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ARTICLE

Methods, Tools, and Technologies

Improving trophic position estimates from amino acid stable isotopes by accounting for physiology and environment

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Abstract

Nitrogen isotope analyses of amino acids (δ^{15}N-AA) are being increasingly used to decipher trophic dynamics. Interpretation of δ^{15}N-AA in consumers relies on the assumption that consumer physiological status and nutritional status of prey have negligible influences on the trophic discrimination factor (TDF), hence a constant TDF value is used in trophic position (TP) equations. Recent experiments have shown that this is not always the case and there is also a need to validate derived TP estimates in the field. We take advantage of the uniquely long time series of environmental monitoring data and archived (frozen) samples from the species-poor Baltic Sea. We analyzed δ^{15}N-AA in similar sized individuals of cod and in its prey herring from four decades, 1980–2018; including time periods where dramatic reduction in condition status of cod has occurred. We expected that TDF in trophic AAs would increase during periods of poor cod condition, resulting in inflated TP estimates. We found that calculated TP and empirical estimates of TDF (difference in δ^{15}N in trophic AAs between cod and herring) for cod increased in recent decades and that this was linked to condition status, herring (prey) lipid content and the hypoxic state of the ecosystem. Statistically adjusting TP for condition and prey lipid content as well as environmental stress (hypoxia) resulted in lower cod TP which better resembled the observed decrease in herring TP in recent decades. TP calculated from stomach analysis data in cod individuals over the same period showed no trend over time and confirmed that adjusted TP estimates mirror the real dietary TP better than unadjusted. By simultaneously measuring condition/nutritional status in both predator and prey it is possible to adjust for them as confounding variables and decipher actual consumer TP, partly overcoming the issues of unknown and variable TDF-values. Our study also highlights the importance of including environmental stressors (here hypoxia) when interpreting TP and reconstructing food webs.

KEYWORDS
Clupea harengus, compound specific stable isotope analyses (CSIA), environmental monitoring, *Gadus morhua*, isotope baseline, isotope fractionation, trophic discrimination (TDF) enrichment (TEF), trophic interactions

INTRODUCTION

Evaluating the impacts of environmental change on ecosystems and implementing sustainable management practices requires a broad understanding of the trophodynamics of food webs. Trophic positioning of consumers is instrumental when assessing food web structure (Post, 2002), and is used in contaminant science for quantifying biomagnification of contaminants (Rolff et al., 1993). Stable isotope ratios of nitrogen (^{15}N:^{14}N), expressed as $\delta^{15}N$ (the isotope ratio in parts per thousand deviations from a standard), are commonly used to estimate trophic position (TP) in consumers since the heavy isotope is enriched with each trophic transfer in a rather predictable way, that is, the difference in $\delta^{15}N$ value measured between consumer and diet is constant (e.g., McCutchan et al., 2003; Minagawa & Wada, 1984). However, this general trophic enrichment (often referred to as trophic discrimination factor, TDF; Bond & Hobson, 2012) is not independent of physiological status of the consumer or nutritional quality of the diet/prey, for example, for starving animals excretory losses are high relative to nitrogen incorporation resulting in higher TDFs and hence higher $\delta^{15}N$-values (e.g., Martínez del Rio & Wolf, 2005). Furthermore, environmental stress may also lead to higher $\delta^{15}N$-values in consumers due to physiological costs associated with, for example, contaminant exposure (e.g., Ek et al., 2015; Shaw-Allen et al., 2005; Staaden et al., 2010). Finally, the $\delta^{15}N$ baseline varies considerably in both space and time, because it depends on the isotopically distinct nitrogen sources used by primary producers (e.g., nitrate vs. fixed nitrogen, wastewater inputs) and the dominant biogeochemical processes involved in N cycling (Rolff, 2000; Vander Zanden & Rasmussen, 1999). Hence, interpreting $\delta^{15}N$ in bulk samples, particularly for higher trophic levels, is complex and requires a broad understanding of the ecosystem.

Analysis of $\delta^{15}N$ in specific amino acids (AA) in consumers has become increasingly popular because it allows for TP and baseline $\delta^{15}N$ estimates, simultaneously, without sampling the base of the food web (Chikaraishi et al., 2009; Ohkouchi et al., 2017). Trophic AAs undergo substantial transamination reactions during consumer metabolism resulting in increases in their $\delta^{15}N$-values due to kinetic isotope effects (hence higher TDF values), whereas source AAs undergo minimal transamination and thus show no or very little change in their $\delta^{15}N$-values (Chikaraishi et al., 2009; Macko et al., 1986; McClelland & Montoya, 2002; O'Connel, 2017). $\delta^{15}N$ in AAs can potentially provide a more accurate TP estimation in food web studies than $\delta^{15}N$ in bulk material (Blanke et al., 2017; Bowes & Thorp, 2015), by incorporating information from both source and trophic AAs. However, the AA approach for calculating TP also builds on fixed TDF values for each AA (difference between consumer and diet $\delta^{15}N$ values, sometimes denoted by Δ). When the difference between $TDF_{trophic}$ and TDF_{source} is combined into a trophic enrichment factor ($TEF = TDF_{trophic} - TDF_{source}$, often erroneously used as a synonym to TDF in the literature), it can, in common with the traditional bulk approach, also affect TP estimates, potentially even more so since assumptions for two TDFs exist. Since $\delta^{15}N$ values in AAs are products of their specific biosynthetic pathways (Chikaraishi & Kashiyama, 2007), any stress (e.g., suboptimal environmental conditions or starvation) that alters AA metabolism may also lead to profound changes in individual AA-$\delta^{15}N$ values (Lübcker et al., 2020; Poupin et al., 2014; Shipley et al., 2022), likely making AA-TP estimates more sensitive to physiological stress than the traditional "bulk" approach (Ek et al., 2018). Previous studies have shown that TDFs are sensitive to several factors including diet protein quality (e.g., Chikaraishi et al., 2015; McMahon et al., 2015), diet lipid content (Blanke et al., 2017), and metabolic balance between production and degradation of AAs (Takizawa et al., 2020), which are all closely associated with altered rates of trans- and deamination of specific AAs (Goko et al., 2018; Macko et al., 1986).

The consequences of stress for AA-TP estimates are largely unknown and may differ depending on which AAs are included. For example, TP estimates might differ if they are calculated using the two most commonly used trophic and source AAs (Glutamic acid and Phenylalanine, respectively; see Chikaraishi et al., 2009) or using multiple AAs (see Nielsen et al., 2015). The latter has been suggested as a more robust approach regarding uncertainties in the TDFs of individual AAs (but see O'Connel, 2017). How estimates of TP based on AAs are affected in real-world ecosystems where individuals can adapt their behavior (e.g., change diet, perform

compensatory feeding, or escape stress) is unknown. There is a need to validate derived AA-TP estimates in field. This can be done by exploring whether the shift in AA-δ^{15}N between predator and important prey(s) is constant over time, and relate potential deviations and hence consequences for AA-TP to data on condition status or environmental conditions.

Here, we take advantage of the uniquely long time series of environmental monitoring data and archived samples from the well-studied and species-poor Baltic Sea to link proxies for physiological status in cod (*Gadus morhua*) and its prey, herring (*Clupea harengus*) with isotope data. During the last four decades, this species-poor ecosystem has gone from a cod-dominated to a forage fish-dominated system, largely as a consequence of overfishing and increased hypoxia (often referred to as a regime shift; Möllmann et al., 2009) with consequences for condition status of both cod and herring (Casini et al., 2010, 2016; Karlson et al., 2020). The fluctuating trends in cod and herring condition status over the last four decades are well documented allowing investigations into the possible consequences for δ^{15}N values in wild populations. Low growth rate could lead to increased rates of catabolic processes, which would increase δ^{15}N values in trophic AAs (e.g., McMahon et al., 2015; McMahon & McCarthy, 2016). Proteins that are metabolized for energy should cause differences in AA-specific TDFs, but the lipid content of prey should also be relevant for TDF estimates because lipids provide an important energy source for the consumer (energy density is approximately twice as high in lipid compared with carbohydrates and protein; Livesey, 1995). When the lipid content in the diet (prey) decreases, available energy from lipids will be reduced and other nutrients will be metabolized to provide energy. Such a shift in metabolic pathways likely alters ^{15}N fractionation, for example, a lipid-rich diet has been linked to a low TDF (Blanke et al., 2017; Chikaraishi et al., 2015).

We expect that over time δ^{15}N values in trophic AAs and derived TP estimates, in both herring and cod, are confounded by the nutritional status of their diet and, in cod, also the hypoxic state of the ecosystem, that is, the environment, influencing its physiology. Hypoxia followed by normoxic recovery induces oxidative stress in fish and upregulates antioxidant enzymes (Luschak & Bagnyukova, 2007) which may influence isotope composition (Beaulieu et al., 2015). More specifically, a low condition status in cod (based on mass–length relationship and lipid content) indicates slow growth and/or physiological stress, and is expected to result in a higher TDF in trophic AAs for and higher TP estimates (Figure 1a). Additionally, cod TP estimates are expected to be influenced by the nutritional status of its prey (e.g., lipid content in herring), and hence the prey's TDF and derived prey-TP (Figure 1b,c). We test the influence of proxies for cod condition status (which partly incorporate environmental stress effects on its physiology) and herring lipid content as a proxy for prey nutritional status on AA-δ^{15}N values (muscle tissue) and TDF and TP estimates over a 40-year period. Since condition status changes have been most pronounced in cod during the most recent 25 years, this period is the focus of the study; we predict that the deteriorated condition will result in inflated TDF and TP estimates in cod during this time. All analyses build on annual time series data of AA-δ^{15}N in cod and herring and condition status data in the same fish.

MATERIALS AND METHODS

Sample preparation

Samples of cod and herring were provided by the Environmental Specimen Bank (ESB, long-term frozen storage, −25°C) at the Swedish Museum of Natural History. Cod were caught south east of the island of Gotland (56°53′ N, 18°38′ E; Appendix S1: Figure S2) and herring were caught outside Landsort (58°42′ N, 18°04′ E; Appendix S1: Figure S2), in the western Gotland basin, approx. 200 km north of the cod sampling site. This is a representative station based on comparison with herring stations further south and closer to Gotland which have shorter time series (Appendix S1: Figures S2 and S3). All fish were caught during the autumn (October–November) and frozen immediately after capture. Adult cod in this size class feeds mainly on pelagic prey such as herring and sprat (*Sprattus sprattus*) (Neuenfeldt et al., 2020). Herring and sprat both feed mainly on zooplankton (Möllmann et al., 2004). We used herring as a proxy for pelagic forage fish prey since this is the species available from ESB, but importantly, herring together with sprat constituted the most important prey to cod diet based on mass in recent decades (Neuenfeldt et al., 2020; Appendix S1: Figure S1) and both herring and sprat show the same trends in condition status (i.e., improvements since the historically low values in the early 1990s; Karlson et al., 2020). Significant correlations in δ^{15}N in source AAs (e.g., phenylalanine) over time for cod and herring would validate the assumption of a stable predator–prey relationship.

Ten similar sized individuals from each species and year (cod 35.3 ± 3.6 cm and herring 18.3 ± 1.1 cm [total length]), with as equal sex ratio as possible, and of the same age (3–5 years for both species), over a 40-year period (cod; 1981–2018, one missing year 2014, herring

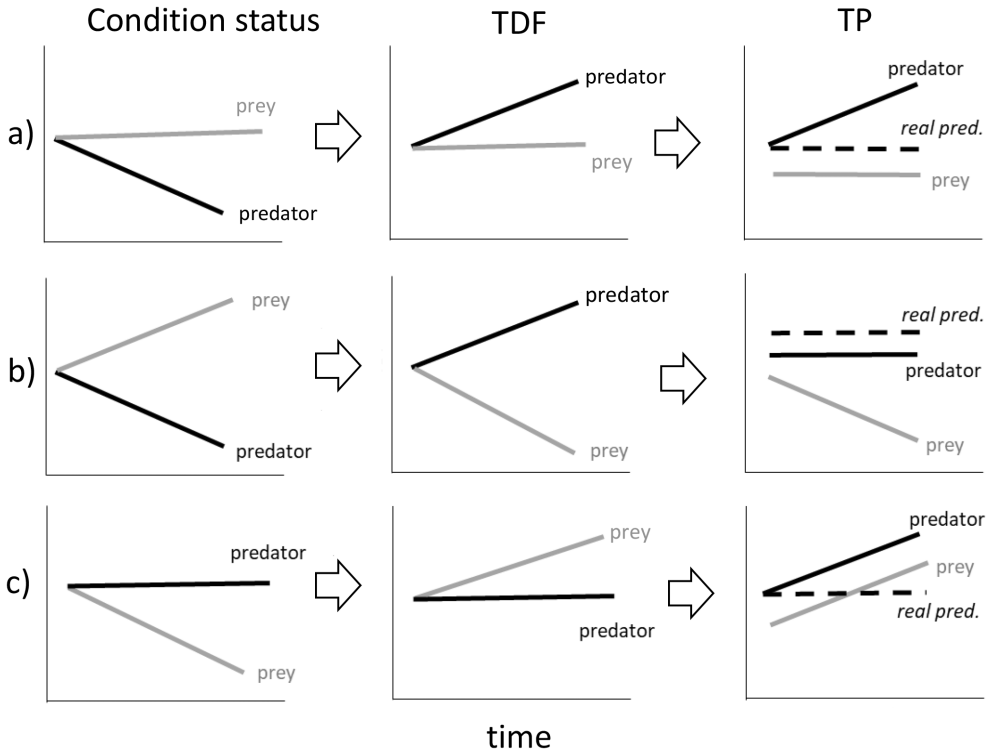

FIGURE 1 Conceptual scenarios (a–c) of temporal relationships between consumer condition status (proxy of physiological or nutritional status; left panel), trophic discrimination factor (TDF, mid-panel) in trophic amino acids (AAs) and in right panel estimated trophic position (TP) as well as "real" predator TP (dotted lines). Scenario a: Declining predator and unchanged prey condition results in increasing predator TDF and inflated TP values. Scenario b: Declining predator and increasing prey condition results in increasing TDF in predators and decreasing TDF in prey. This in turn leads to decreasing TP estimates for prey but no net change in TP for the predator (lower TP for prey counteracts the increase in predator TDF). Scenario c: Declining prey and unchanged predator condition results in increasing TDF and TP for prey with effects from prey resulting in increasing predator TP. In this study we calculate TP in both prey and predator from their respective $\delta^{15}N$ values in source and trophic AAs using fixed literature values of TDF and estimate the AA-TDF in the predator from the difference between predator and prey $\delta^{15}N$ values (only TDF estimates in trophic AAs are expected to be influenced by condition status) during a time period with deteriorated predator condition.

1980–2018; seven missing years 1982, 1993, 1998, 2000, 2006, 2013, and 2015) were sampled for isotope analyses. Twenty milligrams of white muscle tissue was dissected from each specimen from above the lateral line (epidermis and subcutaneous lipid tissue were removed; Pinnegar & Polunin, 1999). The tissue was freeze-dried and homogenized, whereafter composite samples of the material based on the 10 individuals for each year and species were used for AA-$\delta^{15}N$ analyses (3 mg in glass vials). The sample preparation with pooling of individuals within a narrow size range, as for cod and herring in this study, is standard for expensive chemical analyses in the Swedish contaminant monitoring program (Bignert et al., 2014).

As a complement, bulk analyses of $\delta^{13}C$ and $\delta^{15}N$ of the composite samples were analyzed (1 mg sample in tin capsules). Bulk $\delta^{15}N$ values are commonly used to estimate TP in consumers but requires a temporally and spatially matching baseline (Post, 2002); here, we used annual bulk isotope data in blue mussels (*Mytilus edulis trossulus*), the species recommended as an isotope baseline in the area (Karlson & Faxneld, 2021), from two sites separated within 200 km from fish sampling sites (Appendix S1: Figure S2, Kvädöfjärden in the south 1981–2017; collected in autumn, Ek et al., 2021, and Askö in the north, 1993–2016; collected in summer, Liénart et al., 2022) to calculate bulk TP in cod and herring and compared derived time trends with the AA-TP approach (details under data analyses and statistics). $\delta^{13}C$ values are commonly used to link consumers to the primary producers at the base of the food web (e.g., Post, 2002; Trueman et al., 2012). We expected that cod, herring and filter-feeding blue mussels would be ultimately fueled by pelagic carbon (phytoplankton) similar to the predator–prey link for cod and herring which was investigated via $\delta^{15}N$ in phenylalanine (see above).

All samples were sent to the UC Davis Stable Isotope Facility, USA, for analysis. $\delta^{15}N$-AA was analyzed using a Thermo Trace GC 1310 gas chromatograph coupled to

a Thermo Scientific Delta V Advantage isotope-ratio mass spectrometer via a GC IsoLink II combustion interface. AAs were made suitable for gas chromatography by derivatization as *N*-acetyl methyl esters (Corr et al., 2007). Prior to derivatization, AAs were hydrolysed (6 M HCl, 70 min, 150°C under N_2 headspace), redissolved in weaker HCl solution, and purified using cation-exchange chromatography (SCX; Dowex 50WX8 resin; see Appendix S1: Section S1 for details on injection and calibration procedure). Samples were measured in duplicates and replicates of the quality control and assessment materials are measured for every five samples. Analytical precision for δ^{15}N-AA was ±<0.45‰ and for bulk δ^{13}C and δ^{15}N ±<0.14‰. δ^{13}C values were mathematically adjusted for the C:N ratio according to Post et al. (2007) since lipids are depleted in ^{13}C and a higher C/N ratio indicates high lipid content (especially herring muscle has a high lipid content, but chemical lipid removal may influence δ^{15}N values; Pinnegar & Polunin, 1999). Data and metadata are available from Figshare (https://doi.org/10.6084/m9.figshare.24147900.v1).

Data analyses: Condition status of predator and prey

Biological data on length, mass, age, and lipid content (muscle lipid for herring and liver lipid for cod, expressed as % of wet mass; Karlson et al., 2020) were compiled from the ESB database (publicly available at www.sgu.se), and annual mean values were calculated for use as potential explanatory variables in statistical analyses (Figure 2, see below). A condition factor was calculated according to Fulton's condition index ($K = 100 \times \frac{W}{L^3}$), where W is somatic mass (in grams) and L fork length (in centimeters) (Ricker, 1975). Lipids were measured using a method where wet tissue was extracted with a mixture of polar and nonpolar solvents (Jensen et al., 1983). The lipid data were measured from 20 individuals each year in 1981–1996, 10 individuals each year in 1997–2014, and since 2015 a mean value of two pools of 12 individuals each year (comparable over time according to Bignert et al., 2014).

Data analyses: Trophic metrics of predator and prey

TP based on AA-δ^{15}N was calculated for both cod and herring according to Chikaraishi et al. (2009) using Equation (1) ($TP_{Glu\text{-}Phe}$) and Equation (2) using multiple AAs (hereafter TP_{all}).

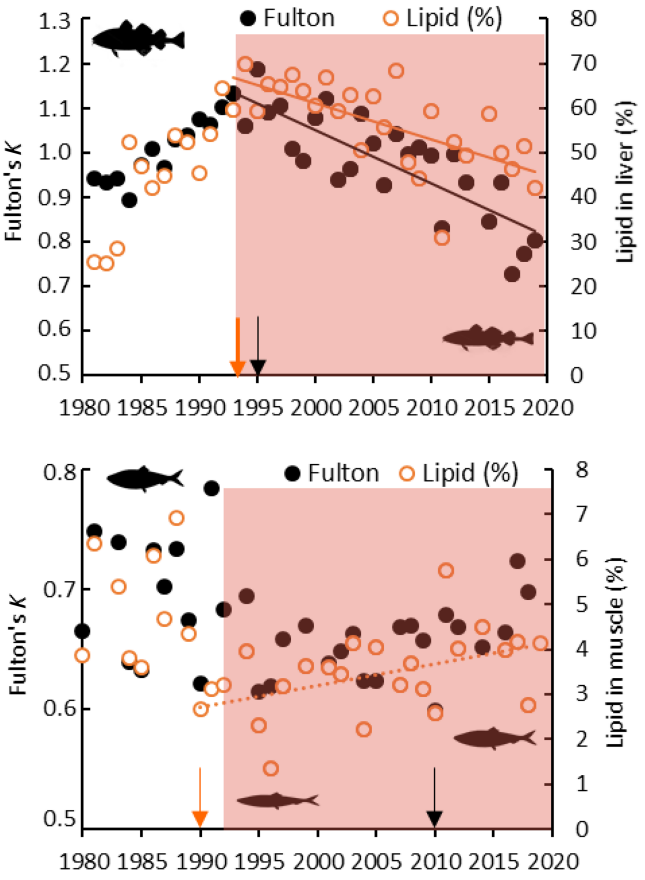

FIGURE 2 Condition status in cod (top) and herring (bottom) 1980–2019. The red shaded area indicates the cod deterioration period (1993 and onward) and arrows correspond to specific "breakpoints" for lipid content and Fulton's *K* for cod and herring (proxies for physiological status, see text for details). Trendlines (dotted for herring and solid for cod) illustrate significant unidirectional trends since breakpoints (see text for details).

$$TP = \frac{(\delta^{15}N_{Glu} - \delta^{15}N_{Phe}) - \beta}{(\Delta_{Glu} - \Delta_{Phe})} + 1. \quad (1)$$

$$TP_{x/y} = \frac{\sum(\delta^{15}N_{xi})/|x| - \sum(\delta^{15}N_{yj})/|y| - \beta}{(\Delta_x - \Delta_y)} + 1. \quad (2)$$

In Equation (2), N_{xi} are the δ^{15}N values of trophic AA_i, and N_{yj} are the δ^{15}N values of source AA_j. The "trophic" AAs are Glutamic acid (Glu), Alanine (Ala), Aspartic acid (Asp), Proline (Pro), Leucine (Leu), Iso-leucine (Ile), and Valine (Val), and the "source" AAs are Phenylalanine (Phe), Glycine (Gly), Serine (Ser), Tyrosine (Tyr), Lysine (Lys), Methionine (Met), Histidine (His), and Threonine (Thr) (O'Connel, 2017). The letters in subscript i and j correspond to the different trophic and source AAs respectively, in Equation (2). β corresponds to the difference between the δ^{15}N values of trophic AAs (x) and source AAs (y) in primary producers, Δ_x and Δ_y are the

^{15}N fixed TDF factors for each AA(s) x and y, respectively. Values for β and $\Delta_x - \Delta_y$ (the trophic enrichment factor, TEF) differ between the equations; in Equation (1) we used 3.4 and 7.6 according to Chikaraishi et al. (2009) and in Equation (2) we used 2.9 and 5.9 (Nielsen et al., 2015). Equation (2) was calculated according to Nielsen's approach where all the source δ^{15}N values are normalized not only relative to δ^{15}N-Phe but also without this normalization since δ^{15}N-Phe has been shown to respond to food limitation (Barreto-Curiel et al., 2017, 2018; Nuche-Pascual et al., 2018) and by including the additional AAs Thr, Gly, and Ser (these three AAs are usually not included as they are considered to respond to physiology and nutritional status; McMahon & McCarthy, 2016; Lübcker et al., 2020; Shipley et al., 2022). Results of both multi-AA approaches were highly correlated (Pearson's $r = 0.92$, $p < 0.01$), so hereafter only TP_{all} without Phe-normalization is shown as the main interest lies in the potential effects of statistical adjustments to remove physiological effects on AA-δ^{15}N values.

Fixed TDF estimates (Δ) are used in the TP equations; we tested whether this general assumption is robust when applied to field data (cf. Figure 1, mid-panel), by calculating the difference between cod and herring ("consumer-diet") δ^{15}N separately for each AA and for each year (hereafter referred to as proxy for AA-TDF in cod) and comparing trends over time and with condition status (see *Statistics*). We expected effects on TDFs for cod in trophic AAs only (Figure 1). Note that this AA-TDF proxy was not possible to calculate for herring since there were no δ^{15}N-AA data in zooplankton (or blue mussels).

Finally, TP based on bulk δ^{15}N (TP_{bulk}) was calculated using the equation by Post (2002), and blue mussels (*Mytilus edulis*) were used as an isotope baseline (see above):

$$TP = \frac{\left(\delta^{15}\,\text{N fish} - \delta^{15}\text{N blue mussel}\right)}{\Delta} + 2, \qquad (3)$$

where Δ is the bulk-TDF of 3.4 and 2 is the position of blue mussels in the food web (primary consumer of phytoplankton). Trends in bulk-TP was compared to the two AA-TP approaches.

Statistics

We used a four-step procedure to test the predictions described in Figure 1 including (1) establishing a period with deteriorating condition status and negative correlation between the estimated proxies for the cod estimate of TDF-AA in trophic AAs and condition status, (2) generalized linear models (GLMs) (identifying the best predictor/s for TP), (3) multiple regression (adjusting TP with best predictor/s), and (4) ground truthing (comparing time trends in adjusted and non-adjusted TP with stomach-based TP [and TP_{bulk}]). Assumptions on the stable predator–prey relationship over time were tested using Pearson correlations with focus on the source AA δ^{15}N-phenylalanine and the bulk δ^{13}C values in cod and herring. The proportion of herring and sprat in cod diet as estimated from stomach analyses was not expected to have increased or decreased during the cod condition deterioration period based on Neuenfeldt et al. (2020). Cod stomach data extracted from 30 to 40 cm cod caught in the northern Baltic proper (SD 27, 28 and 29) were available until 2014, from Neuenfeldt et al. (2020; median 109 individuals per year; Appendix S1: Figure S1). Absence of trends over time in herring and sprat diet in cod using this subset of data was confirmed using Kendall tau correlations (Appendix S1: Figure S1).

Step 1: Defining the deterioration period in condition status and establishing links between cod condition and cod TDF estimates in trophic AAs

We expected to be able to identify a period of deterioration in cod condition in the early 1990s based on a proposed regime shift (e.g., Möllmann et al., 2009) and recent studies on Baltic cod condition and growth (Casini et al., 2016; Mion et al., 2020). We carried out statistical breakpoint analyses in R (R Core Team, 2019) using the strucchange package (Kleiber et al., 2002) to compute breakpoints in time trends of proxies for physiological status (lipid % and Fulton's K over time in both species), and TP based on stomach analyses in cod ($TP_{stomach}$, see details below). Breakpoint analyses were also performed for the derived AA-TDF estimates for cod and the TP values in both species. We expected derived TDFs in trophic AAs and TP estimates to show breakpoints similar to the condition status data (sensu Figure 1). The optimal number, and corresponding year, of breakpoints in a time series was selected based on Bayesian information criterion (BIC) estimates. Unidirectional time trends in condition status, isotope data, and derived trophic metrics were assessed by nonparametric Mann–Kendall tests in time series based on the identified breakpoints for cod condition status.

During the deterioration period, potential negative relationships between lipid % (arcsine transformed) and Fulton's K, and between lipid % and estimates of TDF for each AA in cod, were tested using Pearson correlations (cf. Figure 1a). We expected correlations between cod

condition status and estimated TDFs (in cod) for the trophic AAs only.

Step 2: Testing links between TP-AA and condition status in cod and herring

GLMs were used to test the effects of condition status, prey lipid content, and environmental stress on cod (and herring) TP-AA estimates. TP_{bulk} estimates in cod were not included in this step since the baseline organism, which partly determines the bulk TP values, could not be expected to respond to variations in fish physiology. Lipid % in herring was used as a proxy for cod diet quality, as previously described by Karlson et al. (2020) and lipid % in cod was used as a physiological condition proxy for cod (the interaction between cod and herring lipid % was included in the model). Hypoxic volume for the Baltic Proper, using integrated yearly values from 1980 until 2016 extracted from Savchuk (2018), was used as the variable for environmental stress. Tissue bulk-$\delta^{13}C$ was also included as a predictor (proxy for diet origin for both cod and herring). For cod, we expected different physiological effects during periods of good (1980s) and bad conditions (since early 1990s, exact year defined in step 1); hence, separate GLMs were performed for the two time periods. In contrast to cod, herring had different physiological breakpoints depending on whether lipid % or Fulton's *K* was used (see results), and no separation into time periods was performed when testing the effects of physiological status. Final models were the best combination of predictors based on parsimony and Akaike information criterion (AIC) scores. Analyses were performed in Statistica in the GLZ module (Generalized linear/nonlinear models) using a log-link (residuals were visually inspected for normality).

Step 3: Adjusting TP estimates in cod and herring based on their condition status (results from step 2)

To reduce the amount of variation and hence increase the statistical power to detect true time trends in TP (Figure 1), AA-TP estimates were adjusted for potential confounding factors (the variables selected in the final GLM models based on AIC criteria, step 2) by multiple regression (Ek et al., 2021; Kleinbaum et al., 2008) using the software package PIA (Bignert, 2007). This analysis estimated the TP at the mean value of the covariates. Breakpoint analyses and time trends using Kendall-tau test (cf. step 1) were performed on adjusted TP values and compared with non-adjusted results.

Step 4: Truthing corrected TP estimates (in cod only) using stomach content data

Finally, the adjusted AA-TP data in cod were validated using TP calculated from stomach analyses data in other cod individuals (see above). $TP_{stomach}$ in cod was calculated as the sum of biomass-weighted prey with predefined TP, according to Cortés (1999). We used the four most common prey species which constituted >80% of total stomach content by biomass all years—herring, sprat, the invertebrate predator *Saduria entomon*, and mysid shrimps (*Mysis* spp). TP of the invertivore-feeding prey was set at 3.2 for herring, 3 for *Saduria* and sprat, and 2.3 for *Mysis* (Pauly & Christensen, 1995). Square root-transformed biomass data were used since they correspond to ingestion rate better than raw data (Neuenfeldt et al., 2020). Correlations of $TP_{stomach}$ with TP based on the various isotope approaches were explored using Pearson correlations; we expected the adjusted AA-TPs would be a better predictor of TP as estimated based on stomach content analysis (since unadjusted AA-TP would likely be confounded by altered condition status; Figure 1, step 2 in the statistical approach).

RESULTS

Validating assumptions on stable prey–predator and baseline relationships

For the entire time series, $\delta^{15}N$ in the source AAs Phe and Met as well as the trophic AA Asp covaried significantly and positively in herring and cod (Pearson's $r = 0.4$–0.5, Figure 3a; Appendix S1: Table S1). Correlations with a 1-year lag for cod (as a proxy for slow turnover of muscle tissue in slow growing fish; cf. Trueman et al., 2012) improved correlation coefficients for the source AAs Phe and Met during the deterioration period (Phe from $r = 0.28$, $p < 0.25$ to $r = 0.68$, $p < 0.001$ and Met from $r = 0.25$ to 0.55). Phe-$\delta^{15}N$ in both fish showed decreasing values of about 2 ‰ during the cod deterioration period (Figure 3a; herring: tau $= -0.39$, $p < 0.02$; cod: tau $= -0.31$, $p < 0.04$). Bulk-$\delta^{15}N$ for herring (but not cod) and the shorter blue mussel dataset also showed decreasing values during this time period (Figure 3b; herring: tau $= -0.63$, $p < 0.01$; mussel: tau $= -0.41$, $p < 0.01$). Over the entire time series, bulk $\delta^{15}N$ in both fish correlated significantly and positively with their respective trophic AAs (on average Pearson's $r = 0.8$), and with most of the source AAs but with lower r values, especially for cod (on average 0.2 for cod and 0.5 for herring; Appendix S1: Table S2a,b). $\delta^{13}C$ in both cod and herring covaried with the $\delta^{13}C$ blue mussel baseline over the entire time series (cod: $r = 0.23$, $p < 0.06$; herring: $r = 0.30$, $p < 0.09$; Figure 3c).

Temporal changes in condition status and correlations to TDF proxies (step 1)

Fulton's K and lipid % were positively and significantly correlated for both species (cod: $r = 0.47$, $p < 0.003$; herring: $r = 0.63$, $p < 0.001$). According to breakpoint analyses, lipid % and Fulton's K in cod began decreasing in 1993 and 1995, respectively (Figure 2). Both condition metrics decreased after 1993 (Fulton tau = −0.63, $p < 0.001$; lipid % tau = −0.53, $p < 0.001$) and we defined this year as the start of a deteriorating period for cod in subsequent analyses (vertical arrows in Figure 2). For herring, breakpoints were identified at 1990 (lipid %, increase after 1990, tau = 0.33, $p = 0.026$) and 2010 (Fulton's K, decrease until 2010, tau = −0.34, $p = 0.017$), and the entire time series was used when exploring herring condition status in relation to its TP values (as mentioned in *Materials and methods*: step 3).

The calculated cod AA-TDF breakpoints were found in 1994 (corresponding to the start of the cod deterioration period since year 1993 was missing for herring) for several trophic AAs (Table 1), whereafter significant unidirectional increases followed (Table 1, Figure 4). The magnitude of the shifts in TDF was about twofold for Glu. Lipid % and Fulton's K in cod were significantly negatively correlated with trophic TDF-AA estimates, but not with TDF estimates in source AAs during its deterioration period (Table 2). During the earlier time period, no such correlations were found with only one exception, the TDF for Pro (Table 2).

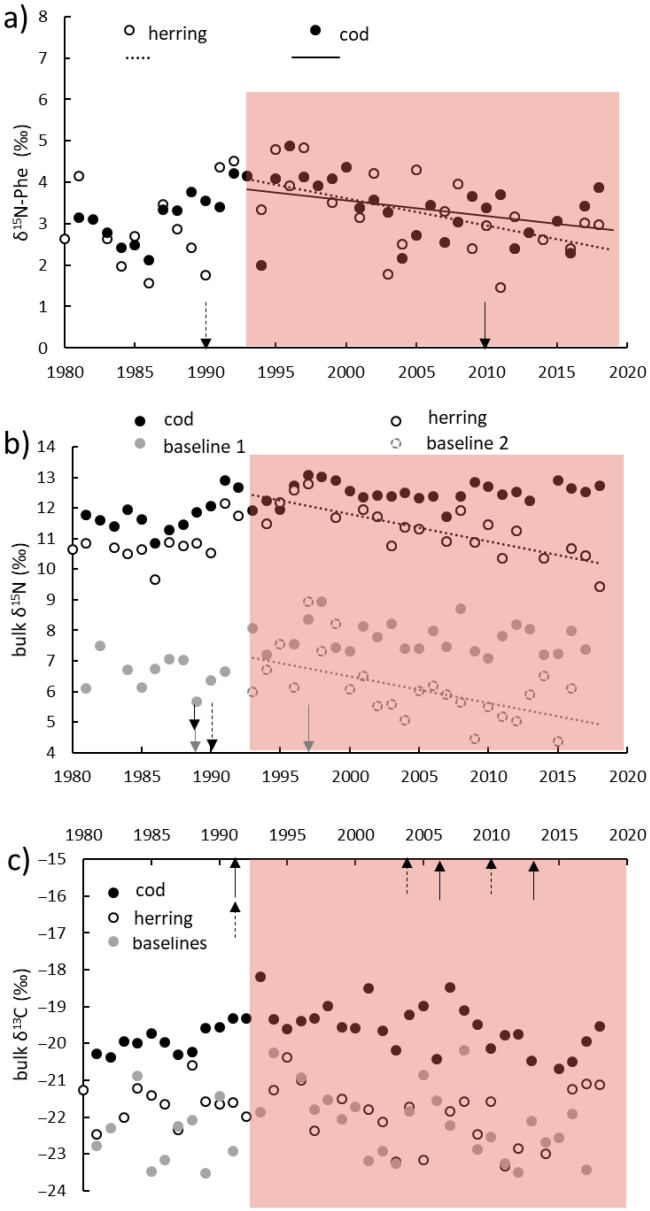

FIGURE 3 Long-term patterns (a) in $\delta^{15}N$ in the source amino acid Phenylalanine (Phe) for cod and herring, (b) in bulk $\delta^{15}N$-values for cod, herring, and the isotope baseline blue mussel (from two locations, see *Materials and methods*), and (c) in the $\delta^{13}C$-values of cod, herring, and blue mussel. Trendlines (dotted for herring and solid for cod) indicate significant unidirectional trends in $\delta^{15}N$-Phe and $\delta^{15}N$-bulk during the cod deterioration period (see text for details) and vertical arrows indicate breakpoints (solid lines for cod, dotted for herring).

TABLE 1 Estimates of cod trophic discrimination factor (TDF), statistical breakpoints (BPs), and unidirectional trends after breakpoint for each amino acid (AA) (second breakpoint for Ala, 2004 or entire time series if there was no breakpoint).

AA-TDF	Mean value (SD)	BP(s)	Tau value	*p*-value
Glu	1.9 (1.2)	1994	**0.67**	**<0.001**
Asp	8.0 (1.1)	1994	**0.67**	**<0.001**
Ala	2.2 (1.4)	1992, 2004	0.38	0.12
Val	0.2 (1.5)	1988	**0.40**	**<0.01**
Pro	1.1 (2.1)	1994	**0.45**	**<0.01**
Ile	1.8 (2.6)	No BP	**0.50**	**<0.001**
Leu	1.1 (1.4)	No BP	**0.42**	**<0.01**
Phe	0.1 (1.0)	No BP	0.03	0.80
Lys	−0.5 (1.0)	1990	0.21	0.18
Gly	2.5 (1.1)	No BP	−0.27	0.04
Ser	0.7 (1.2)	No BP	−0.08	0.54
Met	−0.3 (1.0)	1987	0.37	**0.01**
His	−1.4 (1.6)	No BP	0.05	0.74
Tyr	−1.7 (1.9)	No BP	0.15	0.25
Thr	−1.5 (2.0)	1990	−0.10	−0.53

Note: Values in boldface are lower than the Bonferroni-corrected ($n = 15$ AAs) p value of 0.013. See *Materials and methods* for full names of each AA.

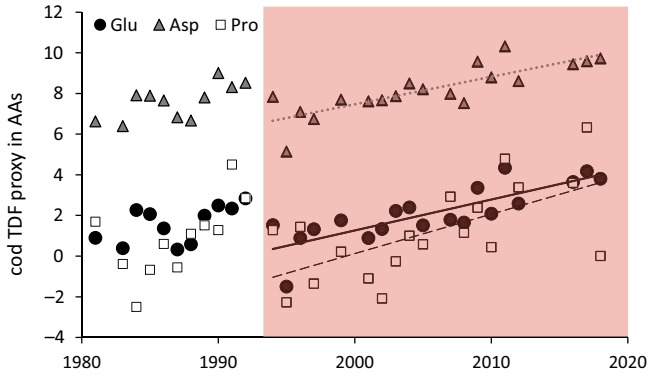

FIGURE 4 Long-term patterns in estimated AA-TDF (trophic discrimination factors for amino acids) in cod (calculated from cod–herring difference in $\delta^{15}N$) for AAs which breakpoints coincided with the start of the cod deterioration period (red shaded area); Glutamic acid (Glu), Aspartic acid (Asp), and Proline (Pro). These AA-TDF estimates for cod had increasing time trends after breakpoints (shown as trendlines, see Table 1 for details) and correlate with the condition status of cod during this time period only (Table 2). Source AA-TDFs showed no time trends or breakpoints coinciding with cod deterioration period (Table 1) and no correlation with condition status (Table 2).

Temporal changes in TP for cod and herring

The breakpoint(s) for the derived TP estimates in cod and herring (Figure 5a,c,e) differed between the methods (trends for adjusted TP-AA estimates in Figure 5d,f are described in step 3 below). In the $TP_{stomach}$ time series (Figure 5b) no breakpoints were detected and there was no unidirectional change in the time series. The temporal trends as assessed from Kendall-tau tests differed among the different TP methods (Figure 5a,c,e). Cod $TP_{Glu\text{-}Phe}$ increased over the entire time period (no breakpoint detected, tau = 0.39, $p < 0.01$), and the trend was more pronounced during the cod deterioration period (Figure 5c, tau = 0.40, $p < 0.01$). During this period TP_{all} also increased (Figure 5e, tau = 0.33, $p < 0.02$) as well as the $TP_{Nielsen}$ estimates (based on Phe-normalized source AAs, see *Materials and methods*; Appendix S1: Figure S4, tau = 0.38, $p < 0.01$). TP_{bulk} in cod showed no breakpoints and no time trend during the entire time period nor during the cod deterioration period when the longer baseline data were used (Figure 5a, tau <0.06 and 0.13, respectively), if the shorter baseline dataset was used, there was, however, an increase over time for TP_{bulk} in cod (tau = 0.48, $p < 0.01$, Figure 5a). Herring showed decreasing TP during the cod deterioration period, using both AA methods ($TP_{Glu\text{-}Phe}$: tau = −0.53, $p < 0.01$; TP_{all}: tau = −0.49, $p = 0.01$), and also for TP_{bulk} when using the longer baseline (TP_{bulk}: −0.57, $p < 0.01$). The two AA-based methods correlated significantly with each other in cod over the

TABLE 2 Pearson correlation results between trophic discrimination factor (TDF) estimates in cod for each amino acid (AA, grouped into trophic and source AAs) and the lipid % (arcsine transformed) and Fulton's *K* in cod during the deterioration period 1994–2018 (no data for herring in 1993) and the earlier time period (1981–1994).

	Lipid %		Fulton's *K*	
AA	**Pearson' *r***	***p***	**Pearson' *r***	***p***
1981–1994				
Glu	0.51	0.088	0.47	0.122
Asp	0.55	0.063	0.60	0.038
Ala	0.43	0.164	0.43	0.166
Val	0.20	0.542	0.27	0.400
Pro	0.27	0.398	0.78	**0.003**
Ile	0.57	0.052	0.41	0.180
Leu	0.54	0.071	0.48	0.118
Phe	−0.17	0.604	0.06	0.850
Lys	−0.06	0.305	−0.06	0.848
Gly	−0.36	0.248	−0.05	0.885
Ser	−0.30	0.349	0.14	0.657
Met	−0.27	0.403	0.03	0.932
His	−0.39	0.216	−0.12	0.718
Tyr	−0.58	0.051	−0.19	0.565
Thr	−0.63	0.029	−0.34	0.284
Deterioration period, 1994–2018				
Glu	**−0.67**	**0.002**	**−0.83**	**<0.001**
Asp	**−0.66**	**0.002**	**−0.78**	**<0.001**
Ala	−0.41	0.078	**−0.61**	**0.005**
Val	**−0.61**	**0.005**	**−0.61**	**0.006**
Pro	**−0.56**	**0.012**	**−0.57**	**0.011**
Ile	−0.46	0.044	**−0.61**	**0.006**
Leu	**−0.67**	**0.002**	**−0.77**	**<0.001**
Phe	−0.46	0.049	−0.44	0.058
Lys	−0.34	0.148	−0.39	0.100
Gly	−0.49	0.035	−0.53	0.018
Ser	−0.32	0.187	−0.48	0.038
Met	−0.28	0.248	−0.30	0.215
His	**−0.63**	**0.004**	−0.34	0.153
Tyr	−0.29	0.224	−0.42	0.071
Thr	0.42	0.076	0.25	0.299

Note: Values in boldface are lower than the Bonferroni-corrected ($n = 15$ AAs) *p* value of 0.013. See *Materials and methods* for full names of each AA.

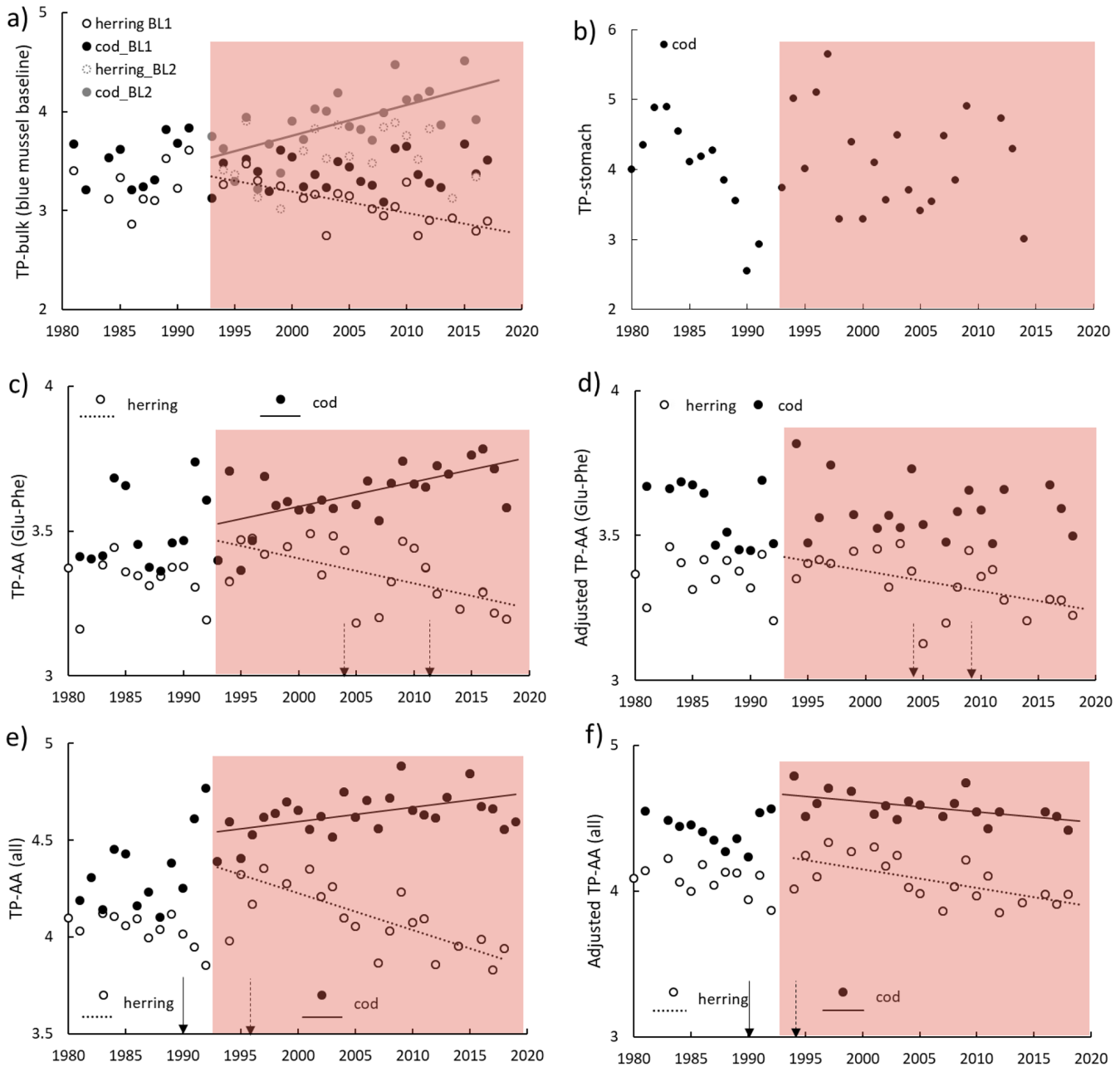

FIGURE 5 Long-term patterns in trophic position (TP) of cod and herring calculated using (a) the bulk method with blue mussel as baseline (two different baseline [BL] datasets varying in location and time length, see *Materials and methods*), (b) the four most common prey items according to stomach analyses in Neuenfeldt et al. (2020), see *Materials and methods*, (c) only Glutamic acid (Glu) and Phenylalanine (Phe) (Equation 1) and (e) all amino acids (AAs) (Equation 2). See Appendix S1: Figure S4 for TP calculated using a reduced selection of normalized source amino acids according to Nielsen et al. (2015). In (d) and (f) TP values after statistical adjustment are presented (see text; for cod adjustment for condition status (lipid content, proxy of physiological status), lipid content of prey (proxy of its nutritional value), and the hypoxic state of the ecosystem (for herring adjustment only for its condition status); compare Appendix S1: Figure S5 for lipid-adjustment only in cod). Trendlines (dotted for herring and solid for cod) illustrate significant unidirectional trend in TP during the cod deterioration period (the red shaded area). Vertical arrows indicate statistical breakpoint(s) for each species. Note that the *y*-axis differs among TP methods for visual purposes; relevant here are trends over time not absolute values.

entire as well as the last time period (Pearson's = 0.79–0.83, $p < 0.05$); however, the TP_{bulk} values never correlated with AA-TP values (neither of the methods) for either of the fish species (Pearson's r ranging 0.11–0.17; only tested for TP_{bulk} estimates using the longer baseline dataset).

Linking consumer condition and prey quality to TP with subsequent adjustments of these confounders (steps 2 and 3)

Herring TP (both AA equations) was best explained by its body condition (Fulton's *K*), while cod TP (both AA equations) was best explained by both herring lipid and its own lipid content as well as their interaction (Table 3; Appendix S1: Table S4). Although the time series before the deterioration period is quite short, the model resulted in the same selection of variables, but with opposite effects (+ vs. −) compared with the deterioration period (Table 3; Appendix S1: Table S4).

Adjusting TP estimates for condition status and additionally nutritional status of prey for cod variables resulted in reduced coefficients of variation in all time series, as would be expected (Appendix S1: Table S3). Adjusting herring TP with its condition status (Fulton's *K*) did not change the decreasing trend during the cod deterioration period (adjusted TP_{all} tau = −0.46, $p < 0.01$; adjusted $TP_{Glu\text{-}Phe}$ tau = −0.34, $p < 0.04$); however, the breakpoints were detected 2 years earlier after adjustment (Figure 5c vs. 5d, and 5e vs. 5f). The increasing TP trend for cod was however detrended after condition (cod lipid) and nutrition status (herring lipid) adjustment (adj $TP_{Glu\text{-}Phe}$ tau < 0.14, $p > 0.4$; adj. TP_{all} tau = −0.08, $p > 0.6$; Appendix S1: Figure S5). The adjusted TP data in cod correlated significantly with hypoxic volume (Figure 6a,b, TP_{all} $r = 0.72$, $TP_{Glu\text{-}Phe}$ $r = 0.56$, $p < 0.01$ in both cases; it was also the second best GLM model for unadjusted data; Appendix S1: Table S4). Corresponding values for unadjusted TP estimates were lower ($r = 0.44$, $p < 0.02$ and $r = 0.33$, $p < 0.10$, Figure 6a,b); hence, hypoxic volume was additionally adjusted for (shown in Figure 5d,f). These additional adjustments resulted in a decreasing trend for cod TP_{all} during the cod deterioration period (tau = −0.39, $p = 0.02$, Figure 5f) similar to the herring TP trend (cf. Figure 5e with 5f). For cod $TP_{Glu\text{-}Phe}$, the additional hypoxia adjustment did not result in a decreasing trend but decreased the correlation coefficient (tau < 0.01, $p > 0.97$, Figure 5d cf. 5c).

Truthing corrected TP estimates using stomach content data (step 4)

The cod TP based on stomach analyses showed no unidirectional trend and no breakpoints during the entire time period. Herring and sprat in stomachs had no unidirectional trend during the cod deterioration period (Appendix S1: Figure S1). Significant (Bonferroni corrected $p < 0.02$) positive Pearson's correlations between $TP_{stomach}$ (stomach data available only until 2014, Figure 5b) and lipid + hypoxia-adjusted TP_{all} were found in cod ($r = 0.52$, Figure 6d, marginally significant at $p < 0.04$ and $r = 0.44$ for $TP_{Glu\text{-}Phe}$, Figure 6c), but no significant correlations were found for unadjusted TP estimates (both methods $r < 0.1$). Lipid only-adjusted TP estimates resulted in marginally significant correlations with $TP_{stomach}$ ($r = 0.43$–0.45, $p < 0.04$, not shown in

TABLE 3 Results of generalized linear models testing the effects of diet origin, condition status (proxies for physiological status) and environmental status for trophic position, TP, derived using the two amino acid (AA)-based equations (denoted by $TP_{Glu\text{-}Phe}$ and TP_{all}) in herring and cod (separate models for each species).

Time series	Best model	Estimate	SE	Wald	*p*
Herring $TP_{Glu\text{-}Phe}$, entire period	Fulton's *K*	−0.31	0.11	7.89	0.005
Herring TP_{all}, entire period	Fulton's *K*	−0.29	0.14	4.27	0.039
Cod $TP_{Glu\text{-}Phe}$, before 1993	Herring lipid	−1.84	0.58	10.23	0.001
Cod $TP_{Glu\text{-}Phe}$, after 1993	Cod lipid	−0.54	0.16	11.39	<0.001
	Herring lipid	−5.57	2.17	6.58	0.010
	Cod lipid × Herr lipid	10.79	3.71	8.45	0.004
Cod TP_{all}, before 1993	Cod lipid	0.94	0.13	55.67	<0.001
	Herring lipid	5.96	1.27	21.88	<0.001
	Cod lipid × Herr lipid	−15.06	2.42	38.62	<0.001
Cod TP_{all}, after 1993	Herring lipid	5.96	1.27	21.88	<0.001
	Cod lipid × Herr lipid	−15.06	2.42	38.62	<0.001
	Cod lipid × Herr lipid	7.45	2.70	7.63	0.006

Note: For cod, time series are divided into before and after the deterioration period starting in 1993 (see text). Best models based on Akaike information criterion (AIC) and the estimates of these variables are shown and then used as covariates in subsequent multiple regression to calculate adjusted TP values (Figure 5d,f). The top five models with AIC scores are presented in Appendix S1: Table S4.

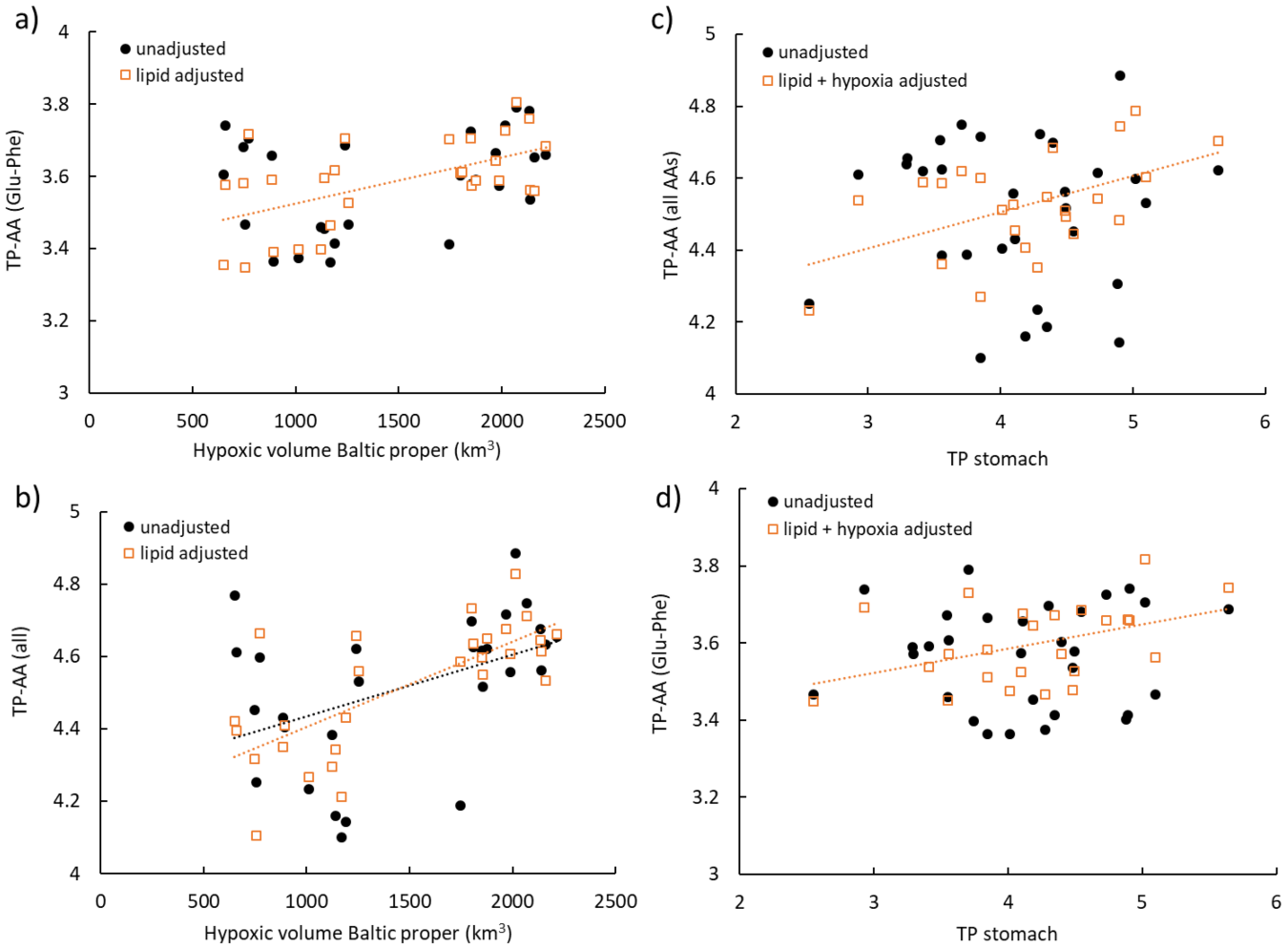

FIGURE 6 Pearson correlations (significant correlations are indicated with trend line, see text for statistics) between cod trophic position estimates ($TP_{Glu\text{-}Phe}$ and TP_{all}) and hypoxic volume in the Baltic proper (a and b) and cod $TP_{stomach}$ data (c and d). In (a) the filled symbols are unadjusted TP_{all} and in (b) $TP_{Glu\text{-}Phe}$ estimates while empty symbols are the corresponding lipid-adjusted TP estimates, that is, adjusted for condition status (proxy for physiological status and/or nutritional value of prey). In (c) and (d) empty symbols represent lipid + hypoxia-adjusted TP_{all} and $TP_{Glu\text{-}Phe}$ estimates (see text for details).

Figure 6). Herring + sprat % in diet was significantly correlated with lipid+hypoxia-adjusted but not with unadjusted TP (Appendix S1: Figure S6).

DISCUSSION

This study demonstrates that the apparent long-term divergent changes in TP for cod and its prey herring, as assessed from the δ^{15}N-AA values (Figure 5c,e), are incorrect. The divergent TP patterns are a result of environmental/physiological stress that has not been accounted for; specifically, fixed TDF does not accurately account for the effect of fluctuating environmental and physiological conditions on TP in wild top consumers (field data).

Stomach content provides the most accurate available TP estimates, and are the most reliable ground truth data with which to compare isotope estimates. The cod $TP_{stomach}$ values did not increase during the deterioration period (Figure 5b), and there was no temporal trend in the diet contribution of the quantitatively most important prey herring and sprat during this time period either (Appendix S1: Figure S1). However, cod TP estimates using all isotope methods showed an increase during this time period. As hypothesized, larger TDF estimates in trophic AAs were observed during the period of poor cod condition in recent decades and correlated with its own deteriorated condition status (Figure 1a, first and mid-panel). The cod deterioration period coincides with slow growth rate (Mion et al., 2020), and slow growing consumers have generally higher TDFs (Martínez del Rio & Wolf, 2005). Therefore, it is likely that the increasing cod TP found using fixed TDFs (same value for all years as used in TP equations) in recent decades is

incorrect (Figure 5c,e). Adjusting for condition status (and nutritional status in its prey, see below) detrended the previously increasing cod TP shown using both TP-AA equations, and when we adjusted for environmental stress (hypoxia), cod TP_{all} (but not $TP_{Glu\text{-}Phe}$) mirrored the decreasing trend for herring TP over the last two decades. Hence, the issue of the generally unknown and variable TDF values in the field could, at least partly, be overcome if other relevant data (non-isotopic) are available, allowing for statistical adjustments of TP. Below, we discuss the possible mechanisms behind differences among the various approaches tested (three different equations with and without adjustments).

Cod TP was likely influenced by the nutritional value of herring. It is well known for fish, including Baltic cod, that prey protein quality influences the bulk TDF—which can be as low as 0.8‰ when the AA composition in prey matches requirements in the predator (Ankjærø et al., 2012). A higher lipid content in prey is also known to be associated with a lower TDF in predators (e.g., Blanke et al., 2017). Even higher TP estimates for cod would likely have been calculated if herring condition status was stable over time. A higher lipid content in recent years for herring may reflect better growth conditions (in contrast to the predator cod) which would result in reduced TDF for herring itself (Martínez del Rio & Wolf, 2005). The increased lipid content in herring in recent decades since the historically low levels during early 1990 (see also Karlson et al., 2020), along with its lower $\delta^{15}N$-values during the same recent time period, may have counteracted expected increases in $\delta^{15}N$-values in cod resulting in a less pronounced inflation in its TP (Figure 1b). Our study shows that prey nutritional value likely invalidates TP estimates higher up the food web.

In addition to prey nutritional value and its own condition-related effects, exposure to hypoxic conditions may have influenced cod trophic AA ^{15}N isotope discrimination and TP-AA estimates. The cod TP-AA trend(s) only reflected the decreasing herring TP-AA in recent decades after adjustment for condition *and* hypoxia. Exposure to hypoxia in the cod in this study (~35 cm) could be a result of summer spawning migrations (up to several months in deep saline but oxygen-poor water; Nielsen et al., 2013). In contrast, pelagic forage fish like herring are not expected to be exposed to hypoxia to the same extent. During hypoxic conditions, oxidative stress with effects on ^{15}N can be expected (Beaulieu et al., 2015). Moreover, exposure to toxic hydrogen sulfide can result in physiological costs from combating toxic exposure (Calow, 1991) and result in increased ^{15}N in trophic AAs (Ek et al., 2018). In addition, indirect effects on cod metabolism can occur due to migration to warmer, shallower water (Claireaux & Chabot, 2016) possibly leading to altered ^{15}N fractionation patterns (Poupin et al., 2014). The correlation between TP estimates and hypoxia was stronger after lipid adjustment of TP (Figure 6) suggesting that both nutritional status of prey and environmental stress influence TDF and hence TP estimates in cod. Importantly, cod $TP_{stomach}$ and AA-TP estimates only showed correlation after adjustments (especially after hypoxia adjustment). Also, the proportion of herring and sprat in diet only correlated with cod TP after adjustment, further demonstrating that our approach of adjusting TP values using environmental data leads to better interpretations of real trophic changes. Together, these findings confirm the need to account for physiological/environmental stress before interpreting TP-AA.

A similar dataset on which to base herring $TP_{stomach}$ does not exist and we can only speculate on the reasons for the observed changes. The sudden increase in herring AA-TP_{all} in the mid-90s may reflect the appearance of the invasive predatory zooplankton *Cercopagis pengoi*, that resulted in nearly one unit increase in TP for young herring compared with the 1980s (estimated from the bulk isotope method; Gorokhova et al., 2005). Decreases in herring in TP in recent years may reflect a diet dominated by lower TP zooplankton, although with no abrupt breakpoint in diet like in the early 1990s. Regarding real changes in cod diet, benthic invertebrates constituted a larger proportion of diet in the 1980s than in the last few decades (although remaining at consistently low levels during the cod deterioration period, Neuenfeldt et al., 2020), indicating that the breakpoint in 1990 for TP_{all} (adjusted and non-adjusted), agreeing with the lowest$TP_{stomach}$ value is reflecting a real diet change preceding the cod deterioration period (and may hence be the ultimate cause of the bad condition). $\delta^{13}C$ values in both fish correlated over time with mussel $\delta^{13}C$ values, demonstrating large-scale patterns in the biogeochemistry of the pelagic ecosystem (Figure 3), for example, reflecting the Suess effect or increased terrestrial carbon inputs, as discussed for Baltic mussels by Liénart et al. (2022). Changes in the biogeochemical $\delta^{15}N$ signal over time were evident for both cod and herring source AA-$\delta^{15}N$ and in the bulk $\delta^{15}N$ of blue mussel baselines. Blooms of N-fixing cyanobacteria are depleted in ^{15}N and this signal is mirrored in the entire Baltic food web after the summer (Karlson et al., 2015). The nonlinear trend in source AA-$\delta^{15}N$ of fish may partly reflect long-term patterns in surface accumulations of cyanobacterial blooms which have increased in recent decades but these were also common in the early 1980s (Kahru & Elmgren, 2014). Another not mutually exclusive explanation for low $\delta^{15}N$ in the beginning of the time series is the generally lower eutrophication status at that time (Savchuk,

2018). The late autumn sampling of mussel baseline 1 may explain why the decrease in $\delta^{15}N$ was harder to detect in these mussels compared to mussel baseline 2 which were sampled in August. Effects of changes at the bottom of the food web on cod physiology would be an interesting topic to explore further using source AAs. It is promising that the results from this study indicate that source AAs are not associated with breakpoints or trends in physiological status of consumers (but see Nuche-Pascual et al., 2021).

The main purpose of this study was not to determine definitive TP values in the study species but to demonstrate how TP is influenced (although in a correlative, time series approach) by factors usually not accounted for in food web studies. As proposed in the three example scenarios in Figure 1, altered condition status can result in incorrect ecological interpretations of TP over time. Therefore, we have not focused on interpreting the absolute TDF values in cod, here estimated from cod–herring differences in $\delta^{15}N$ for each AA, or the absolute TP values in fish. The correlations between TDFs in trophic AAs with lipid content (Table 2) are used as mechanistic support (mid-panel in Figure 1) for the GLM results, prior to performing the adjustments of TP using non-isotope data (lipid content, hypoxia data). However, it is worth highlighting that the TDF proxy for Glu in cod was only about 2‰ while literature values used in our equations are much higher. Similarly, the low TDF estimate for Glu has however previously been found in a number of piscivorous fish and in sharks, and may be related to the high protein content of prey higher up in the food web (Hoen et al., 2014). It is also worth noting that the two AA-based methods for TP calculation result in between-species differences of only about 0.5 units. If one applies a lower enrichment factor of 6.7 instead of 7.6 to the Glu-Phe equation, as recommended by Nielsen et al. (2015), the temporal trends naturally remain identical but the absolute TP estimates increase and approach average values of 4 for cod and 3.6 for herring (see Appendix S1: Figure S4), which are more realistic estimates for cod but not for herring. Bradley et al. (2015) found an even lower enrichment factor of 5.7‰ in their review, which would increase herring TP even more. TP in Baltic fish would also increase if taking into account that the cyanobacterial blooms drive production in the summer in the Baltic (Karlson et al., 2015), meaning a lower offset between source and trophic AAs (β-value) in cyanobacteria compared with other phytoplankton (see e.g., Ramirez et al., 2021) should be used in equations. Experiments manipulating stress exposure could identify which AAs respond to certain stressors (e.g., hypoxia) and which AAs should be included in TP calculations to provide the most reliable (i.e., reflecting true diet) TP estimates. In a recent meta-analysis it was demonstrated that the TDFs of some AAs are higher in brackish water, probably due to osmoregulation, suggesting those AAs should be omitted from TP calculations (Nuche-Pascual et al., 2021).

Because warm water holds less oxygen, it is possible that many other seas, gulfs, and bays may soon experience hypoxic conditions or cyanobacterial blooms similar to the Baltic. And therefore, the potential for stress, physiology and nutrition to affect $\delta^{15}N$ will be even more important in the future. Using complementary techniques such as otolith chemistry (Limburg & Casini, 2019) or enzymatic biomarkers would help to test the extent of physiological stress and/or hypoxia or other environmental stress such as contaminant exposure. Takizawa et al. (2020) recently proposed that $\delta^{13}C$ values of individual AAs mirror the pathways of de novo synthesis, hence a combined analysis of $\delta^{15}N$ and $\delta^{13}C$ of individual AAs will better reflect the metabolic status of consumers and will ultimately improve estimates of mass and energy transfers at the ecosystem level (Goko et al., 2018).

To conclude, care should be taken when reconstructing food webs based on $\delta^{15}N$ values in AAs if there is no information about consumer condition status and prey $\delta^{15}N$ and nutritional value. This study suggests an approach of adjusting the TP values in a target fish using other environmental data partly overcomes the issues of unknown and variable TDF values in the field. Specifically, it demonstrates the importance of including physiological status metrics, not only in prey as proxies of diet quality/nutritional status but also in the predators themselves, as well as information about environmental conditions, to avoid erroneous interpretations of TP in food web studies.

AUTHOR CONTRIBUTIONS

Agnes M. L. Karlson initiated the study, acquired funding, and wrote the first draft of the paper. Caroline Ek and Douglas Jones contributed equally to the study by commenting on the study approach and writing. All authors conducted statistical analyses.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data (Karlson et al., 2024) are available from Figshare: https://doi.org/10.6084/m9.figshare.24147900.v1.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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