid content; further studies are required to examine this relationship. The low variability in TEFs of Asp, Glu, Ile, Pro, Val, and Ala with changes in protein content indicated that isotope discrimination remained relatively constant despite changes in dietary protein ranging from 40% to 60%, and only TEF_{Leu} decreased with higher protein content. In muscle, unexpectedly, Phe and Lys TEFs varied as a function of protein content despite that these AAs are believed to reflect baseline isotope ratios with minimum changes across trophic levels and diet compositions. Hence, careful consideration of whether these AAs are reflecting an isotopic baseline is warranted.

Regarding the effect of diet quality, we found that the TEFs of source and trophic AAs did not differ significantly between diets varying in protein digestibility in liver tissue. In muscle, the TEFs of Phe, Lys, and Met were sensitive to changes in protein quality, while Gly TEF exhibited low variability between treatments, indicating that Gly in muscle tissue may function as a robust source AA in teleosts, unlike other taxa for which a greater degree of variability has been observed (McMahon & McCarthy, 2016). Among trophic AAs, only TEFs of Glu, Ile, and Leu showed differences between diets differing protein digestibility. TEF_{Glu} exhibited a large range of values, which indicates that TEF_{Glu} varies substantially in teleost muscle in response to changes in protein quality.

Our results differ from the current paradigm that considers Phe to reflect baseline isotopic values because we found variable isotopic fractionation with differing diet content and protein quality in muscle (but not in liver tissue). Further, the observed variability in AAs TEFs between liver and muscle tissues indicates isotopic fractionation is variable between these tissues, and should not be assumed to be universal. In our study, the observed differences in TEF_{AA} between liver and muscle are likely driven by tissue-specific functional roles and nutritional requirements relative to the availability of dietary AAs. Concurring with reviews of the premises underlying the application of stable isotope measurements in bulk tissues (Martínez del Río et al., 2009) and AAs (Ohkouchi et al., 2017), more experimental studies that consider AAs metabolism in response to dietary AA profiles and nutrient requirements are clearly needed for a better understanding of the causes underlying

differences in TEFs between tissues. Our study highlights the need for carefully examining animal nutritional physiology before formulating diets, as well as independently evaluating the effect of dietary nutrients (e.g., protein quantity and quality, fatty acid, and carbohydrate content) in experimental feeding studies. Considering these aspects will help disentangle the variability in N isotopic fractionation in association with specific dietary protein attributes and will help us to identify the mechanisms that drive isotopic fractionation in bulk tissues and AAs.

CHAPTER 3. The relationship between amino acid $\delta^{15}N$ trophic enrichment factors, dietary components and ecological factors in teleost fish: a meta-analysis

3.1. Introduction

Ecologists use data derived from nitrogen (N) compound-specific stable isotope analysis (CSIA) in amino acids (AAs) to estimate animal trophic position (TP) (Popp et al., 2007; Chikaraishi et al., 2007,2009). The CSIA-AA approach is based on the premise that AAs $\delta^{15}N$ values quantified from consumer tissues provide data of both primary producers and diet (McClelland and Montoya, 2002; Popp et al., 2007; Chikaraishi et al., 2009). The N isotopic discrimination between a consumer's AAs relative to its diet is known as trophic enrichment factor (TEF_{AA}). Trophic discrimination factors (TDF) is another parameter necessary to estimate the TP of a consumer by calculating the isotopic difference between the TEF_{AA} of a trophic and a source AA quantified from the same animal tissue (Popp et al., 2007; Chikaraishi et al., 2009). Hence, calculating TP requires accurate estimates of TEFs. Chikaraishi et al. (2009) proposed that TP estimation following this equation:

$$TP_{x/y} = (\delta^{15}N_x - \delta^{15}N_y - \beta_{x/y})/ (TEF_x - TEF_y) + 1 \quad (7)$$

where x and y are trophic and source AA, respectively, $\beta_{x/y}$ is the difference between the $\delta^{15}N$ values of *x* and *y* in primary producers and TEF_x and TEF_y are the trophic enrichment factors for trophic AA and source AA, respectively.

AAs have been classified into these two broad groups based on the degree of the isotopic discrimination observed per trophic step. The source AAs reflect the isotopic baseline because they show limited or near to zero isotope discrimination with each trophic level and should thus reflect the isotopic composition at the base of the food web (e.g. Phe≤0.4‰), whereas trophic AAs reflect a consumers´ trophic step due to their substantial enrichment in ^{15}N with each trophic level (e.g. Glu=8.0‰) (Chikaraishi et al., 2009). Early in the application of CSIA-AA, source AAs included phenylalanine (Phe), lysine (Lys), methionine (Met), glycine (Gly), serine (Ser), threonine (Thr), tyrosine (Tyr), while trophic AAs included glutamic acid (Glu), alanine (Ala), isoleucine (Ile), leucine (Leu), valine (Val), aspartic acid (Asp), proline (Pro) (Popp et al., 2007). Thr was subsequently re-classified as a "metabolic" AA because its isotope discrimination does not follow the definition of source nor trophic AA, and its $\delta^{15}N$ values are depleted in ^{15}N, especially for high trophic level consumers (Germain et al., 2013). Ser and Gly have been considered "metabolic" AAs as well due to a high level of variability in empirical TEF estimates, which renders their utility as source AA questionable (McCarthy, Benner, Lee & Fogel, 2007;

McMahon & McCarthy, 2016). Based on the consistency in TEF estimates across trophic levels and taxa, Phe and Glu have been considered the canonical source and trophic AA, respectively (Chikaraishi et al., 2009; O´Connell, 2017), but other studies have advocated for the use of a suite of select AAs for characterizing the baseline isotopic composition and estimating TP (Nielsen et al., 2015).

Identifying the main factors driving N isotopic fractionation in AAs across tissues, species and trophic levels is key for the use of CSIA-AA in food web studies. Estimation of TP depends on the precise and accurate estimation of β and TEF_{AA} values, which can vary substantially between consumer-prey relationships (Nielsen et al., 2015). The variability in δ^{15}N-AA and TEF_{AA} values in consumer tissues have been associated with specific metabolic pathways in AAs of primary producer (Hare et al., 1991) and consumer´s tissues (Hare et al., 1991; Chikaraishi et al., 2007; Chikaraishi et al., 2009; O´Connell, 2017). Ecological and physiological factors also influence AA isotopic fractionation, such as habitat type (Dale et al., 2011), ontogenetic stage (Dale et al., 2011), wild caught vs. captive animals (McClelland and Montoya, 2002; Chikaraishi et al., 2007; McMahon and McCarthy, 2016), taxa (Nielsen et al., 2015; McMahon and McCarthy, 2016), feeding habits (Bloomfield et al., 2011; Hoen et al., 2014), mode of N excretion (Dale et al., 2011; Germain et al., 2013), dietary protein quantity (Nuche-Pascual et al., 2018) and dietary protein quality (Nakashita et al., 2011; McMahon et al., 2015, Nuche-Pascual et al., 2018). Several studies have concluded that the TP of marine consumers, in particular, those feeding at higher TP, might be underestimated (Dale et al., 2011; Nielsen et al., 2015).

Recent literature surveys have reported high variability (rather than stability) in empirical estimates of TEFs of source and trophic AAs. In a meta-analysis of TEF values across taxa, McMahon and McCarthy (2016) found TEFs of -0.1 $\pm$ 1.6‰ and 6.4 $\pm$ 2.5‰ for Phe and Glu, respectively. In a meta-analysis of TEF_{AA} and stomach-content derived TP estimates of captive and wild marine organisms that differed in diet type (carnivorous vs. omnivorous vs. herbivorous) and mode of nitrogen excretion (urea vs. ammonia). Nielsen et al. (2015) found that diet and mode of N excretion influenced TEF_{AA} values and hence TP estimates derived from CSIA-AA, and that TP estimates for higher trophic level species tended to underestimate the trophic position. The level of variation reported for each AA challenges the application of universal values for TEFs across taxa and trophic levels in ecological studies. In these two previous meta-analyses, authors encouraged more research on the influence of nutrition and animal physiology in isotope discrimination in AA, and highlighted the need for taxon-specific TEF_{AA} values. Focusing on a single taxonomic group allows eliminating known factors that contribute to the variation in isotope discrimination between taxa, such as mode of nitrogen excretion (Nielsen et al., 2015) and poikilothermic vs endothermic metabolism (Thomas and Crowther, 2014).

Fishes have complex life cycles in which the early life stages (larvae and early juveniles) undergo distinct

physiological and development processes that are linked to bioenergetic requirements and metabolic processes (Kamler, 1991). In particular, the digestive capacity, bioenergetic balance, and efficient protein metabolism, that characterizes the early life stages of fish, enable rapid growth and development (Finn et al., 2002). During early life stages, fish require high protein consumption to sustain high protein accretion (fast growth) (NRC, 2011), protein synthesis is efficient and protein turnover can be high (see reviews by Houlihan et al., 1995; Concienciao, 1997). Meta-analyses of isotope discrimination in bulk tissues have identified that life stage and habitat type influence TEF values (Vanderklifft and Ponsard, 2003; Sweeting et al., 2007; Madigan et al., 2018). However, only a few CSIA-AA studies have evaluated the role of life stage and habitat type on AA TEFs (Dale et al., 2011). AA metabolism is linked to N isotopic discrimination (O´Connell, 2017), therefore, TEF $\delta^{15}N$ values can vary as a function of life stage. Thus, depending on fish life stage, nutrient requirements and metabolic rates, fish would select prey that would determine nutrient (i.e. protein and lipids) intake metabolic pathways, AA isotopic discrimination, and variability in TEF_{AA} (McCutchan et al., 2003; Dale et al., 2011; Bradley et al., 2016; O´Connell, 2017).

To date, most experimental studies on CSIA-AA have not considered the role of nutrient requirements and feeding regime on AA TEF estimates. The first control feeding study in fishes showed that fish fed with a dietary protein of extreme levels of protein quantity and quality have large isotopic fractionations in trophic AAs (e.g. McMahon et al., 2015). In specific, the TEFs of Gly, Asp, Glu, Ile, Leu, Val, Ala differed significantly in juvenile mummichog (*Fundulus heteroclitus*) fed with a very low protein diet (vegetarian) in comparison to those fed with higher protein content (clam and squid; McMahon et al., 2015). In another control feeding experiment with juveniles of totoaba (*Totoaba macdonaldi*) fed with isoenergetic diets, that used two protein sources and varied protein content (by 3%), Barreto-Curiel et al. (2018), found that TEFs of Phe, Lys, Met, Gly, Glu, Pro, Val differed significantly.

Recently, Nuche-Pascual et al. (2018) fed juvenile Pacific yellowtail (*Seriola lalandi*) with treatments that varied in dietary protein content (by 10%) and quality (i.e., digestibility %), considering species-specific nutrient requirements, and found that both protein quantity and quality influences Lys and Leu TEFs in liver tissue, and Phe, Lys, Ile, Leu TEFs in muscle tissue.

Proteins and lipids are the major organic components of fish body tissues, that serve as the main energy substrates (Tocher, 2003; NRC, 2011); fish have no carbohydrates requirements *per se* (NRC 2011). Protein (i.e., AAs) and lipids (i.e., fatty acids) are used to meet energetic and metabolic balance through anabolic and catabolic processes. Therefore, the availability of dietary protein and lipids may influence the degree of AA catabolism and possibly impact N isotope discrimination in AAs. Protein and lipid requirements (where

requirements are defined as the minimum amount of protein or lipids needed to maximized growth; e.g. Dacosta-Calheiros, Arnason and Bjornsdottir, 2003; NRC, 2011) are determined by essential AA and fatty acid requirements, respectively. Since both protein and lipid assimilation and availability drive biomass gain and regulate the metabolism of AAs (NRC, 2011), it is necessary to evaluate the role of dietary lipid content on TEF_{AA} and not only the protein content.

It is widely recognized that "fish eat to satisfy their energy and nutrient requirements," and food consumption varies in response to diet quality and quantity to meet energetic and nutrient requirements for AAs, fatty acids and vitamins (Cho and Kaushik, 1990; NRC, 2011) and to achieve energy balance (NRC, 2011). The fish feeding regime, i.e. under a fixed amount of food or satiation (a physiological process that results in the termination of food ingestion; e.g. Ritter, 2004; Saravanan et al., 2012) determine the quantity of food consumed, and protein amount assimilated and catabolized. Hence, feeding regime can alter the catabolic activity of AA and influence isotopic discrimination. This is especially the case in fish fed low protein quantity or quality diets (Saravanan, Schrama, and Figueiredo-Silva, 2012).

We conducted a meta-analysis that compiled TEF_{AA} estimates from studies on captive teleosts subjected to controlled feeding experiments to (a) evaluate the role of various nutritional and ecological factors and (b) quantify the degree of variability in TEF estimates of source and trophic AAs. We also evaluated whether the level of variation in AA-specific TEFs of a single taxonomic group (teleosts) is lower than when considering multiple taxa, which has important implications for the selection of TEF values for estimating TP of natural populations. Specifically, we assessed the relationship between the TEF_{AA} and (i) dietary protein (DP) and dietary lipid (DL) content, and (ii) DP and DL relative to taxon-specific estimates of protein and lipid requirement under three levels (low vs. optimum vs. high). We also evaluated whether TEF_{AA} varied as a function of (iii) diet type (fish vs. invertebrates vs. plant-based feeds), (iv) feeding regime (fixed vs. satiation), (v) life stage (larvae vs. early juvenile vs. subadult vs. adult) and (vi) aquatic habitat type (marine vs. brackish vs. fresh). We hypothesized that TEFs of source AAs would not differ as a function of nutritional and ecological parameters. For trophic AAs, we hypothesized that TEFs would increase with high DP levels relative to protein requirement, because fish should catabolize excess dietary protein resulting in higher excretion of ^{15}N-depleted nitrogen (Martínez del Río and Wolf, 2005). In addition, we hypothesized that AA TEFs would be lower for early life stages since most of the protein consumed is efficiently assimilated for growth, leading to lower AA catabolism and hence lower isotope discrimination.

3.2. Methods

We compiled literature of controlled laboratory feeding experiments reporting δ15N-AA values for fish muscle tissue and diets, from which TEFs were reported or could be calculated using published data (Supplementary Table 1, hereafter Table S1). When TEFs were not reported, we calculated TEFAA values as follows:

$$TEF_{AA} = \delta^{15}N\text{-}AA_{tissue} - \delta^{15}N\text{-}AA_{diet} \quad (8)$$

where $\delta^{15}N\text{-}AA_{tissue}$ and $\delta^{15}N\text{-}AA_{diet}$ represent the nitrogen isotopic composition of each AA in a consumer's muscle tissue and the diet, respectively (Popp et al., 2007). TEF_{AA} from each dietary experiment was included as an individual data point. We selected experiments in which fish tissues reached isotopic equilibrium, which was evaluated based on author analysis or by estimating the relative weight gain (WR) achieved during each feeding experiment. A minimum three-fold increase in weight was considered as indicative of equilibrium (Herzka, 2005; Nuche-Pascual et al., 2018). We included all the AAs reported in at least two studies, except for Thr, because this AA exhibit very depleted $\delta^{15}N$ values in contrast to the other AAs (Hare et al. 1991), and is not considered an adequate tracer for baseline or TP (e.g. Germain et al., 2013; McMahon et al., 2015).

We evaluated the relationship between TEF_{AA} and five nutritional characteristics of the diet, and three important ecological factors in fish. Nutritional characteristics included the dietary protein and lipid content reported in each study, and same content relative to species-specific protein and lipid requirements. We evaluated the relationship between protein and lipid content or TEFs AA by using regression analysis, and compared our results with other feeding experiments on specific fish species, when protein and lipid content were reported explicitly such as McMahon et al., 2015; Blanke et al., 2017; Nuche-Pascual et al., 2018; Barreto-Curiel et al., 2018 (see Tables S3 and S4). Method previous to CSIA-AA analysis differed among studies. Lipid extraction previous to analysis was explicitly reported by the method followed by Blanke et al. (2017), Nuche-Pascual et al. (2018) and Barreto-Curiel et al. (2018). In contrast, lipid extraction prior to CSIA-AA analysis was not explicitly reported by McMahon et al. (2015).

To examine the role of protein and lipids content relative to dietary requirements, each dietary treatment was classified into one of the three categories: low, optimum, or high protein or lipid level relative to the species' requirements. A diet was considered to contain an optimum dietary protein level if it was within ±5% of the protein requirement reported in the literature. A 10% difference in dietary protein content on most fish nutrition studies results in a strong influence on growth performance (Catacutan et al., 2001; Nuche-Pascual et al., 2018). Diets classified as containing a 'high' protein level had ≥5% protein content than a specific species' requirement,

and those with 'low' protein level had ≤5% or less protein content than the requirement. A diet was considered to contain an optimum lipid level when it contained ±3% of the lipid requirement reported in the literature, whereas a high and low lipid content had ≥ and ≤ 3% of lipid requirement, respectively. Species-specific protein and lipid requirements were obtained from the literature when available. If unavailable, published genus or family-specific protein and lipid requirements were used (Table S1).

The feeding regime used in each experiment was classified as either fixed feeding rate, when a pre-established quantity of feed was provided or as satiation feeding, in which food is provided until fish are apparently satiated (Ritter, 2004; Saravanan et al., 2012). Food types used during feeding experiments were classified into three categories based on the predominant protein source: plant, invertebrate or fish.

Ecological factors included life stage and aquatic habitat. The life stage of the fish during the feeding experiment was classified into four categories: larvae, early juvenile, subadult and adult stages. Each species' habitat was classified as marine, brackish or freshwater, based on their predominant environment.

To evaluate whether the variability of our obtained TEF_{AA} values of teleosts was lower than that reported when multiple taxonomic groups were considered, we compared it with the TEF_{AA} estimated using data from 73 feeding experiments conducted on mold, bacteria, fungus, insects, crustaceans, mollusks, amphibians, reptiles, teleosts, elasmobranchs, and mammals (McMahon and McCarthy 2016). In our meta-analysis, we examined 32 consumer-prey feeding experiments on teleosts, which is 23 more teleost than were included in McMahon and McCarthy´s (2016) meta-analysis. We compared our TEF_{AA} estimates for teleosts with the global TEFs reported by McMahon and McCarthy (2016). In addition, we compared our TEF_{AA} values for teleosts with TEFs that we calculated from McMahon and McCarthy (2016)'s dataset but excluding all teleosts (i.e., mean TEFs for all other taxa).

3.2.1 Statistical analysis

For comparative purposes, both the mean and median TEF for each AA were estimated for each source and trophic AA (Figure 7). The mean and median are both central tendency indicators (Miller, 1991), and although the mean and standard deviation (SD) are the more commonly used indicators, they are sensitive to outliers. Errors were calculated for the mean (SD) and the median value (median absolute deviation; MAD). The calculation of SD assumes a normal distribution and values are influenced by sample size. In contrast, the median

is not influenced by outliers, and the MAD does not assume a normal distribution and it is not influenced by the sample size (Leys et al., 2013). Given that the sample size TEFs for some AAs, such as Met and Ser, was limited, the median was considered a robust central tendency indicator for comparison with mean values. MAD is defined as the median of the absolute deviations from the overall median (Huber, 1981) and was estimated following Leys et al. (2013):

$$MAD=b\, M_i\, (\, |\, x_i-M_j\, (x_j)|\,) \qquad (9)$$

where x_i refers to each of the original observations (i.e. the TEF_{AA} values from each feeding experiment), x_j refers to the number of observations (i.e. the number of TEFs included in the estimate), and M_i is the median, M_j is defined as the absolute value of (x_i-M_i). The constant b =1.4826 is applied when the data have an underlying normal distribution (Rousseeuw and Croux, 1993), or b=1/Q (0.75) in cases when the data are non-normal (normality was previously evaluated for each TEF_{AA} in separate tests) and where Q (0.75) represents the value of the third quartile (Huber, 1981). Only TEFs of Phe, Gly, Asp, and Leu were not normal. Mean and median values, as well as SD and MAD, were similar for most TEFs of source and trophic AAs, indicating that there were few extreme values influencing mean TEFs. Hence, to report our results we refer solely to the mean values.

As mentioned before, regression analysis was used to examine the relationship between TEF_{AA} and dietary percent protein and lipids. Levene's test was used to test the homogeneity of variance between source AA and trophic AA (separate tests). Since the assumption of homogeneity of variance was met, one-way ANOVAs were used to test for differences in mean TEF_{AA} for each nutritional and ecological factor. Statistical analyses were carried out using STATISTICA V 7. Categories where only one TEF_{AA} value was reported in all the studies surveys (i.e., n=1) were included in graphs for comparative purposes only but excluded from statistical analyses.

We evaluated the variation in our TEF_{AA} values of teleosts with TEF values reported by McMahon and McCarthy (2016; we named it "TEF global"), and another TEFs that we calculated from their published data set. The latter excludes teleost TEFs (and it was referred as 'non-teleost TEF') to evaluate the variation between teleost and other taxonomic groups. We calculated the mean and SD TEFs for each AA. A Levene's test was used to test for homogeneity of variances between global TEF, non-teleost TEFs and our teleosts TEFs for each AA. A student t-test was applied to test for differences in mean TEFs when the variances were homogeneous, and a non-parametric statistical test was used when variances were not homogeneous. Statistical analyses were carried out using STATISTICA V 7.

3.3. Results

We found 9 studies published between 2009 and 2018 that included 11 teleosts consumer species and 32 individual consumer-diet feeding experiments reporting a total of 236 AA-specific consumer-diet relationships (Table S1). We compiled TEFs for the five AAs initially classified as source AAs (Phe, Lys, Met, Gly, and Ser) and 7 trophic AAs (Asp, Glu, Ile, Pro, Val, Leu, and Ala). We found no consistency in the number of source and trophic AAs reported in the nine studies analyzing TEF_{AA} values in teleost; only the TEFs for Glu were reported in all studies and experiments. We recovered a total of 32 TEF_{Glu} published in control feeding experiments.

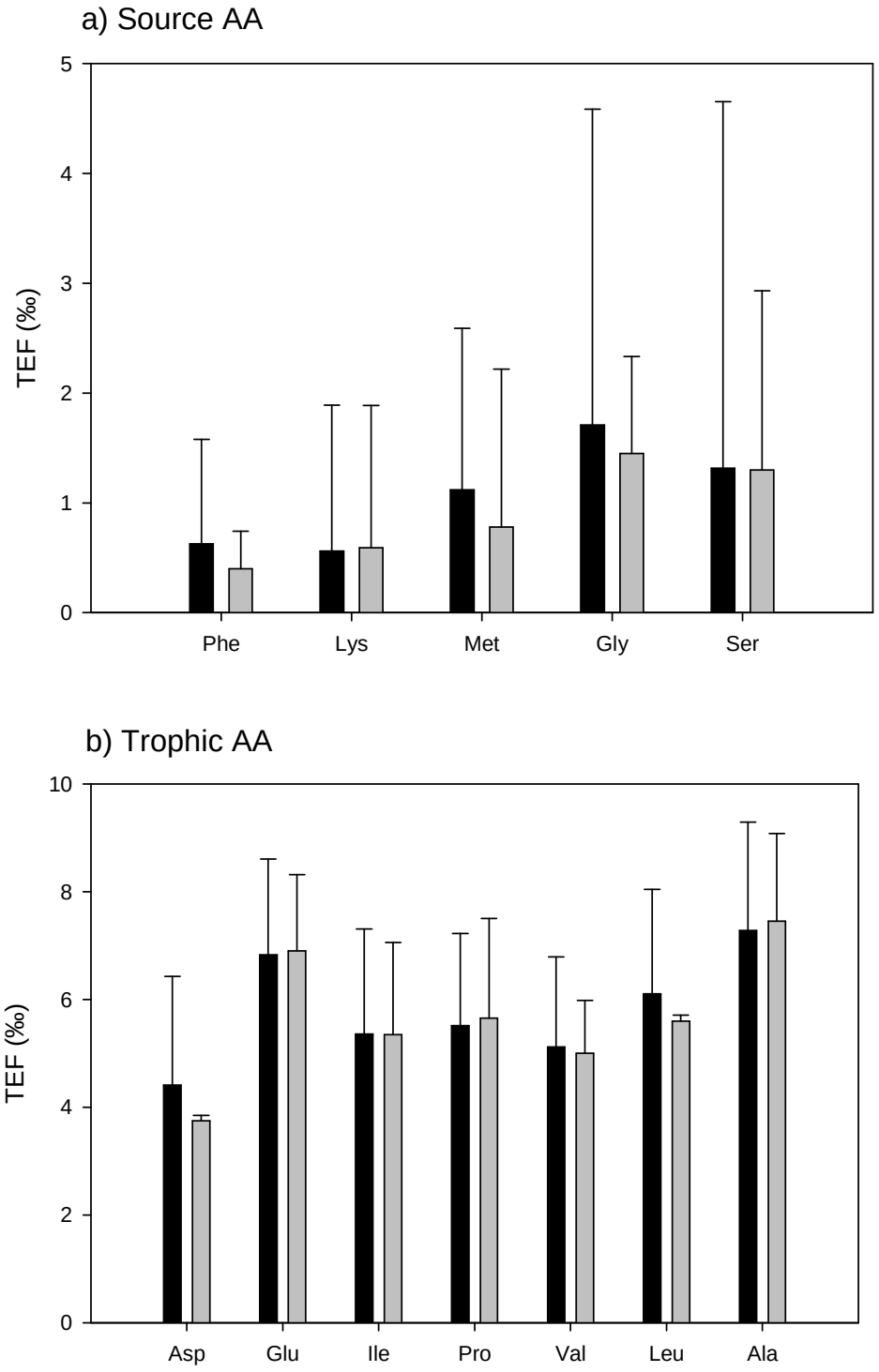

Figure 7. Mean (black bars) and median (grey bars) trophic enrichment factors (TEFs) for amino acids measured in fish muscle tissue. Errors are represented as Standard deviation and Median Absolute Deviation for mean and median values, respectively. Phe, phenylalanine; Lys, lysine; Met, methionine; Gly, glycine; Ser, serine; Asp, aspartic acid; Glu, glutamic acid; Ile, isoleucine; Pro, proline; Val, valine; Leu, leucine; Ala, alanine. The number (n) of TEF_{AA} estimates in published studies varied (Phe=31, Lys=18, Met=11, Gly=20, Ser=9, Asp=17, Glu=32, Ile=18, Pro=20, Val=20, Leu=20, Ala=20).

3.3.1 Dietary protein and lipid content

The dietary protein content for all feeding experiments ranged from 8 to 71% of the diet. Only a single feeding experiment included a very low protein content (5%) treatment; the other experiments had 40% or higher protein in the diets. The mean TEFs for each of the seven trophic AAs was highest for fish fed with the lowest (i.e. 5%) protein treatment in comparison to fish fed with ≥ 40% protein diets. Regressions analyses between protein percent and each source AA TEFs were not significant (Figure 8a). In contrast, regression analysis between dietary protein content and TEF values for two trophic AAs, in specific, Glu and Ala, were significantly and negatively related ($p=0.011$ and $p=0.027$, respectively; Figure 8b), and no significant regression analysis was found for the other five trophic AAs ($p>0.05$). Percent of dietary lipid used in the feeding experiments ranged between 2 to 24% of the diet. We found no significant relationships between lipid content and either source or trophic AA TEFs (Figure 9).

When we analyzed the relationship between TEFs vs. dietary protein content were considered, we found significant negative relationships for Lys ($R2=0.95$, $p=0.025$, $TEF_{Lys}=3.12-0.02 \bullet$ % protein), Glu ($R2=0.95$, $p=0.024$, $TEF_{Glu}=11.37-0.07 \bullet$ %protein), Ile ($R2=0.94$, $p=0.029$, $TEF_{Ile}=9.81-0.06 \bullet$ %protein), Pro ($R2=0.91$, $p=0.044$, $TEF_{Pro}=7.42-0.01 \bullet$ %protein), and Leu ($R2=0.99$, $p=0.003$, $TEF_{Pro}=10.53-0.07 \bullet$ %protein) for feeding experiments on mummichog (Fundulus heteroclitus; McMahon et al., 2015), but not for Pacific yellowtail (Nuche-Pascual et al., 2018) nor for totoaba (Barreto-Curiel et al., 2018).

With regard to dietary lipid content, significant relationships were only found between percent lipid and TEFs of Met ($R2=0.66$, $p=0.049$, $TEF_{Met}=-2.20+0.24 \bullet$ %lipid) for Pacific yellowtail (Nuche-Pascual et al., 2018). There was no relationship between dietary lipid content and TEFs in the experiments conducted on mummichog.

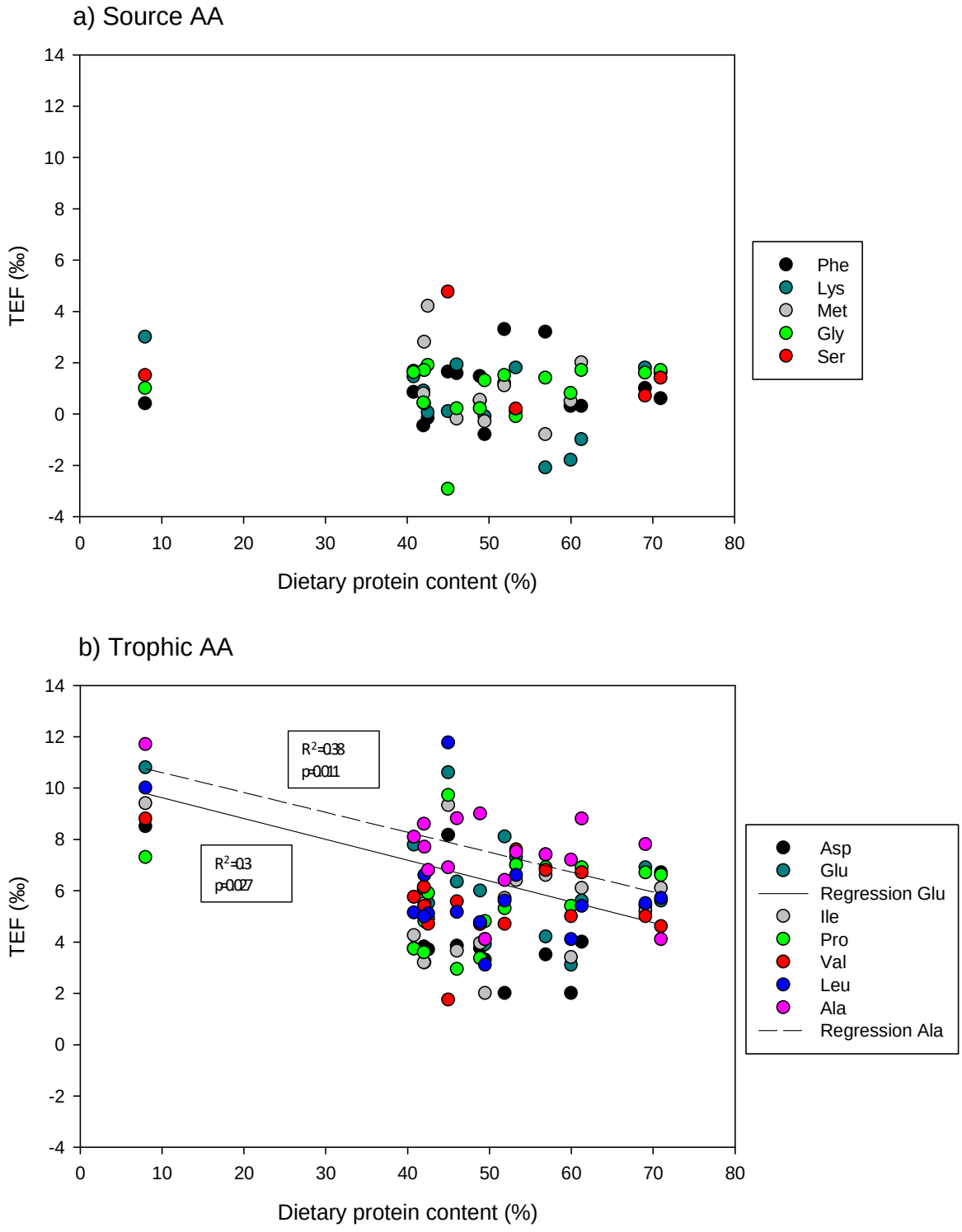

Figure 8. Nitrogen trophic enrichment factors (TEFs) of source and trophic AAs in muscle of fish fed different levels of dietary protein. TEFAA values are represented individually for each consumer-diet combination. Regression analysis yielded no significant relationships between percent dietary lipids and AA-specific TEFs

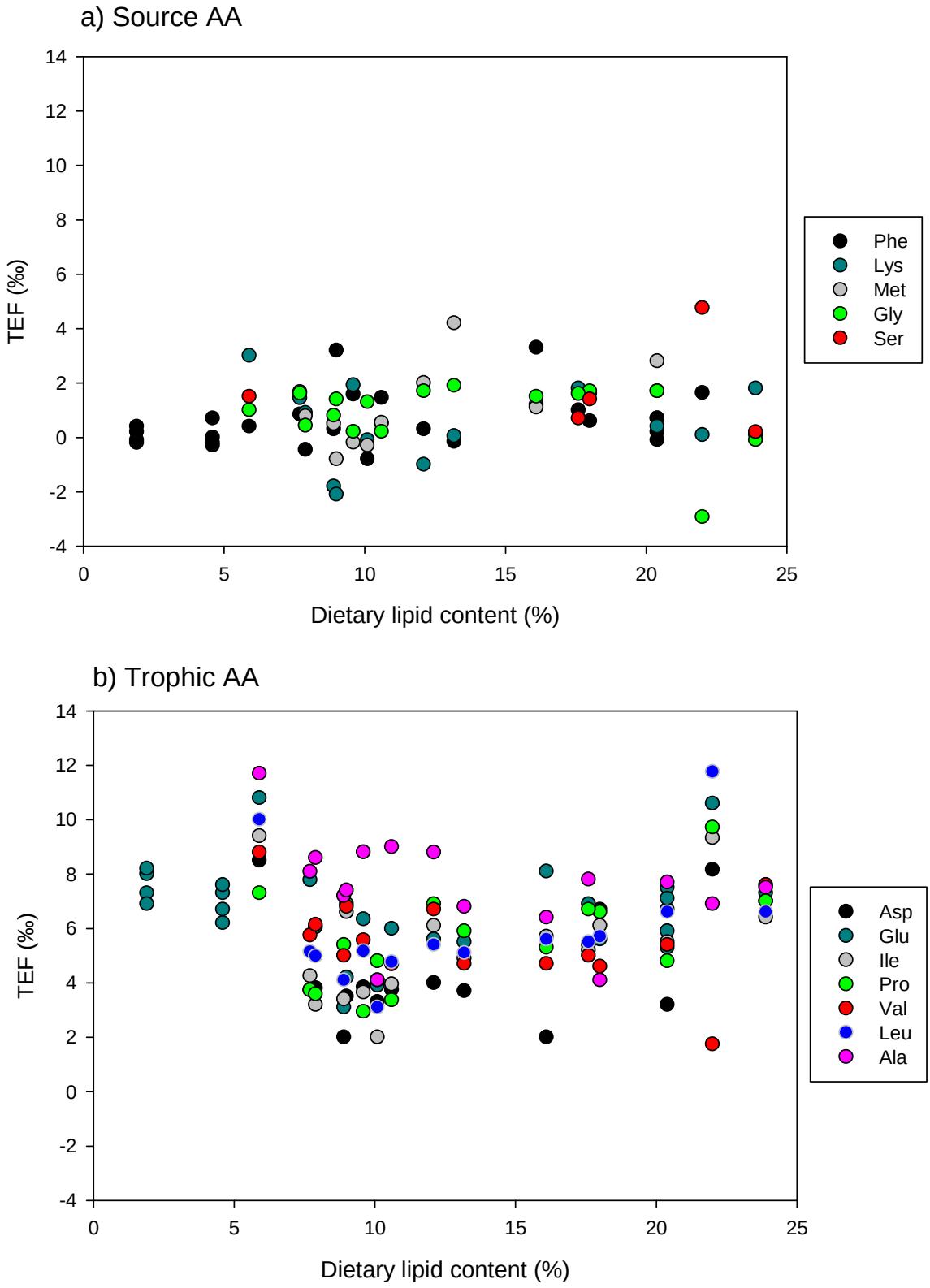

Figure 9. Nitrogen trophic enrichment factors (TEFs) of source and trophic AAs in muscle of fish fed different levels of dietary lipid content. TEF_{AA} values are represented individually for each consumer-diet combination. Regression analysis yielded no significant relationships between percent dietary lipids and AA-specific TEFs.

3.3.2 Dietary protein and lipid content relative to nutritional requirements

The $\delta^{15}N$ TEF_{AA} values were relatively variable among groups (source or trophic) and between categories (high, optimum or low; Figure 10). Among source AAs, the mean TEF_{Ser} exhibited the highest value (up to 3‰) for the diet with low protein content category, and the lowest TEF_{Ser} (0.2‰) with the optimum protein content category. TEF_{Lys} was the only AA that showed a negative value among source AAs. In specific, the TEF Lys was -0.3 ppm in fish fed with a high protein level, but not with the low and optimum protein levels (1.0 and 1.1‰, respectively). However, there were no significant differences in mean TEF_{AA} values among high, optimum or low protein content relative to requirement ($p>0.05$). The mean TEF of Phe remained relatively constant regardless of protein level (0.7, 1.1 and 1.1‰ for the low, optimum and high protein levels, respectively; Figure 10a). Among trophic AAs, Asp showed the lowest mean TEF for each of the three dietary protein level categories (5.2, 4.0 and 4.3‰ for low, optimum and high protein diets, respectively). TEF_{Glu} was the only AA that decreased as protein level increased in the diets (from 7.7 to 5.1‰; Figure 10b), although differences were not statistically significant (ANOVA, $F=166.9$, $df=13.0$, $p=0.130$).

Our analysis between AA TEFs values and lipid content showed some clear patterns for some source and trophic AAs between categories. Among the source AAs, only TEF_{Lys} varied significantly in fish fed with diets of low to high dietary lipid content categories (ANOVA, $F=3.9$, $df=13.0$, $p=0.47$); and there was a 1.8‰ difference between lipid content categories for the mean TEF Lys (Figure 11a). TEFs Met and TEF Gly showed a clear trend: TEF_{Met} increased with increasing dietary lipid content (from 0.3 to 1.7‰ from the low to the high category, respectively), whereas TEF_{Gly} decreased with increasing dietary lipid level (from 1.3 to 0.7‰ from the low to high lipid level diets, respectively). Among trophic AAs, no significant differences were observed in TEFs among the three lipid content categories despite that similar patterns were observed among some AAs. In particular, TEFs of Asp, Ile, and Leu increased as a function of dietary lipids content relative to fish requirements (Figure 11b); TEF_{Asp} ranged from 3.1‰ (low category) to 5.2‰ (high category), TEF_{Ile} ranged between 4.1‰ (low category) to 6.0‰ (high category), and Leu ranged from 4.9‰ (low category) to 6.7‰ (high category). TEFs of Glu, Val, and Ala did not show a clear trend and TEF_{Pro} was relatively consistent among lipid content categories.

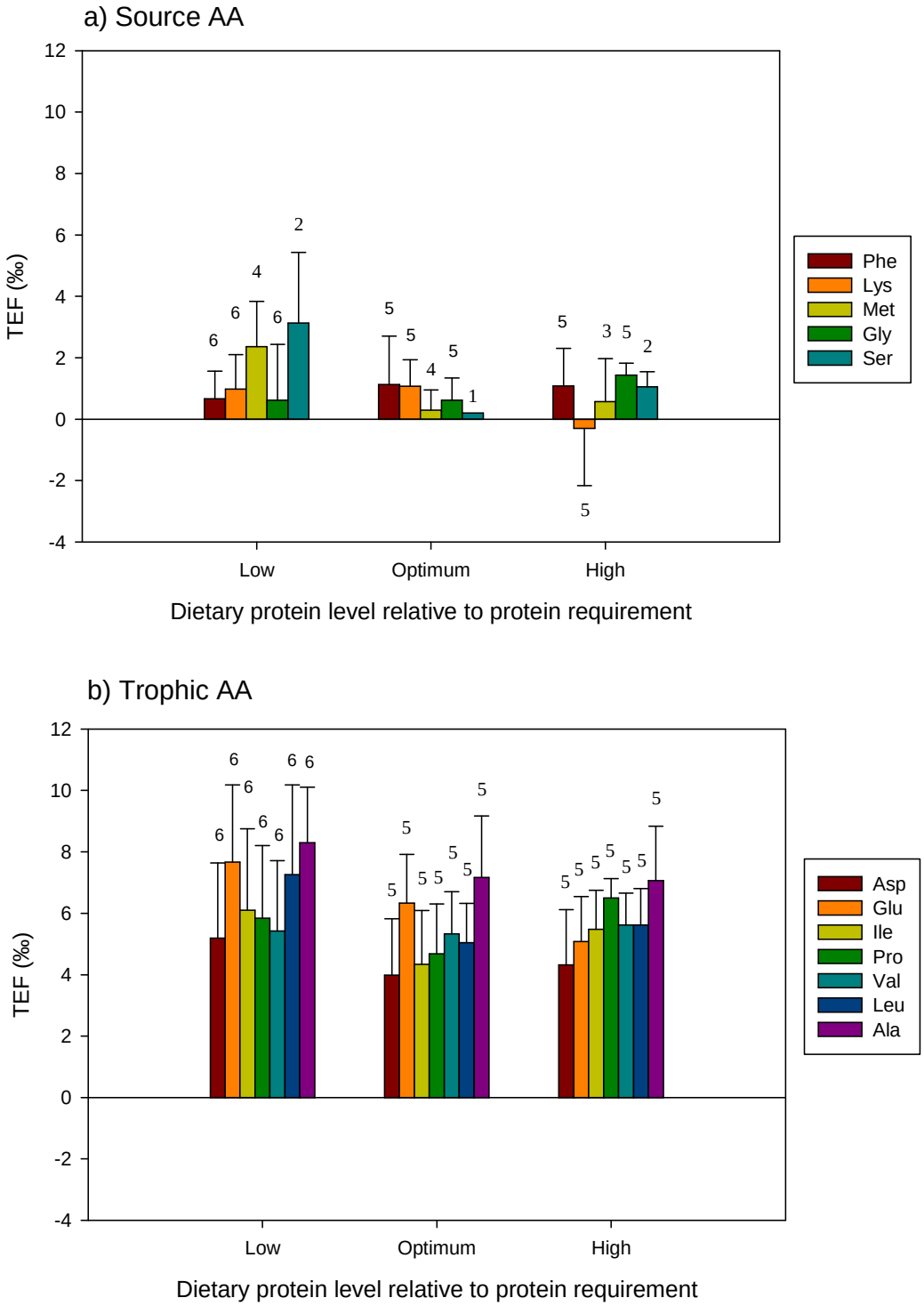

Figure 10. Mean TEFs of source and trophic amino acids for fish fed different dietary protein content relative to taxon-specific protein requirement. Error bars represent the standard deviation. The number of TEF estimates included in each mean is presented above the error bars. Optimum level= ±5% species-specific protein requirement, low level=≤5% species-specific protein requirement and high level=≥5% species-specific protein requirement.

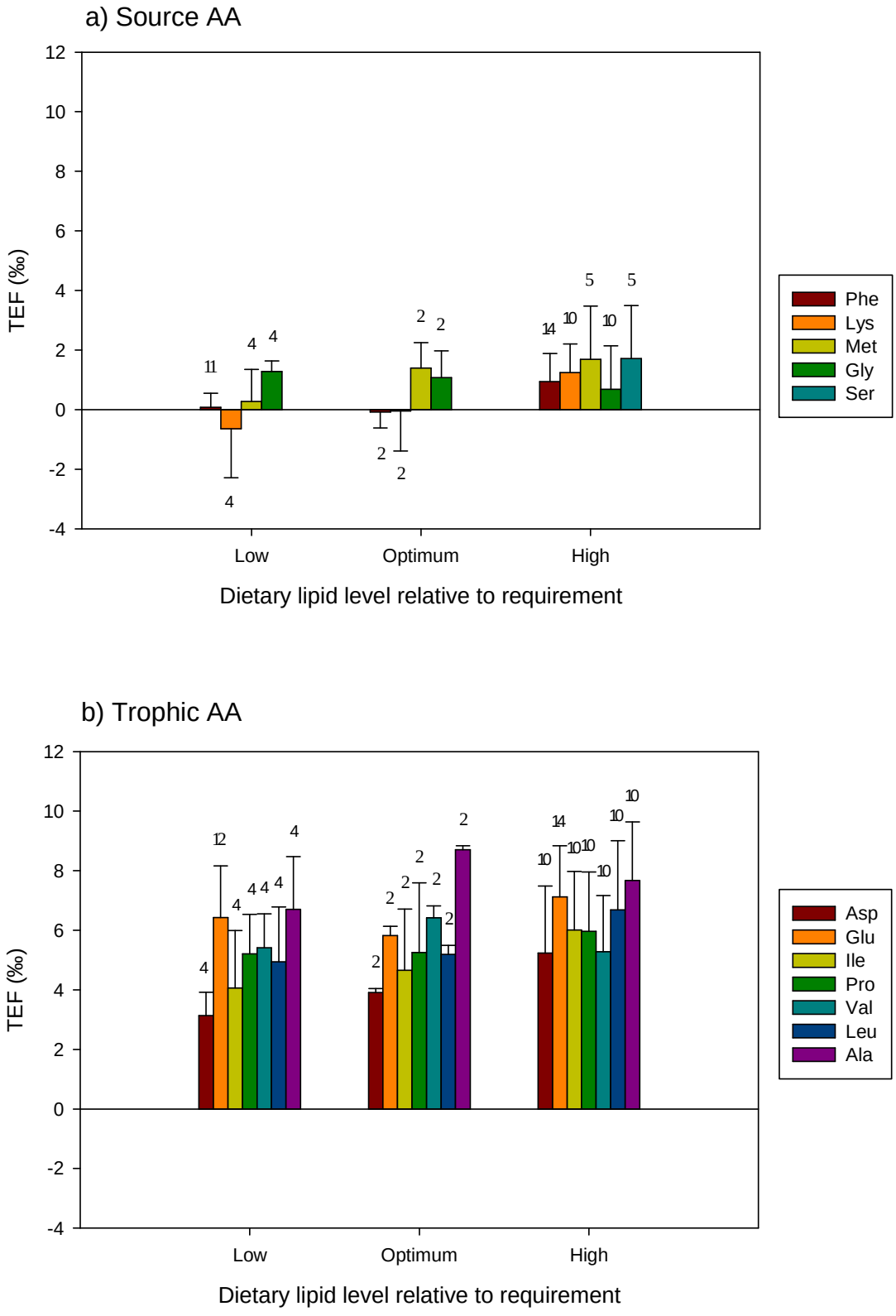

Figure 11. Mean TEFs of source and trophic amino acids for fish fed different dietary lipid level relative to taxon-specific requirement. Error bars represent the standard deviation. The number of TEF estimates included in each mean is presented above the error bars. Optimum level= ±3% species-specific lipid requirement, low level=≤3% species-specific lipid requirement and high level=≥3% species-specific lipid requirement.

3.3.3 Feeding regime

Only 2.5% of feeding experiments were performed using a fixed feeding regime vs. 97.5% that used satiation. Among source AAs, only TEF_{Lys} differed significantly between feeding regime categories. The TEF_{Lys} of fish fed a fixed feeding regime was significantly lower than under satiation feeding conditions ($F=11.6$, $p=0.004$; Figure 12a). Among trophic AAs, the TEFs of Asp and Glu exhibited significant differences between fixed and satiation feeding regimes. In both cases, the TEFs values from feeding experiments using a fixed feeding protocol were significantly lower ($F=8.33$, $p=0.012$ for TEF_{Asp}, and $F=10.46$, $p=0.003$ for TEF_{Glu}; Figure 12b).

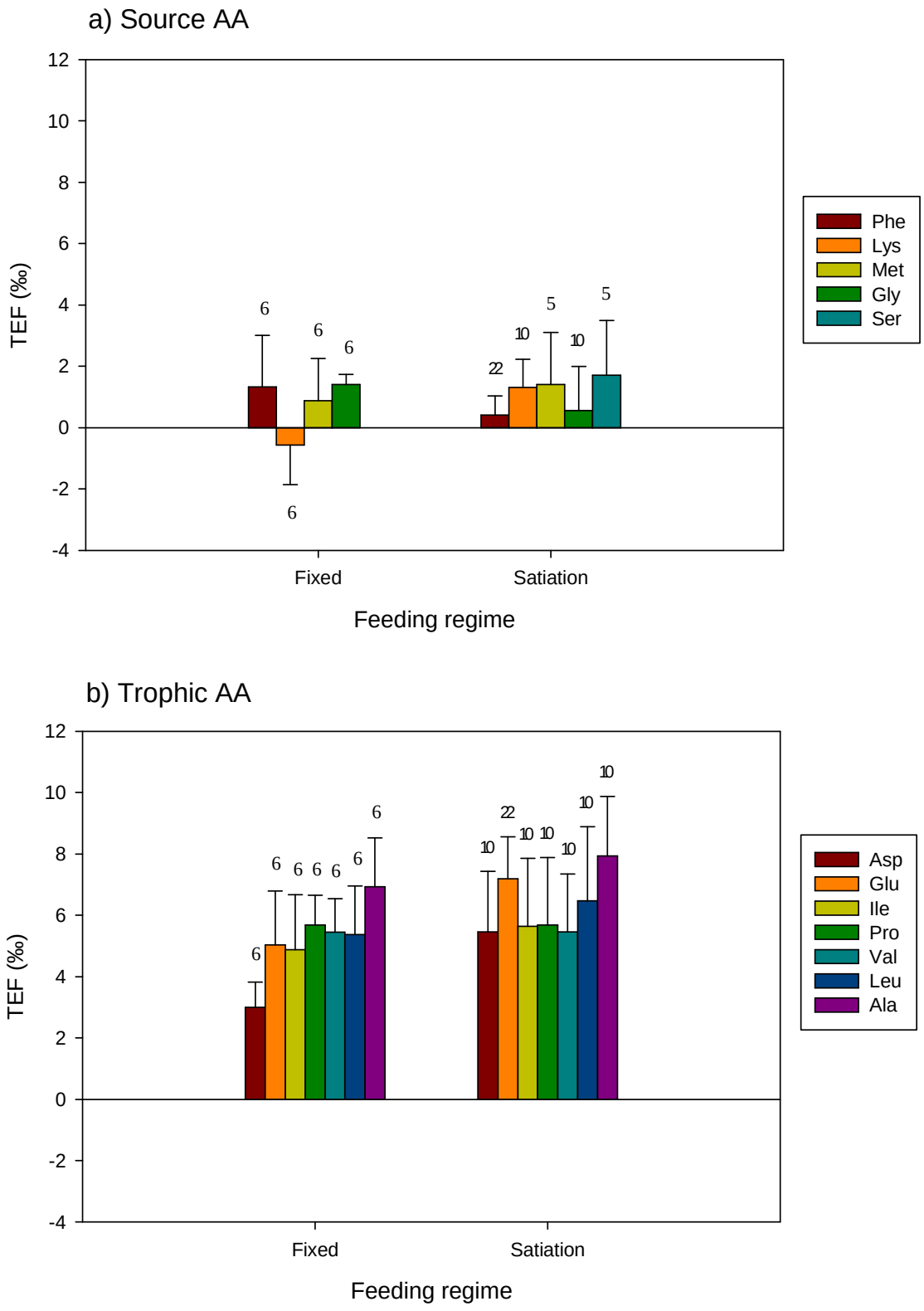

Figure 12. Mean TEFs of source and trophic amino acids for fish fed different feeding regimes. Error bars represent the standard deviation. The number of TEF estimates included in each mean is presented above the error bars.

3.3.4 Diet type

Most of the consumer-diet feeding experiments (177 out of 236) used fish as the main component of their diet, while 48 used invertebrates and only 11 incorporated plants as the main protein source (Table S1). TEF_{Gly} showed significant differences between experiments conducted with a fish or invertebrate-based diets ($F=7.87$, $p=0.012$); the mean TEF with the fish-based diets was 4‰ lower (Figure 13a). Although no other source TEF_{AA} exhibited significant differences among diet type categories, the mean TEF of Lys differed by 2.7‰. The TEFs of trophic AAs did not show significant differences among diet type categories, but the mean TEFs of Asp, Glu, Ile, and Pro were from 3.9 to 8.5‰, from 6.4 to 10.8‰, from 5.0 to 9.4‰ and 5.2 to 7.3‰, respectively, when comparing fish-based diet to a plant-based diet (Figure 13b).

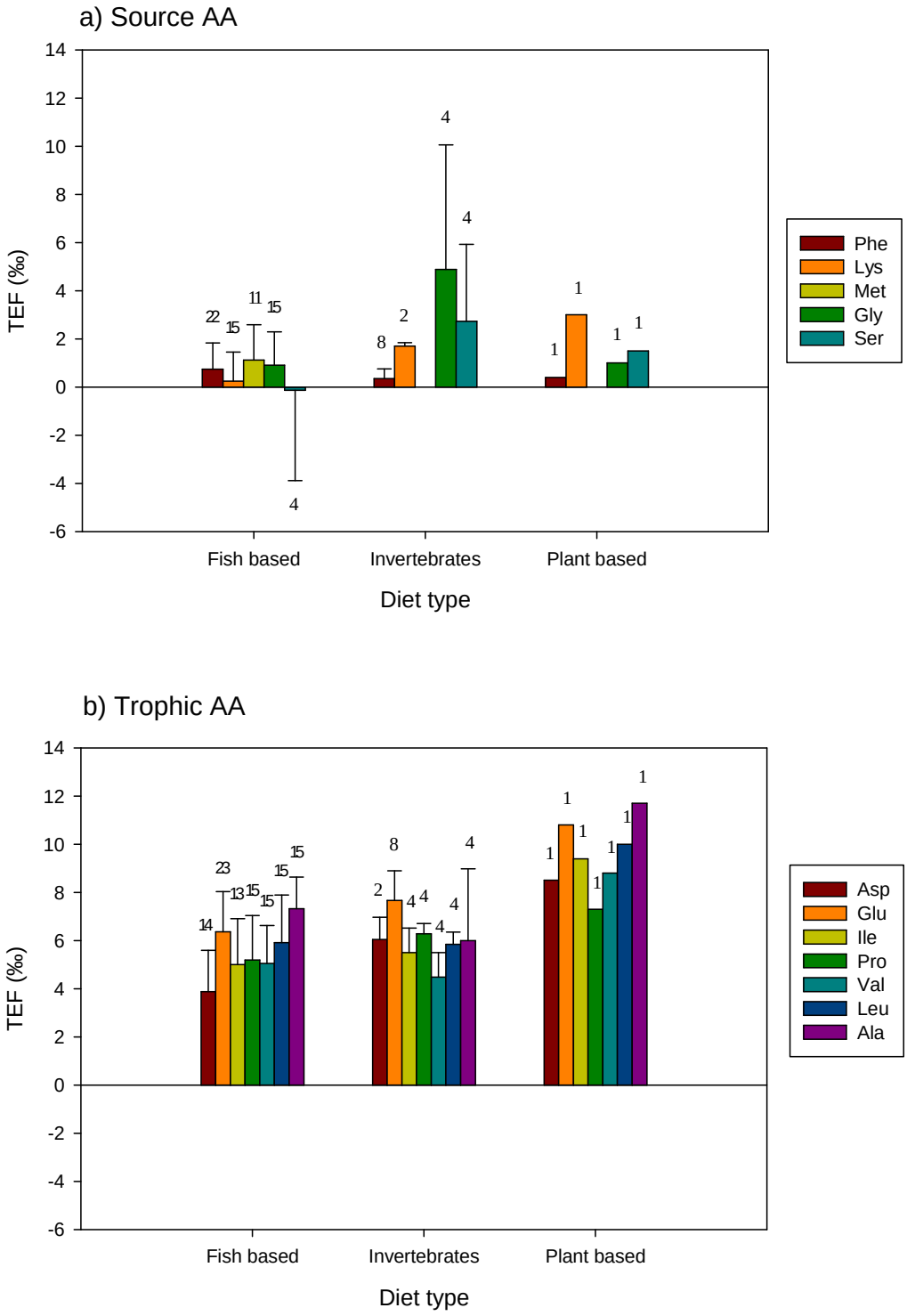

Figure 13. Mean TEFs of source and trophic amino acids for fish feed different diet types. Error bars represent the standard deviation. The number of TEF estimates included in each mean is presented above the error bars.

3.3.5 Life stage

79% of the feeding experiments in fish were conducted on early juvenile fishes, that produced data to generate empirical estimates of TEF_{AA}. Among source AAs, only TEF_{Gly} showed significant differences between the larval and early-juvenile life stages ($F= 24.6$, $p=0.0003$); the TEF_{Gly} for larvae was significantly higher (8.1‰) than for early-juvenile (0.9‰; Figure 14). TEF_{Ser} exhibited negative values in subadult (-4.2‰) and adult (-1.3‰) stages, although these data were not included in the statistical analysis given the low sample size (n=1). For trophic AAs, there were no significant differences in TEF_{AA} among life stages, and mean values for specific AA varied by less than 3.2‰.

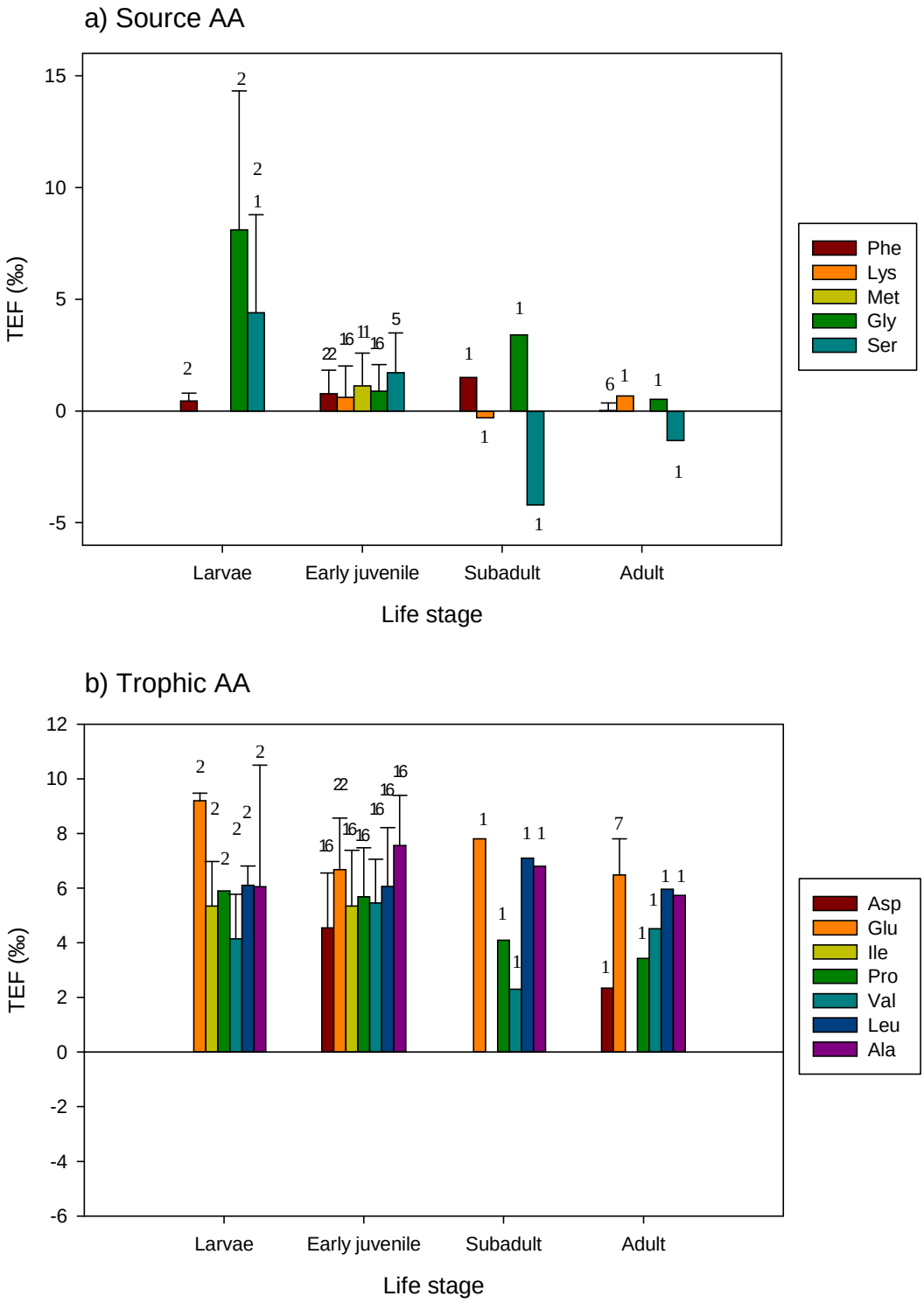

Figure 14. Mean TEFs of source and trophic amino acids for fish of different life stages. Error bars represent the standard deviation of TEFAA values. The number of TEF estimates included in each mean is presented above the error bars.

3.3.6 Aquatic habitat

Most of the feeding experiments (157 out of 236) were conducted on fish species from marine habitats, and 24 feeding experiments in freshwater fishes. The mean TEF of Lys was significantly different (i.e.by 1.6 ‰) between fish from marine vs. brackish habitats ($F=6.14$, $p=0.025$); marine fishes exhibited less isotope discrimination (Figure 15a). Among trophic AAs, the TEF of Asp of marine fishes (3.3‰) was significantly lower ($F=66.3$, $p=0.0002$) than those fishes inhabiting brackish habitats (7.2‰). The TEFs of Ile ($F=10.6$, $p=0.006$), Pro ($F=15.13$, $p=0.0012$) and Leu ($F=7.9$, $p=0.0117$) also showed significant differences between marine and brackish habitats; marine TEFs had lower values for all trophic AAs (4.6, 4.9 and 5.5‰ for Ile, Pro and Leu vs 7.3. 7.5 and 7.9 for Ile, Pro and Leu, respectively; Figure 15b).

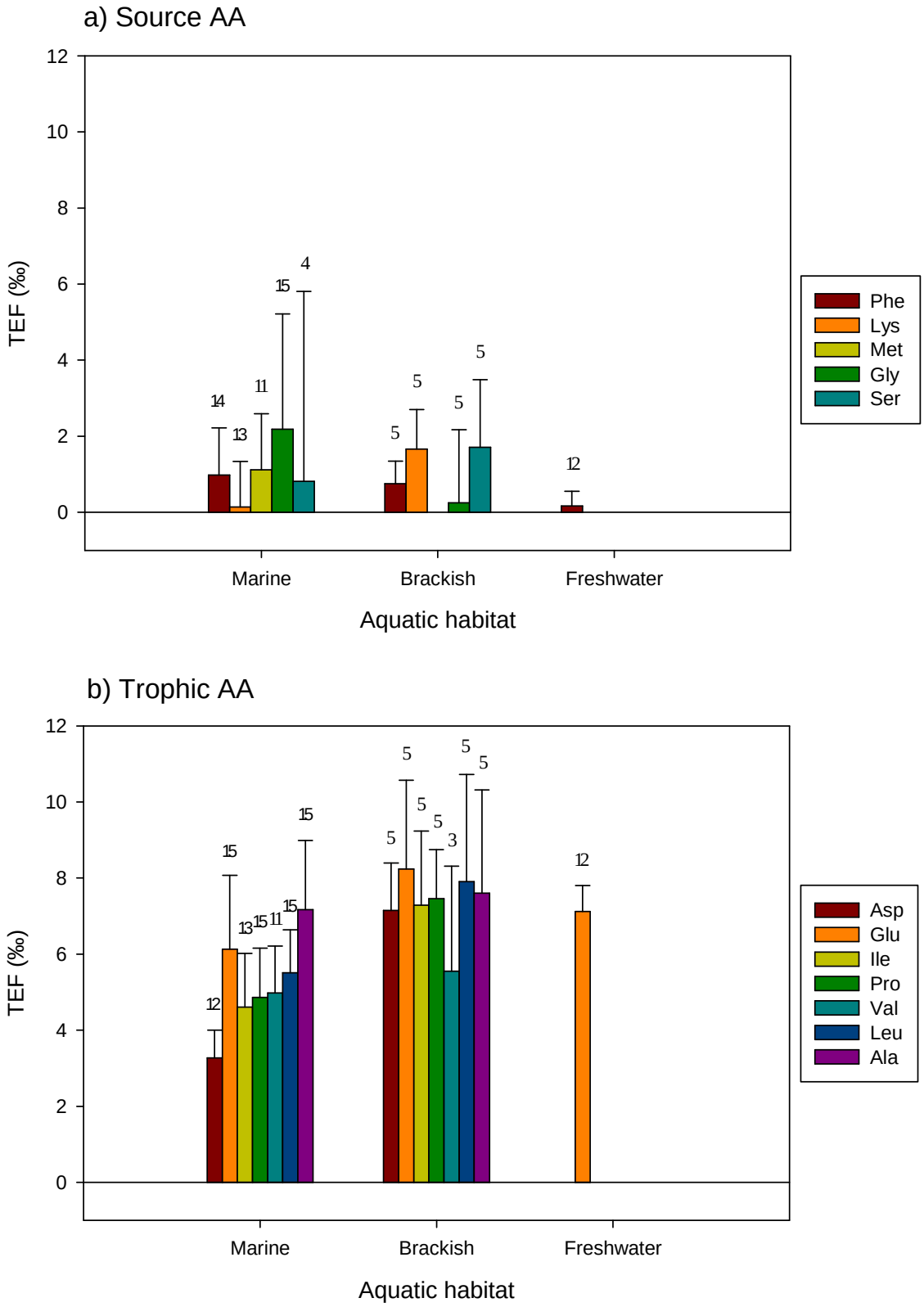

Figure 15. Mean TEFs of source and trophic amino acids for fish that differ in their dominant habitat. Error bars represent the standard deviation. The number of TEF estimates included in each mean is presented above the error bars.

3.3.7 Comparison of teleost, global and non-teleost TEFs

Among source AAs, the means and SDs of TEF Met remained stable among TEFs teleosts (mean±SD, 1.1±1.5‰), global (1.2±2.1‰) and non-teleost (1.2±2.1‰), while the other source AAs exhibited different patterns. The mean TE_{FPhe} (0.6‰) from teleost (TEF teleost) was higher than the mean global TEF_{Phe} (0.0‰) (Figure 16a), while the mean TEF_{Lys} (0.6‰) was lower than the mean TEF global. Statistical differences were found in the mean global TEFs of Lys (Z=2.6, p=0.009) and the mean non-teleosts TEFs of Lys (Z=3.064, p=0.002). The variability (quantified as the SD of the mean) in TEFs of Phe, Lys, and Met was lower in teleosts, and higher but relatively similar between global and non-teleost values (Figure 16b). Gly and Ser had the highest mean and SD values among source-AAs, and the largest differences between TEFs teleost, and TEF global or non-teleost; the mean and SD of TEF_{Gly} for teleosts were lower (1.7‰±2.9‰ vs 3.8‰± 4.8‰ for Gly and 1.3±3.3‰ vs 3.5 ± 5.1‰ for Ser, respectively). However, statistical differences were only found for TEF_{Gly} (Z=2.5, p=0.014).

Among trophic AAs, Ala had the highest differences in the mean TEF between teleost, global and non-teleost (ca. higher or equal 2.0‰; Figure 16a). However, statistical differences between means were only found for Asp (Z=3.4, p=0.001). The SD for teleost TEFs of trophic AA were 1.5-2.0‰ lower when considering teleosts vs global values.

Patterns of mean and SD between TEFs (telost, global, non-teleost) for each AA was relatively constant among trophic AAs. Statistical differences were found between the mean TEFs of Asp (Z=2.9, p=0.004), Pro (Z=2.3, p=0.020), Leu (Z=2.5, p=0.014); means were higher for teleosts. Among trophic AAs, Asp had the same SD TEF for teleosts and non-teleosts (2.0‰), while the SD for Glu, Ile, Pro, Val, Leu, and Ala were lower when considering only teleosts.

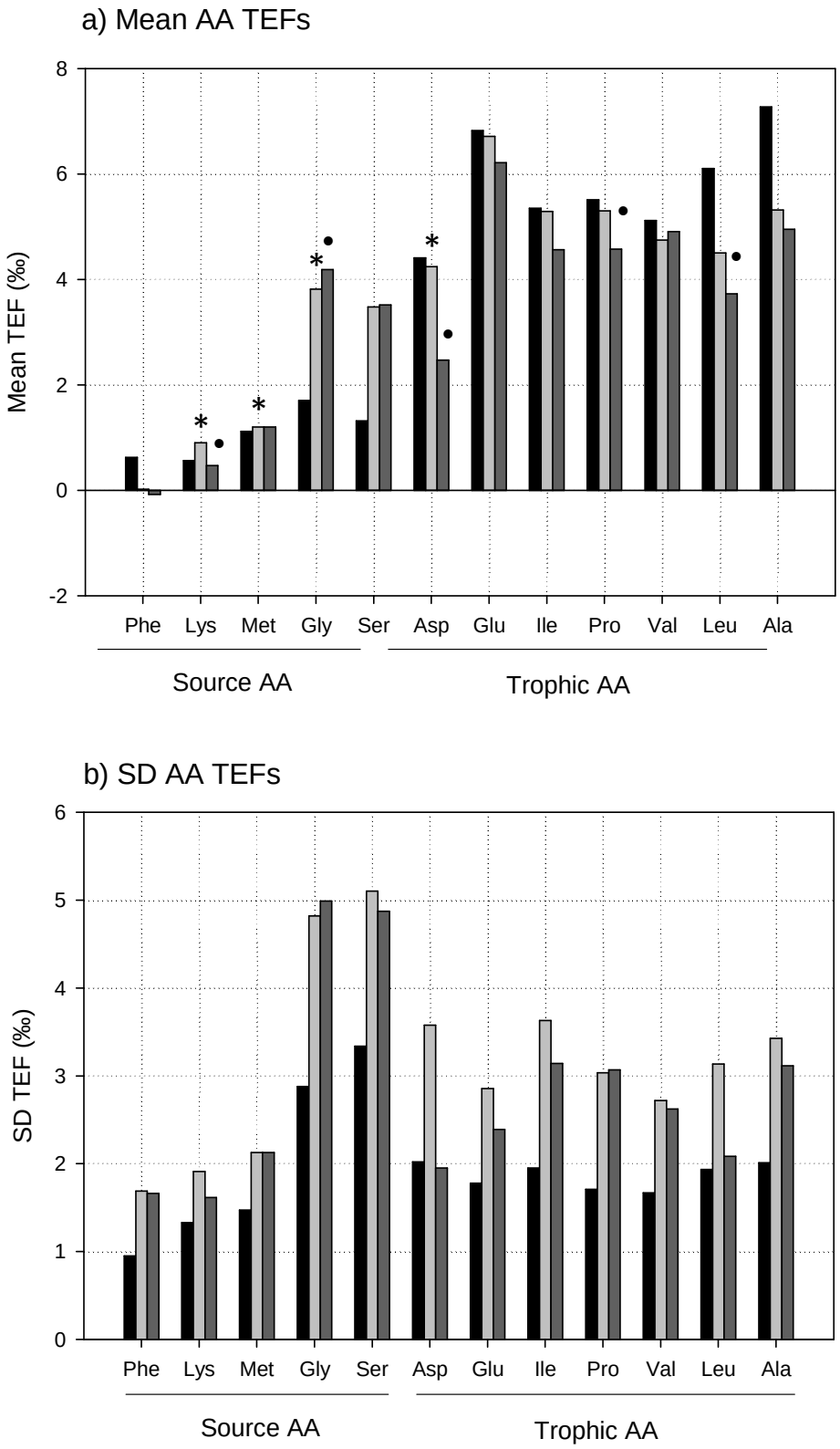

Figure 16. Mean (a) and SD (b) TEFs of source and trophic AAs derived from teleosts (this study; black bars), global values means estimated from McMahon and McCarthy (2016) (light grey bars) and non-teleosts means calculated from McMahon and McCarthy (2016) (dark grey). Symbols * and • in (a) represent significant differences between mean teleosts vs mean global values, and mean teleosts vs mean non-teleosts values, respectively.

3.4. Discussion

The results of our study reveal important patterns of variation in $\delta^{15}N$ TEF estimates from muscle tissues in teleost, and in relation to key nutritional factors (such as protein and lipid content, and diet type) and ecological factors (such as habitat, feeding regime or diet availability, and life stages). The novel results of our meta-analysis also illustrate the variability in mean TEFs from a single taxonomic group (i.e., teleosts) and in comparison with two sets of TEFs estimated from many different terrestrial and aquatic taxons (TEF global; derived from McMahon and McCarthy, 2016) and those that excluded teleost to evaluate quantitatively the variability in TEFs, and improve the application of CSIA-AA to calculate trophic level in teleost fishes.

3.4.1 Nutritional factors

3.4.1.1. Dietary protein and lipid content vs. TEFs

We found that control feeding experiments (n=32) used a broad range of percent protein in fish diets (8 to 71%), and some of these studies did not control for rearing conditions, nutritional characteristics of the diets, and requirements of the target fish species. Keeping these potential sources of variability in mind, we found that linear regressions between each source TEF_{AA} and percent protein were not significant, indicating that source AAs are not dependent on dietary protein content because the TEF values were relatively similar at the lowest and highest dietary protein content. In particular, for the canonical source AA, negative TEF_{Phe} values were found at relatively optimal levels, ~ 60% of protein, and the highest TEF values (~3‰) at extreme low protein content (<10%), but there was not a clear pattern of variation across protein percentages. Diets with very low protein content put fish under nutritional stress (Schreck et al., 2001), yet Phe N isotope fractionation is limited. Overall, a higher level of variability was observed in source AA TEF values within 40 and 60% of dietary protein content than at higher or lower protein contents (Figure 8); however, these results are biased toward the number of studies using 40-60% protein range (n=8) in comparison to those using higher or lower protein contents (n=1). Similarly, McMahon et al. (2015) reported a lack of relationship in TEFs of Phe, Gly and Ser for mummichog fed diets that varied in percent protein (range 8 to 71%). Barreto-Curiel et al. (2018) did not find a significant relationship between percent protein (40.8 to 48.9%) and source AAs TEFs for the carnivorous totoaba (*Totoaba macdonaldi*), and Nuche-Pascual et al., (2018) did not find a significant relationship between the TEFs of Phe, Lys, Met and Gly for the carnivorous Pacific yellowtail (*Seriola lalandi*) fed with diets differing in percent protein (42.1 to 61.3%). In general, the studies to date indicate a lack of relationship between TEFs of source AA and percent protein.

For trophic AAs, our results indicated that TEFs of Glu and Ala were negatively correlated with percent protein. McMahon et al. (2015) reported a significant negative relationship between the TEFs of Glu, Ile, Pro and Leu and percent protein, while Nuche-Pascual et al. (2018) and Barreto-Curiel et al. (2018) did not find a relationship between these trophic AAs TEFs and percent protein for Pacific yellowtail and totoaba, respectively. The range of dietary protein content in the experiments of McMahon et al. (2015) included a broad range of values; the inclusion of a very low protein diet (8%) for which high isotope discrimination was observed for Glu, Ile, Pro, and Leu may explain the reported negative relationship. High TEF_{AA} values may reflect the high deamination (catabolism) of endogenous AAs for use as energy substrates when the dietary energy is insufficient to meet energetic requirements (Goto et al., 2018).

We found that the percent of dietary lipids ranged from 2 to 24%, which is broad to dietary lipid ranges used in fish nutritional requirement (e.g. Miller et al., 2005). No significant correlations were found in TEFs for any of the source or trophic AA TEFs with lipid content. AA-specific TEFs were the highest at 6% and 22% of lipids, hence, not variation is linked to lipid content. These results agree with previous studies. In four freshwater fish species, Blanke et al. (2017) found no relationship between percent lipids and TEFs Phe and Glu (the only AAs they reported) for fish fed three non-formulated diets that varied in percent lipids that varied by 1.9%, 4.6%, and 20.4%. Together, our findings indicate that most source and trophic AAs TEFs do not seem to be sensitive to dietary lipid content, perhaps because the availability of dietary lipids, and lipid metabolism, do not require nitrogen. In fact, lipids are composed of chains of C, H, O, and the route for lipid synthesis in fish includes oxidative decarboxylation of pyruvate (carbohydrate source) and oxidative degradation of some amino acids (protein source) to obtain acetyl-CoA as carbon sources (Tocher, 2003). Therefore, lipid availability does not seem to influence the metabolism of AAs, and consequently N isotope discrimination.

High variation in TEFs of source and trophic AAs vs. percent of protein and lipids were observed (Figures 7 and 8), and only TEFs of Glu and Ala showed a negative relationship with protein content. Considering this variability, the lack of relationships between AA TEF and percent protein for many AAs, and between AA TEF with percent lipids for fish fed diets differing percent in protein and lipids, we suggest that other factors, such as species or culture conditions, may be contributing to TEFs variability.

3.4.1.2. Dietary protein and lipid content relative to nutritional requirements

TEF_{Phe} were found to vary significantly in muscle tissue of Pacific yellowtail fed formulated diets prepared with

a single protein source with varying dietary protein levels (Nuche-Pascual et al., 2018). The authors reported a higher mean TEF_{Phe} (3.3‰) in fish fed diet with an optimum protein level, compared to diets with a low (1.7‰) and high (0.3‰) protein levels. When considering the protein requirements of the mummichog, higher TEF_{Phe} (1.0‰) were found in fish fed diets with a high protein level, compared to fish fed on a diet with optimum level (0.1‰) and low level (0.4‰) feeds. The lack of consistency between the results in this meta-analysis and other single studies, as well as the high level of variability in AA TEFs as a function of protein level, warrants further research. Evaluation of dietary protein and specific-AA availability relative to species-specific requirements may allow a better understanding of the mechanisms producing variations of AA TEF values.

The National Research Council (2011), published the *Nutrient requirements of fish and shrimp*, and indicate that fish fed diets that do not contain an optimal dietary protein and lipid content given species-specific nutrient requirements may undergo AA catabolism (or anabolic processes linked to protein accretion) in order to meet nutrient and energy requirements. Consequently, this may influence isotope discrimination (O'Connell 2017; Nuche-Pascual et al., 2018). Our analysis showed that TEFs of source AAs did not differ significantly between diets classified as containing low, optimum or high protein content categories, which support the hypothesis that source AA TEFs are independent of the amount of dietary protein. Note that there is nitrogen isotopic fractionation in source AAs: TEFs varied from -3‰ to +5‰ considering all source AA (Figure 8a), and for Phe, TEF values vary from -2‰ to +3‰. However, nitrogen isotopic fractionation in source AAs is generally limited and appears to be independent of protein content. The lack of differences in source AA TEFs has been attributed to similar deamination processes in terrestrial and aquatic consumers (Popp et al., 2007, Chikaraishi et al., 2009; Chikaraishi et al., 2015; McMahon and McCarthy, 2016; O´Connell, 2017). Hence, similar deamination processes explain the low isotopic fractionation in fish Phe, Met, Lys, Gly, and Ser, and perhaps the limited metabolic processes for these source AAs restrict the level of isotopic fractionation in fish regardless of dietary protein content.

Trophic AAs did not vary significantly among fish classified as having been fed low, optimum and high protein levels. The lack of statistical differences in trophic AAs TEFs was also reported by Nuche-Pascual et al. (2018). In contrast, classifying diets reported by McMahon et al. (2015) based on the protein content of the feeds and the requirements of the mummichog leads to different results. For example, there were significant differences between dietary protein levels for Asp, Glu, Ile, Val, Leu, and Ala, which were largely driven by the high TEFs of the low protein diet. Feeding an omnivorous fish with such low protein level feed (8.0%) is unrealistic from a nutritional and ecological perspective (i.e., estimated protein requirement is 52%; Prinslow et al., 1974, Table 4S) and may yield TEF_{AA} values that may not be realistic or applicable to natural populations.

Although trophic AA TEFs did not vary statistically based on their classification as high, optimum or low protein, TEF_{Glu} values clearly decreased from 7.7 to 5.1‰ as protein level increased (Figure 10), which is approximately equivalent to a third of a trophic level assuming $TDF_{Glu\text{-}Phe}$=7.6 (Chikaraishi et al., 2009). This pattern in TEF_{Glu} was also observed between the lowest (TEF_{Glu}=7.8‰) and the highest (TEF_{Glu}=6.0‰) dietary protein content relative to requirement for a carnivorous fish (i.e., totoaba) fed formulated feeds prepared with poultry by-product meal and fish meal as protein sources (Barreto-Curiel et al., 2018). Glutamic acid is a major energy substrate and nitrogen reservoir and is central to AA metabolism (e.g. O´Connell, 2017), and is involved in the transamination of many AAs (Cammarata and Cohen, 1950). The higher mean TEF_{Glu} (7.7‰) found for the low protein level in the meta-analysis suggests that fish catabolized higher amount of endogenous Glu to meet energy requirements (Goto et al., 2018), leading to more isotopic discrimination. Therefore, variation patterns observed for Glu TEFs could be related to catabolism of Glu relative to nutrient requirements. McMahon et al. (2015) also found differences in TEF_{Glu} in fish fed diets differing in protein level, although the highest TEF_{Glu} (10.8‰) was observed in the lowest protein level diet and the lowest TEF_{Glu} (5.6‰) in the highest dietary protein level. However, the range of protein levels used in that study may not be ecologically realistic and may have led to nutritional stress at the lowest protein level, and thus these results should be taken with caution.

To our knowledge, the relationship between percent lipid relative to dietary lipid requirements on isotopic discrimination has not been evaluated, neither in teleosts or non-teleosts. The availability of dietary lipids can have a direct impact in the metabolism of AA (Tocher, 2003; NRC, 2011), and hence TEFs. Results from our meta-analysis showed significant differences only in TEF_{Lys} between fish classified as having been fed low (-0.6‰) and high (1.2‰) lipid levels; fish fed diets with an optimum lipid level did not differ significantly. In addition, TEF_{Lys} varied in fish fed low (-1.8‰) and high lipid level (1.2‰) formulated feeds (Nuche-Pascual et al., 2018). Lysine is involved in the synthesis of carnitine, that has a role in the transport of long-chain fatty acids from the cytosol into the mitochondria in mammals and fish tissues (Vaz and Wanders, 2002; Li et al., 2009). Higher dietary lipid content, would thus require more fatty acids transporters, leading to higher catabolism of Lys for carnitine synthesis resulting in higher TEF_{Lys}. However, Barreto-Curiel et al. (2018) did not find a consistent pattern in TEF_{Lys} between low (1.5‰), optimum (0.9‰) and high (1.9‰, 0.5‰) lipid levels. These discrepancies in estimated TEFs of source AAs warrant the need for more well-controlled nutritional studies of dietary lipid content relative to lipid requirements to estimate isotopic discrimination in consumers.

Similar to dietary protein levels, trophic AAs did not vary significantly among low, optimum and high dietary lipid levels. A comparable degree of transamination and deamination between fish fed low, optimum and high lipid levels might have caused the lack of differences observed in the trophic AA TEFs among dietary lipid levels in spite of the metabolic complexity of the trophic AAs (O´Connell, 2017). The lack of a clear pattern in TEFs of

trophic AAs such as Glu has been reported among low (7.8‰), optimum (6.0‰) and high (6.3‰, 5.6‰) dietary lipid levels in totoaba (Barreto-Curiel et al., 2018). In contrast, a large differences in TEF_{Glu} was reported by Nuche-Pascual et al. (2018) among low (3.1‰) and high (8.1‰) dietary lipid levels. These inconsistencies between studies highlight the variability in patterns of isotope discrimination found when considering single studies conducted under particular dietary conditions, compared with a meta-analysis that encompasses a range of species and experimental conditions.

Importantly, in our meta-analysis, Phe, Lys, Met, Ser, and Glu TEFs varied substantially between studies (i.e., up to 4.1‰, 3.0‰, 3.4‰, 3.3‰ and 5.5‰, respectively) within a single dietary protein level. TEFs of Lys, Met, Ser, Glu also varied within a dietary lipid level (i.e., up to 3.6‰, 4.4‰, 4.6‰, 5.5‰, respectively). This is likely due to the grouping of data from experiments that included diets varying in AA profiles, lipid sources, species, and culture conditions. These differences between experiments could be masking the relationship between nutrient availability and fish nutritional requirements and, therefore, the experiments with a single species contribute to a better understanding of the biochemical, nutritional and ecological mechanisms that ultimately result in the TEFs of trophic AAs.

3.4.1.3. Feeding regime

Among source AAs, only the mean TEF of Lys was statistically significantly higher in fish fed to apparent satiation (1.3‰) compared with a fixed feeding regime that has negative TEF values (-0.6‰). Among the trophic AA, only Asp and Glu differed significantly between fixed (mean TEFs of 3.0 and 5.0‰, respectively) and satiation regimes (5.5 and 7.2‰, respectively) by 2‰ for Asp and Glu. These significant isotopic differences and patterns suggest that Lys, Asp, and Glu are more sensitive to these two feeding regimes than any other source and trophic AAs. When fish are fed with fixed feeding regime and diet limited in protein (i.e., EAAs) or energy content relative to requirements they cannot increase food and protein consumption to compensate for EAA or energy deficiencies. In consequence, AAs from the diet would be retained in muscle for protein accretion and catabolism of AAs would remain low. In contrast, fish fed to satiation have a large availability of nutrients, that stimulate higher food ingestion and protein metabolism for EAA and NEAAs (Saravanan et al., 2012). Consequently, catabolism of AAs in excess and nitrogen isotopic fractionation would increase at excess protein content, especially for NEAA. Glu is a major metabolic energy source (Wu, 2009), and considered the canonical trophic AA; Asp is a major gluconeogenic precursor, an important energy source (Li et al., 2009), a precursor for arginine synthesis (Wu, 2009), and this AA can contribute to food intake suppression (see below). Therefore, if

fish are fed the same type of dietary protein but with different feeding regimes (fix vs satiation), it cause significant differences in the isotopic fractionation in Lys, Glu, and Asp. If this is true, these results suggest that fish that are exposed to high prey availability in the wild would have higher N isotopic values compared with fish experiencing limited prey availability during less favorable environmental conditions. However, this pattern cannot be simply extrapolated to wild fish solely from our results, because our meta-analysis includes results from various control feeding experiments that used different dietary protein type and content. For instance, in mummichog fed to satiation with a diet low in protein content (8%, Veggie-Pro) the TEFs of Glu (10.8‰) and Asp (8.5‰) were higher than for diets high in animal protein that were also fed to satiation (5.6‰ and 6.7‰, respectively; McMahon et al. 2015). In another study included in our meta-analysis, Barreto-Curiel et al. (2018) found higher a TEF_{Glu} (7.8‰) in totoaba fed to satiation with a diet low in protein content relative to requirements, a lower TEF_{Glu} (6.0‰) in fish fed a diet with an optimum protein content, and similar TEF_{Asp} values for diets of low and optimum protein content relative to requirements (3.7‰ and 3.6‰, respectively). Laeger and Morrison (2013) found that Lys, Arginine, and Glu were the main AAs (from a total of 20 AAs that constitute proteins in humans) contributing to the suppression of food consumption after an intragastric infusion of individual AAs; however, to our knowledge, this physiological response has not been examined in fish.

Under satiation feeding regimes, animals consume amounts of protein and energy depending on the diet composition until energy and/or nutrient requirements are satisfied (e.g. Morrison et al., 2012; Saravanan et al., 2012). If animals are fed a low protein (with moderate to low energy content) diet relative to their protein requirements, they will typically increase their food consumption, which should increase the catabolic activity of those AA leading to higher TEF_{AA} values (Martínez del Río and Wolf, 2005). Under a fixed feeding regime, however, the amount of food ingested and thus the amount of protein and lipids consumed are fully controlled by the diet composition. Therefore, feeding regimes, fixed vs. satiation, are important factors that influence AA TEF variability, especially for Lys, Glu, and Asp, because these regimes stimulate different levels of food ingestion, AA catabolism and N isotopic fractionation link to dietary protein type and content.

3.4.2 Ecological factors

3.4.2.1. Diet type

The TEFs of source AA (except Gly TEF) varied, but were not statistically significantly different between diet types classified as fish-based and invertebrate-based feeds. This result could be possibly explained by the high quality of the dietary protein (in terms of higher digestibility and suitable EAA profile) between fish- and

invertebrates-based diets: in other words, squid, clams, and fish have similar AA profiles from a nutritional perspective (Kader et al., 2010, 2012). These patterns may indicate relatively similar levels of catabolism of source AAs, and hence, similar TEF_{AA} values.

Gly TEFs were significant higher (4.9‰) in fish fed with invertebrate-based diets compared to those fed fish-based diets (0.9‰) and plant-based diet (no statistically tested but values are ~1‰), that indicate that the Gly nitrogen isotopic fractionation is highly linked to differences in dietary AA profiles. We exclude the TEF for plant-based feeds in statistical analysis due to the small sample size (n=1). TEF_{Gly} ranged by ca. 11‰ in feeding experiments fed invertebrate-based diets (McMahon et al., 2015; Chikaraishi et al., 2009) and 6‰ in those fed fish-based diets (Bloomfield et al., 2011; Bradley et al., 2014; Hoen et al., 2014; Bio-Vita diet from McMahon et al., 2015; Barreto-Curiel et al., 2017; Nuche-Pascual et al., 2018; Barreto-Curiel et al., 2018). These results, together with the high variability for Gly TEFs previously reported for terrestrial and aquatic animals (e.g. mean±SD 3.9±4.9‰; McMahon and McCarthy, 2016) are consistent with the indication that glycine is not adequate source AA for TP estimates in natural populations, as has been previously suggested by McCarthy et al. (2007), Germain et al. (2013) and McMahon and McCarthy (2016).

Trophic AAs did vary between invertebrates and fish-based diets but mean values were not statistically different. Comparison of mean TEFs within studies that used both invertebrates and fish as the primary protein source (McMahon et al., 2015; Blanke et al., 2017), indicate that differences of only 1.7‰ and 2.3‰, respectively. This level of variation in the TEF of Glu is lower than the mean TEF global value of 6.4‰ reported by McMahon and McCarthy (2016), suggesting that Glu-based estimates of TP is affected by diet type.

On the other hand, the TEFs for fish fed a plant-based diet were 2-6‰ higher than the mean values for diets that were fish or invertebrate based. These results were obtained for an omnivorous species in a single experiment that were fed a low protein diet under satiation regimes (McMahon et al., 2015), and may not reflect the level of isotopic discrimination of herbivorous species. Herbivorous fish have longer digestive tracts, different digestive enzyme capacity and nutritional requirements that those feeding at higher trophic levels (Elliot and Bellwood, 2003; NRC, 2011), and warrants more future studies examining the functional role of digestive processes of fish with different feeding habits.

3.4.2.2. Life stage

Most of the feeding experiments included in our analysis were conducted on juvenile fishes (79.6%), hence, larvae, subadults, and adults are poorly represented in our study. Among source AAs, TEF_{Gly} and TEF_{Ser} exhibited high variability between larval to adult and subadult stages, respectively, but only TEF_{Gly} showed statistically significant differences between larvae and early juvenile stages. These results suggest that Gly and Ser have an important function in protein synthesis and obtaining energy for growth in an early stage since these AAs are involved in collagen synthesis and gluconeogenesis (Walton and Cowey, 1982). This result is consistent with a pattern derived from other studies: high TEF_{Gly} values (8.8‰) was reported for larvae by Chikaraishi et al., (2009) compared to juveniles (1.8‰, 0.9‰ and 1.4‰ for McMahon et al., 2015, Nuche-Pascual et al., 2018, and Barreto-Curiel et al., 2018, respectively). In fish larvae, EAAs are primarily retained for protein synthesis, and NEAAs are preferentially catabolized as energy substrates (Conceicao et al., 2002). Gly is an NEAA that can be easily catabolized since it is the simplest AA (Li and Wu, 2018). High TEF_{Gly} may be the result from higher Gly catabolism to meet nutrient and energy requirements for protein and glucose synthesis for rapid grow at an early stage. TEFs of Phe, Lys, and Met did not differ among larvae, early juvenile and adult stages. These results suggest that the catabolism of these AAs remained relatively consistent among life stages and that empirical TEF estimates derived from one life stage may be applicable to another. However, source AAs in subadult and adult stages were represented by one feeding experiment (except for Phe in the adult stage, n=6) and thus they were not considered for statistical analysis. Despite the long experimental periods required for obtaining empirical TEF estimates in subadult and adult fish (Herzka, 2005), more studies on subadult and adult fish need to be conducted.

Mean trophic AAs TEFs did not vary significantly with life stages, and a clear pattern was not distinguished. Limited differences (up to 0.4‰) in TEFs of trophic AAs such as Glu, for example, were observed between feeding experiments with larvae (Chikaraishi et al., 2009). High differences by up to ~5.2‰ were reported in mean TEF_{Glu} values among four to six treatments within two studies with juveniles omnivorous and carnivorous fishes (McMahon et al., 2015 and Nuche-Pascual et al., 2018, respectively), and lower differences (2.0‰ and 1.9‰) in mean TEF_{Glu} values among treatments in other two studies with juveniles (Blanke et al., 2017, and Barreto et al., 2018, respectively). While our meta-analysis is biased towards juvenile stages, because fish are easier to grow and reach isotopic equilibrium faster than adults (Herzka, 2005), the presumably low variation in trophic AA among fish life stages suggest that N isotopic fractionation in muscle remained relatively constant during the experimental period of growth and under their respective dietary treatments. For many fish species in the wild, an increase in $\delta^{15}N$ values (trophic AA values) is expected as fish grow and feed on prey of higher trophic positions (Dale et al., 2011). However, the results of our analysis cannot provide insights about fish

ontogenetic changes in diet in natural populations because fish were under the same dietary treatment for a relatively short period of time in their life cycle. Further stage-specific feeding studies would require same individuals under dietary treatments for a longer period of times, that may include changes in dietary treatments during ontogeny. These type of experiments can elucidate whether differences in AA metabolism throughout development leads to variation in TEFs for trophic AAs.

3.4.2.3. Aquatic habitat

Marine and brackish habitats differ in salinity, with potential implications for osmoregulation, especially for fishes inhabiting brackish water that typically experience fluctuating salinities, and must expend energy to osmoregulate in order to maintain ionic balance and osmotic homeostasis (e.g. Edwards and Marshall, 2013; Marshall, 2013). Therefore we would expect that AAs that are involved in osmoregulation, such as Gly (Powell et al., 1982), would vary among brackish and marine and freshwater. However, among source AAs, only TEF_{Lys} had significant differences between marine and brackish habitats. The mean TEF_{Lys} for brackish fish (1.7‰) was significantly higher than for marine fish (0.1‰). Marine and brackish habitats differ in salinity, with potential implications for osmoregulation, especially for fishes inhabiting brackish water that typically experience fluctuating salinities, and must expend energy to osmoregulate in order to maintain ionic balance and osmotic homeostasis (e.g. Edwards and Marshall, 2013; Marshall, 2013). Lys is involved in maintaining osmotic pressure and acid-base balance in the body fluids (Chiu et al., 1988). This may lead to higher needs of Lys and thus higher catabolism in fish inhabiting fluctuating salinity waters and may explain the higher TEF_{Lys} values in fish typically found in brackish habitat. TEFs of Phe did not differ significantly among marine, brackish and freshwater. This result suggests that the metabolism, in particular the catabolism of Phe, is similar regardless the habitat, maybe because Phe is involved in the synthesis of tyrosine via hydroxylation which is a precursor of important hormones and neurotransmitters (Chang et al., 2007) in fish that does not involve C-N cleavage and isotopic fractionation (Chikaraishi et al., 2007). The other source AAs may not vary between marine and brackish habitats because they are not involved in processes to obtain energy during osmotic regulation (Li et al., 2009) and thus the catabolic activity of these AAs is similar in fish that are normally found in different aquatic habitats.

Among trophic AAs, Asp, Ile, Pro, and Leu had significant higher TEFs (7.2‰, 7.3‰, 7.4‰ and 7.9‰, respectively) in fish that inhabit brackish habitat compared marine species (3.3‰, 4.6‰, 4.9‰, 5.5‰, respectively). The catabolism of Asp, Ile, Pro, and Leu can contribute to meet additional energy requirements involved in osmoregulation (Bystriansky et al., 2007; Walton and Cowey, 1977) which may explain higher mean

TEFs for Asp, Ile, Pro, and Leu in fish that are found in estuaries than in marine habitats. Limited studies have evaluated the aquatic environment on isotopic discrimination. Vanderklift and Ponsard (2003) found lower mean TEF_{bulk} values in marine organisms than in freshwater and terrestrial in a meta-analysis including vertebrates and invertebrates. These values are consistent with our results. Vanderklift and Ponsard (2003) attributed these differences to the mode of nitrogen excretion, however, in our meta-analysis we controlled this potential source of variation using the same taxonomic group and thus the same mode of nitrogen excretion, therefore more studies are required to evaluate the effect that the osmoregulation may have on TEF_{AA} values.

3.4.2.4. Comparison of teleost, global and non-teleost TEFs

Teleosts, global and non-teleosts had comparable mean values for Phy, Lys and Met, with differences ≤ 1‰. A universal TEF value for Phe and other source AA is desirable to easily estimate animal trophic levels for any taxa. Importantly, the SD of TEFs was lower when considering a single taxonomic group (teleosts), which implies that the selection of TEFs on a taxon-specific basis may yield more precise estimates of TP. The low isotopic fractionation for the global mean TEF_{Phe} (close to 0‰) is due to the main catabolic pathway of Phe, which is the hydroxylation to form tyrosine which does not involve cleavage of a C-N bond resulting in low isotopic fractionation (Chikaraishi et al., 2009). Although the differences in mean TEF_{Phe} were limited, the mean TEF for non-teleosts was negative. This mean TEF was heavily influenced by studies on insects, for which negative TEF_{Phe} have been reported (29% of the values for non-teleosts were for insects). Met showed remarkable stability among these three TEFs (teleost, global and non-teleost), and may therefore serve as a robust source AA. However, Met is difficult to measure in consumers due to the low amount of this AA in top predator tissues (Reid et al., 2005), and the limitations of some derivatization techniques. In contrast, mean Gly and Ser TEFs differed between the three estimates, which is consistent with the high level of variation reported for these AA.

Among trophic AAs, TEFs of Asp showed significant differences in mean TEFs between teleosts and global TEFs, whereas TEF_{Glu}, did not differ significantly. This result is encouraging, for a universal TEF_{Glu} value for estimating trophic levels would simply the CSIA approach. However, the variability (2.9‰) in TEF estimates of Glu should also be considered in the equation used for calculating TP proposed by Popp et al. (2007) and Chikaraishi et al. (2009), and future models based on likelihood probabilities would improve its application. The TEFs of Asp, Pro, and Leu were significantly higher in teleosts than in non-teleosts, and SD were ca. 1.5‰ (Figure 16b). In addition, Ala and Leu were particularly variable among these trophic AA.

3.5. Summary

The results of this work suggest that some TEF show a relationship with nutritional and ecological factors, which could play an important role in contributing to variation in AA-specific $\delta^{15}N$ values in natural populations. Among source AAs, TEF_{Phe} did not differ significantly with any of the factors evaluated in this meta-analysis, but it did show limited variation as a function of protein content, diet, life stage and aquatic habitat. Overall, the limited variation in TEF_{Phe} supports the concept of Phe as the canonical source AA for TP estimates. Values of TEF_{Met} were relatively stable. However it is a difficult AA to measure and is therefore less represented in the set of CSIA-AA studies that have reported empirical estimates of its isotope discrimination. Lys TEFs differed with lipid content, diet and aquatic habitat, implying it is not a robust source AA. Glycine and serine showed large variability as a function of nutritional and ecological factors. This pattern has been previously observed, which supports the suggestion of other authors that Gly and Ser are no longer source AA.

Among trophic AAs, Glu, Asp, Ile, Leu and Val showed differences in isotope discrimination that were related to the type of diet. Mean TEF_{Glu} values varied significantly with feeding regime and aquatic habitat between controlled feeding experiments, however, the mean TEFs for teleosts, all taxa (global values) and non-teleosts were similar. However, the SD of the TEFs was relatively high (ca. 2‰) which suggests that there might be intra-individual and species-specific mechanisms that influence isotope discrimination. Lower variation in our teleosts AA TEFs compared to global estimates including all taxa and estimates for non-teleosts may indicate that species-specific estimates should yield more precise estimates of TP. However, it is necessary to perform further controlled laboratory feeding experiments to evaluate the relationship between potential nutritional and ecological factors that may affect fish metabolism and thus isotopic fractionation within the same species.

CHAPTER 4. General conclusion

The source and trophic AAs classification lies in the metabolism of AAs resulting from different degrees of transamination and deamination of AAs related to the AAs requirements and dietary supply.

Isotopic discrimination within the tissues of the consumer is driven by biochemical, physiological and ecological processes that produce variation in the trophic enrichment factors of AA. Nutritional and ecological factors may be correlated in multiple-species estimations which difficult the understanding of the potential sources of variation and the use of the universal TEF values.

Laboratory experiments under controlled conditions are necessary to reduce and have a better understanding the sources of variation of TEFs estimation. In these studies, animal nutrition and physiology should be an important framework in feeding experiments for the understanding the potential sources of variation of isotopic discrimination and that leads to more accurate TEF_{AA} values estimates in the global application of CSIA-AA to ecosystem studies.

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Annex

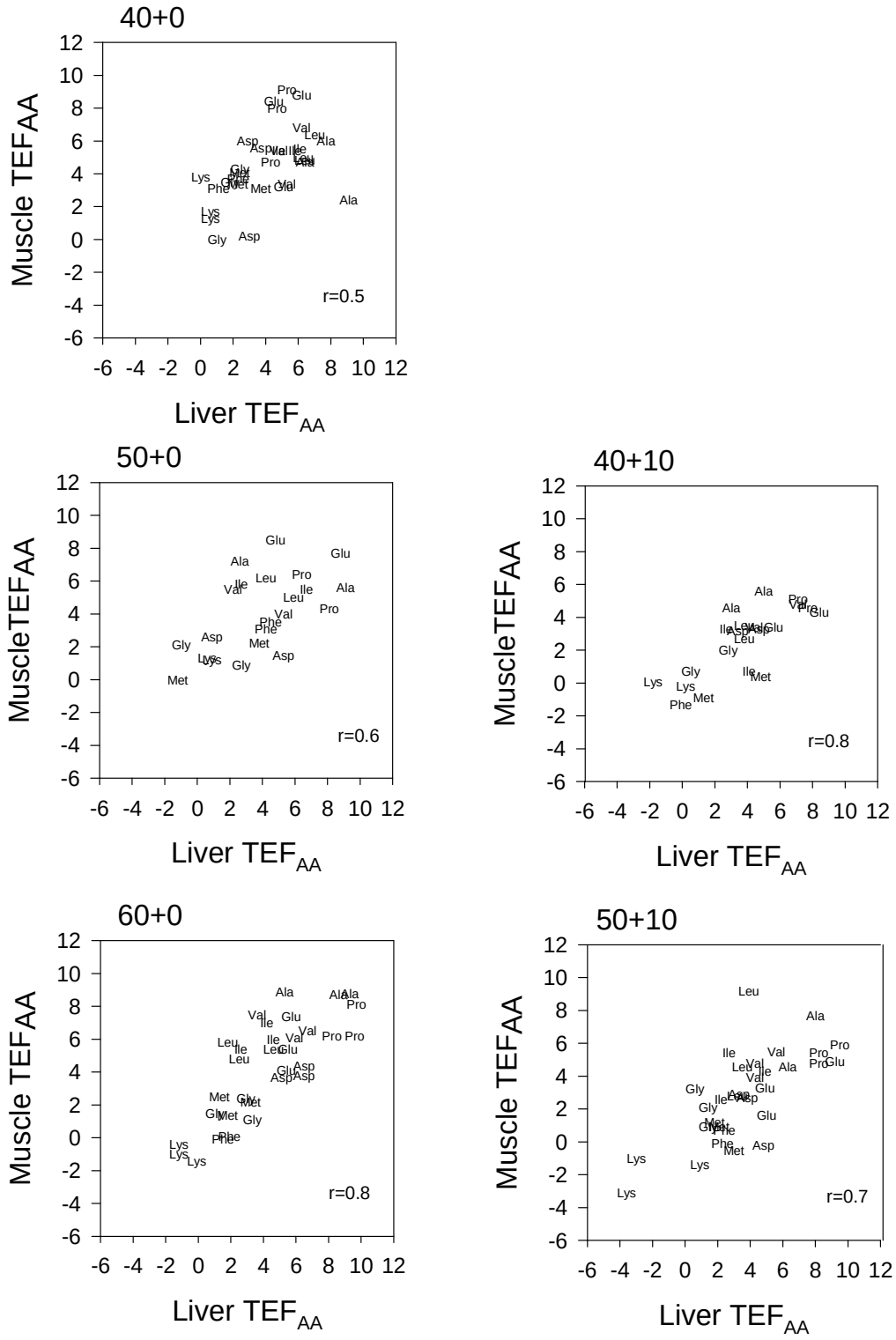

Figure S1. Correlation of TEF_{AA} between liver and muscle tissues in diets varying in protein quantity and quality. Symbols represent measurements of each AA in individual fish (Phe=phnylalanine, Lys=lysine, Met=methionine, Gly=glycine, Asp=aspartic acid, Glu=glutamic acid, Ile=isoleucine, Pro=proline, Val=valine, Leu=leucine, Ala=alanine). Dietary treatments are described in table 2.

Table S1. Average of the standard deviations calculated from the duplicate measurements of CSIA-AA made on each sample (n = 2) analyzed in this study.

Component	Diet	Liver	Muscle
Alanine	0.5	0.6	0.5
Aspartic acid	1.0	0.4	0.3
Glutamic acid	1.5	0.5	0.5
Glycine	0.5	0.5	0.4
Isoleucine	0.2	0.4	0.4
Leucine	0.3	0.2	0.3
Lysine	1.0	0.7	0.6
Methionine	0.8	0.6	0.4
Phenylalanine	0.7	0.4	0.5
Proline	0.3	0.3	0.2
Valine	0.6	0.5	0.3

Table S2. Precision and accuracy of nitrogen CSIA-AA. Average and standard deviations (SD) calculated from the two measurements of the laboratory´s internal standards: two mixtures of pure AA (n=79; UCD AA1 and UCD AA2), and two secondary quality assurance materials: a fish muscle and whale baleen (n=86). One mixture was used for isotopic calibration of measurements (UCD AA 1), while the other was not involved in corrections and served as the primary QA standard (UCD AA 2).

Component	UCD AA1 SD of $\delta^{15}N$	UCD AA1 Average of measured $\delta^{15}N$	Known $\delta^{15}N$ for UCD AA1	UCD AA2 SD of $\delta^{15}N$	UCD AA2 Average of measured $\delta^{15}N$	Known $\delta^{15}N$ for UCD AA2	MMS (Fish muscle) SD of $\delta^{15}N$	RWB (whale baleen) SD of $\delta^{15}N$
Ala	0.9	-6.82	-6.72	1.2	40.65	41.40	1.4	1.3
Asp	0.5	-2.32	-2.34	0.7	-2.51	-2.29	1.1	0.6
Glu	0.7	-4.24	-4.17	1.2	47.79	47.60	1.2	1.0
Gly	0.7	0.81	0.82	1.1	0.93	0.73	1.0	1.0
Ile	0.7	2.39	2.53	0.7	-3.79	-3.53	1.2	1.2
Leu	0.4	9.21	9.24	0.6	-5.07	-4.29	1.0	0.9
Lys	0.9	-0.92	-1.36	1.1	0.14	0.47	1.5	1.3
Met	1.0	-1.80	-1.69	n.m.	n.m.	n.m.	1.1	1.1
Phe	0.5	-1.24	-1.14	0.7	1.53	2.06	1.2	1.1
Pro	0.4	-1.51	-1.44	0.7	-4.93	-4.11	0.8	0.6
Val	0.8	5.22	5.30	1.0	-6.78	-6.62	1.3	1.1

Table S3. Mean and standard deviation (SD) trophic enrichment factors (TEF) of amino acids estimated for muscle tissue in published controlled feeding experiments in teleosts. Phe = phenylalanine; Lys = lysine; Met = methionine; Gly = glycine; Asp = aspartic acid; Glu = glutamic acid; Ile = isoleucine; Pro = proline; Val = valine; Leu = leucine; Ala = alanine

Reference	Common name species (*scientific name*)	Diet	TEF (mean ± SD) (‰)										
			Phe	Lys	Met	Gly	Ser	Asp	Glu	Ile	Pro	Val	Leu	Ala
Chikaraishi et al. (2009)	Japanese halibut (*Paralichthys olivaceus*)	Rotifers	0.7	NA	NA	12.5	1.3	NA	9.4	6.5	5.9	5.3	5.6	2.9
Chikaraishi et al. (2009)	Scorpion fish (*Sebastes schlegli*)	Rotifers	0.2	NA	NA	3.7	7.5	NA	9	4.2	5.9	3	6.6	9.2
Bloomfield et al. (2011)	Black bream (*Acanthopagrus butcheri*)	Commercial diet made with fish meal	1.64	0.09	NA	-2.93	4.76	8.16	10.6	9.33	9.72	1.74	11.76	6.9
Bradley et al. (2014)	Bluefin tuna (*Thunnus orientalis*)	sardine (*Sardinops sagax*), squid (*Doryteuthis opalescens*), gelatin	1.5 ± 0.3	-0.3 ± 0.4	NA	3.4 ± 0.2	-4.2 ± 0.3	NA	7.8 ± 0.2	NA	4.1 ± 0.8	2.3 ± 0.3	7.1 ± 0.7	6.8 ± 0.9
Hoen et al. (2014)	Opakapaka (*Pristipomoides filamentosus*)	haddock, trevally, saithe, mackerel, octopus, anchovy, squid, krill	NA	0.67	NA	0.53	-1.32	2.34	3.86	NA	3.43	4.51	5.96	5.74
McMahon et al. (2015)	Mummichogs (*Fundulus heteroclitus*)	Squid	0.6 ± 0.6	1.6 ± 1.9	NA	1.7 ± 0.7	1.4 ± 1.7	6.7 ± 0.7	5.6 ± 1.1	6.1 ± 1	6.6 ± 0.4	4.6 ± 0.4	5.7 ± 0.6	4.1 ± 0.8
McMahon et al. (2015)	Mummichogs (*Fundulus heteroclitus*)	Clam	1 ± 0.6	1.8 ± 0.7	NA	1.6 ± 0.2	0.7 ± 0.4	5.4 ± 0.3	6.9 ± 0.3	5.2 ± 0.3	6.7 ± 0.8	5 ± 0.3	5.5 ± 0.5	7.8 ± 0.2
McMahon et al. (2015) Bio-Vita	Mummichogs (*Fundulus heteroclitus*)	omnivorous commercial diet (Bio-Vita)	0.1 ± 0.3	1.8 ± 0.3	NA	-0.1 ± 0.5	0.2 ± 0.5	7 ± 0.2	7.3 ± 0.3	6.4 ± 0.5	7 ± 0.6	7.6 ± 0.3	6.6 ± 0.3	7.5 ± 0.4
McMahon et al. (2015)	Mummichogs (*Fundulus heteroclitus*)	vegetable commercial diet (Vegi-Pro)	0.4 ± 0.4	3 ± 0.5	NA	1 ± 0.2	1.5 ± 0.9	8.5 ± 0	10.8 ± 0.2	9.4 ± 0.1	7.3 ± 0.4	8.8 ± 0.5	10 ± 0.3	11.7 ± 0.7
Barreto-Curiel et al. (2017)	Pacific yellowtail (*Seriola lalandi*)	Commercial diet made with fish meal	-0.16 ± 0.58	0.05 ± 0.1	4.2 ± 1.24	1.9 ± 0.05	NA	3.7 ± 0.86	5.5 ± 1.38	4.9 ± 0.35	5.9 ± 0.02	4.7 ± 0.34	5.1 ± 0.19	6.8 ± 0.27

Table S3. (continued)

Reference	Common name species (*scientific name*)	Diet	TEF (mean ± SD) (‰)											
			Phe	**Lys**	**Met**	**Gly**	**Ser**	**Asp**	**Glu**	**Ile**	**Pro**	**Val**	**Leu**	**Ala**
Blanke et al. (2017)	Guppy (*Poecilia reticulata*)	Shrimp	-0.1	NA	NA	NA	NA	NA	8	NA	NA	NA	NA	NA
Blanke et al. (2017)	Zebrafish (*Danio rerio*)	Shrimp	0.4	NA	NA	NA	NA	NA	7.3	NA	NA	NA	NA	NA
Blanke et al. (2017)	Fathead minnow (*Pimephales promelas*)	Shrimp	-0.2	NA	NA	NA	NA	NA	6.9	NA	NA	NA	NA	NA
Blanke et al. (2017)	Bluegill (*Lepomis macrochirus*)	Shrimp	0.2	NA	NA	NA	NA	NA	8.2	NA	NA	NA	NA	NA
Blanke et al. (2017)	Guppy (*Poecilia reticulata*)	Perch	0	NA	NA	NA	NA	NA	7.3	NA	NA	NA	NA	NA
Blanke et al. (2017)	Zebrafish (*Danio rerio*)	Perch	-0.3	NA	NA	NA	NA	NA	6.2	NA	NA	NA	NA	NA
Blanke et al. (2017)	Fathead minnow (*Pimephales promelas*)	Perch	-0.2	NA	NA	NA	NA	NA	6.7	NA	NA	NA	NA	NA
Blanke et al. (2017)	Bluegill (*Lepomis macrochirus*)	Perch	0.7	NA	NA	NA	NA	NA	7.6	NA	NA	NA	NA	NA
Blanke et al. (2017)	Guppy (*Poecilia reticulata*)	Swordfish	0.7	NA	NA	NA	NA	NA	6.7	NA	NA	NA	NA	NA
Blanke et al. (2017)	Zebrafish (*Danio rerio*)	Swordfish	0.2	NA	NA	NA	NA	NA	7.5	NA	NA	NA	NA	NA
Blanke et al. (2017)	Fathead minnow (*Pimephales promelas*)	Swordfish	-0.1	NA	NA	NA	NA	NA	5.9	NA	NA	NA	NA	NA
Blanke et al. (2017)	Bluegill (*Lepomis macrochirus*)	Swordfish	0.7	NA	NA	NA	NA	NA	7.1	NA	NA	NA	NA	NA

Table S3. (continued)

Reference	Common name species (*scientific name*)	Diet	TEF (mean ± SD) (‰)											
			Phe	Lys	Met	Gly	Ser	Asp	Glu	Ile	Pro	Val	Leu	Ala
Nuche-Pascual et al. (2018)	Pacific yellowtail (*Seriola lalandi*)	Formulated diet made from fish meal (40 protein+0 non-digestible protein	1.7 ± 0.6	0.4 ± 0.4	2.8 ± 0.8	1.7 ± 0.7	NA	3.2 ± 0.5	5.3 ± 0.9	5.5 ± 0.7	4.8 ± 0.8	5.4 ± 0.7	6.6 ± 0.2	7.7 ± 1.4
Nuche-Pascual et al. (2018)	Pacific yellowtail (*Seriola lalandi*)	Formulated diet made from fish meal (50 protein+0 non-digestible protein)	3.3 ± 0.3	1.2 ± 0.1	1.1 ± 1.6	1.5 ± 0.8	NA	2 ± 0.7	8.1 ± 0.6	5.7 ± 0.3	5.3 ± 1.5	4.7 ± 1.1	5.6 ± 0.6	6.4 ± 1.1
Nuche-Pascual et al. (2018)	Pacific yellowtail (*Seriola lalandi*)	Formulated diet made from fish meal (60 protein+0 non-digestible protein)	0.3 ± 0.5	-1 ± 0.5	2 ± 0.5	1.7 ± 0.7	NA	4 ± 0.4	5.6 ± 1.7	6.1 ± 0.8	6.9 ± 1.1	6.7 ± 0.8	5.4 ± 0.3	8.8 ± 0.1
Nuche-Pascual et al. (2018)	Pacific yellowtail (*Seriola lalandi*)	Formulated diet made from fish meal (40 protein+10 non-digestible protein)	-0.8 ± 0.6	-0.1 ± 0.2	-0.3 ± 0.9	1.3 ± 0.9	NA	3.3 ± 0.0	3.9 ± 0.6	2 ± 1.8	4.8 ± 0.4	4.1 ± 1	3.1 ± 0.4	4.1 ± 1
Nuche-Pascual et al. (2018)	Pacific yellowtail (*Seriola lalandi*)	Formulated diet made from fish meal (50 protein+10 non-digestible protein)	0.3 ± 0.4	-1.8 ± 0.7	0.5 ± 0.9	0.8 ± 1.3	NA	2 ± 1.9	3.1 ± 1.7	3.4 ± 0.8	5.4 ± 0.6	5 ± 0.9	4.1 ± 0.6	7.2 ± 2.4
Nuche-Pascual et al. (2018)	Pacific yellowtail (*Seriola lalandi*)	Commercial diet formulated with fish meal	3.2 ± 1.6	-2.1 ± 0.6	-0.8 ± 1.3	1.4 ± 1.3	NA	3.5 ± 1.6	4.2 ± 1.4	6.6 ± 1.5	6.9 ± 1.4	6.8 ± 0.4	7.4 ± 0.9	7.4 ± 2.9
Barreto-Curiel et al. (2018)	Totoaba (*Totoaba macdonaldi*)	Formulated fish meal 40% protein	0.84 ± 0.48	1.45 ± 0.05	1.67 ± 0.14	1.62 ± 0.19	NA	3.74 ± 0.1	7.78 ± 0.51	4.25 ± 0.64	3.73 ± 0.58	5.75 ± 0.61	5.14 ± 0.49	8.09 ± 0.03
Barreto-Curiel et al. (2018)	Totoaba (*Totoaba macdonaldi*)	Formulated fish meal 43% protein	-0.46 ± 0.36	0.9 ± 0.11	0.78 ± 0.45	0.43 ± 0.13	NA	3.81 ± 0.1	6.04 ± 0.13	3.19 ± 0.19	3.59 ± 0.25	6.14 ± 0.18	4.98 ± 0.29	8.6 ± 0.34
Barreto-Curiel et al. (2018)	Totoaba (*Totoaba macdonaldi*)	Formulated fish meal 46% protein	1.58 ± 1.03	1.92 ± 0.12	-0.19 ± 0.84	0.21 ± 0.01	NA	3.84 ± 0.1	6.34 ± 0.23	3.65 ± 0.28	2.94 ± 0.01	5.57 ± 0.46	5.16 ± 0.34	8.81 ± 1.35
Barreto-Curiel et al. (2018)	Totoaba (*Totoaba macdonaldi*)	Formulated fish meal 49% protein	1.46 ± 0.2	0.51 ± 0.22	0.54 ± 0	0.21 ± 0.2	NA	3.75 ± 0.3	5.99 ± 0.78	3.95 ± 0.54	3.36 ± 0.01	4.69 ± 0.14	4.76 ± 0.27	9 ± 0.39

Table S4. Nutritional and ecological factors for published controlled feeding experiments in teleosts that report AA-specific TEFs

Reference	Common name species (scientific name)	Dietary protein content (%)	Dietary protein requirements (%) (Reference)	Protein level relative to requirement	Dietary lipid content (%)	Dietary lipid requirements (%) (Reference)	Lipid level relative to requirement	Feeding regime	Diet type	Life stage	Aquatic habitat
Chikaraishi et al. (2009)	Japanese halibut (*Paralichthys olivaceus*)	NA	52.78 (Webster & Lim. 2002)	NA	NA	8.78 (Zhang et al. 2001)	NA	NA	Invertebrates	Larvae	Marine
Chikaraishi et al. (2009)	Scorpion fish (*Sebastes schlegli*)	NA	48.6 (Kim et al. 2001)	NA	NA	8.5 (Lee, 2001)	NA	NA	Invertebrates	Larvae	Marine
Bloomfield et al. (2011)	Black bream (*Acanthopagrus butcheri*)	45	52 (Partridge and Jenkins 2002)	Low	22	13.23 (Om et al. 2001)	High	Satiation	fish based	Early juvenile	Brackish
Bradley et al. (2014)	Bluefin tuna (*Thunnus orientalis*)	NA	61.9 (Biswas et al. 2009)	NA	NA	17.9 (Biswas et al. 2009)	NA	NA	fish based	Subadult	Marine
Hoen et al. (2014)	Opakapaka (*Pristipomoides filamentosus*)	NA	42.8 (Miller et al. 2005; Maldonado-García et al. 2012; Hernández et al. 2014)	NA	NA	10 (Catacutan et al. 2001; Miller et al. 2005)	NA	NA	fish based	adult	Marine
McMahon et al. (2015) Squid	Mummichogs (*Fundulus heteroclitus*)	71	52 (Prinslow et al. 1974)	High	18	4 (Prinslow et al. 1974)	High	Satiation	Invertebrates	Early juvenile	Brackish
McMahon et al. (2015) Clam	Mummichogs (*Fundulus heteroclitus*)	69.1	52 (Prinslow et al. 1974)	High 8	17.6	4 (Prinslow et al. 1974)	High	Satiation	Invertebrates	Early juvenile	Brackish
McMahon et al. (2015) Bio-Vita	Mummichogs (*Fundulus heteroclitus*)	53.3	52 (Prinslow et al. 1974)	Optimum 9	23.9	4 (Prinslow et al. 1974)	High	Satiation	fish based	Early juvenile	Brackish
McMahon et al. (2015) Vegi-Pro	Mummichogs (*Fundulus heteroclitus*)	8	52 (Prinslow et al. 1974)	Low 10	5.9	4 (Prinslow et al. 1974)	High	Satiation	plant based	Early juvenile	Brackish

Table S4. (continued)

Reference	Common name species (scientific name)	Dietary protein content (%)	Dietary protein requirements (%) (Reference)	Protein level relative to requirement	Dietary lipid content (%)	Dietary lipid requirements (%) (Reference)	Lipid level relative to requirement	Feeding regime	Diet type	Life stage	Aquatic habitat
Barreto-Curiel et al. (2017)	Pacific yellowtail (*Seriola lalandi*)	42.55	50 (Masumoto et al., 2002)	Low	13.18	12 (Masumoto et al. 2002)	High	Satiation	fish based	Early juvenile	Marine
Blanke et al. (2017)12	Guppy (*Poecilia reticulata*)	NA	39 (Kithsiri et al. 2010)	NA	1.9	9.47 (Kithsiri et al. 2010)	Low	Satiation	Invertebrates	Adult	Freshwater
Blanke et al. (2017)	Zebrafish (*Danio rerio*)	NA	46.5 (Siccardi et al. 2009)	NA	1.9	11.7 (Siccardi et al. 2009; Hölttä-Vuori et al. 2010)	Low	Satiation	Invertebrates	Early juvenile	Freshwater
Blanke et al. (2017)	Fathead minnow (*Pimephales promelas*)	NA	36 (Lochmann & Kumaran, 2006)	NA	1.9	15.2 (Lochmann & Kumaran, 2006)	Low	Satiation	Invertebrates	Adult	Freshwater
Blanke et al. (2017)	Bluegill (*Lepomis macrochirus*)	NA	41.5 (Webster et al. 1997)	NA	1.9	9.73 (Webster et al. 1997)	Low	Satiation	Invertebrates	Early juvenile	Freshwater
Blanke et al. (2017)	Guppy (*Poecilia reticulata*)	NA	39 (Kithsiri et al. 2010)	NA	4.6	9.47 (Kithsiri et al. 2010)	Low	Satiation	fish based	Adult	Freshwater
Blanke et al. (2017)	Zebrafish (*Danio rerio*)	NA	46.5 (Siccardi et al. 2009)	NA	4.6	11.7 (Siccardi et al. 2009; Hölttä-Vuori et al. 2010)	Low	Satiation	fish based	Early juvenile	Freshwater
Blanke et al. (2017)	Fathead minnow (*Pimephales promelas*)	NA	36 (Lochmann & Kumaran, 2006)	NA	4.6	15.2 (Lochmann & Kumaran, 2006)	Low	Satiation	fish based	Adult	Freshwater
Blanke et al. (2017)	Bluegill (*Lepomis macrochirus*)	NA	41.5 (Webster et al. 1997)	NA	4.6	9.73 (Webster et al. 1997)	Low	Satiation	fish based	Early juvenile	Freshwater

Table S4. (continued)

Reference	Common name species (scientific name)	Dietary protein content (%)	Dietary protein requirements (%) (Reference)	Protein level relative to requirement	Dietary lipid content (%)	Dietary lipid requirements (%) (Reference)	Lipid level relative to requirement	Feeding regime	Diet type	Life stage	Aquatic habitat
Blanke et al. (2017)	Guppy (*Poecilia reticulata*)	NA	39 (Kithsiri et al. 2010)	NA	20.4	9.47 (Kithsiri et al. 2010)	High	Satiation	fish based	Adult	Freshwater
Blanke et al. (2017)	Zebrafish (*Danio rerio*)	NA	46.5 (Siccardi et al. 2009)	NA	20.4	11.7 (Siccardi et al. 2009; Hölttä-Vuori et al. 2010)	High	Satiation	fish based	Early juvenile	Freshwater
Blanke et al. (2017)	Fathead minnow (*Pimephales promelas*)	NA	36 (Lochmann & Kumaran, 2006)	NA	20.4	15.2 (Lochmann & Kumaran, 2006)	High	Satiation	fish based	Adult	Freshwater
Blanke et al. (2017)	Bluegill (*Lepomis macrochirus*)	NA	41.5 (Webster et al. 1997)	NA	20.4	9.73 (Webster et al. 1997)	High	Satiation	fish based	Early juvenile	Freshwater
Nuche-Pascual et al. (2018)	Pacific yellowtail (*Seriola lalandi*)	42.1	50 (Masumoto et al. 2002)	Low	20.4	12 (Masumoto et al. 2002)	High	Fixed	fish based	Early juvenile	Marine
Nuche-Pascual et al. (2018)	Pacific yellowtail (*Seriola lalandi*)	51.9	50 (Masumoto et al. 2002)	Optimum	16.1	12 (Masumoto et al. 2002)	High	Fixed	fish based	Early juvenile	Marine
Nuche-Pascual et al. (2018)	Pacific yellowtail (*Seriola lalandi*)	61.3	50 (Masumoto et al. 2002)	High	12.1	12 (Masumoto et al. 2002)	Optimum	Fixed	fish based	Early juvenile	Marine
Nuche-Pascual et al. (2018)	Pacific yellowtail (*Seriola lalandi*)	49.5	50 (Masumoto et al. 2002)	Optimum	10.1	12 (Masumoto et al. 2002)	Low	Fixed	fish based	Early juvenile	Marine
Nuche-Pascual et al. (2018)	Pacific yellowtail (*Seriola lalandi*)	60	50 (Masumoto et al. 2002)	High	8.9	12 (Masumoto et al. 2002)	Low	Fixed	fish based	Early juvenile	Marine
Nuche-Pascual et al. (2018)	Pacific yellowtail (*Seriola lalandi*)	56.9	50 (Masumoto et al. 2002)	High	9	12 (Masumoto et al. 2002)	Low	Fixed	fish based	Early juvenile	Marine

Table S4. (continued)

Reference	Common name species (scientific name)	Dietary protein content (%)	Dietary protein requirements (%) (Reference)	Protein level relative to requirement	Dietary lipid content (%)	Dietary lipid requirements (%) (Reference)	Lipid level relative to requirement	Feeding regime	Diet type	Life stage	Aquatic habitat
Barreto-Curiel et al. (2018)	Totoaba (*Totoaba macdonaldi*)	40.8	47 (Minjarez-Osorio et al. 2012)	Low	7.70	8 (Minjarez-Osorio et al. 2012)	Low	Satiation	fish based	Early juvenile	Marine
Barreto-Curiel et al. (2018)	Totoaba (*Totoaba macdonaldi*)	42.0	47 (Minjarez-Osorio et al. 2012)	Low	7.9	8 (Minjarez-Osorio et al. 2012)	Optimum	Satiation	fish based	Early juvenile	Marine
Barreto-Curiel et al. (2018)	Totoaba (*Totoaba macdonaldi*)	46.1	47 (Minjarez-Osorio et al. 2012)	Optimum	9.6	8 (Minjarez-Osorio et al. 2012)	High	Satiation	fish based	Early juvenile	Marine
Barreto-Curiel et al. (2018)	Totoaba (*Totoaba macdonaldi*)	48.9	47 (Minjarez-Osorio et al. 2012)	Optimum	10.6	8 (Minjarez-Osorio et al. 2012)	High	Satiation	fish based	Early juvenile	Marine