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Cover image: Woman collecting ascideans at Ponta Mazondue, Inhaca Island, Mozambique (© José Paula, 2005)

Diet dynamics and feeding strategies of *Hilsa kelee* (Cuvier, 1829) and *Valamugil buchanani* (Bleeker, 1853) in the Pangani Estuary, Tanzania: Insights from stomach contents and fatty acid biomarkers

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Abstract

Stomach contents and fatty acid (FA) biomarkers were used to investigate the diet and feeding strategies of the marine fish species *Hilsa kelee* and *Valamugil buchanani*, across three Pangani estuarine zones (about 7 km apart). The three zones depict heterogeneous distribution of trophic resources along the longitudinal estuary gradient. Despite ecological index and FA trophic niche widths indicating high overlap of trophic resources, the permutational multivariate analysis of variance revealed significant interspecific variations in diet and FA compositions. This highlights the importance of using statistically supported tools when drawing inferences on the diet dynamics of estuarine fish. The dominant diatom and detritus diets reflected the high herbivory C22:6(n-3):C20:5(n-3) and omnivory C18:1(n-9):C18:1(n-7) FA trophic indices of *V. buchanani*. The diatoms and copepods dominating the diet of *H. kelee* concurred with the species' higher carnivory C22:6(n-3):C20:5(n-3) and lower omnivory C18:1(n-9):C18:1(n-7) FA biomarkers. The diet niche breadth index, the Amundsen feeding strategy diagrams, and the FA trophic niche suggest that *V. buchanani* exhibits a generalist feeding strategy, and *H. kelee* a mixed feeding strategy that is intermediate between a specialist and a generalist. Due to these differences, it is suggested that *V. buchanani* demonstrates a higher degree of resilience to anthropogenic disturbances than *H. kelee* in the Pangani estuary.

Keywords: Fish, diet, fatty acid profiles.

Introduction

Estuaries are characterized by variations in primary food sources due to temporal fluctuations of river discharge and tidal seawater flow. The myriad of primary food sources derived from autochthonous and allochthonous sources contribute to high estuarine productivity (McLusky and Elliott, 2004; Antonio *et al*., 2012). Such frequent fluctuations in food sources and physico-chemical parameters allow fish assemblages in estuaries to demonstrate different feeding strategies for satisfying their nutritional requirements (Elliott *et al*., 2002). Specifically, the mangrove-fringed estuaries, apart from demonstrating diverse and abundant food resources, provide refuge areas to juvenile marine fish species (Igulu *et al.,* 2014). Due to this, many marine fish species live in estuaries at different stages of their life cycles (Mbande *et al.,* 2005). The ecological status of many estuaries has been modified by anthropological activities and the food webs that link estuarine and coastal ecosystem boundaries have been impaired (Pasquaud *et al*., 2010; Selleslagh *et al.,* 2012). There is a compelling need to understand the functions and ecological roles of individual estuarine systems in relation to the species with

estuarine-ocean connectivity for maintaining healthy coastal ecosystems. An essential step towards achieving this is to study the dietary niches and the feeding strategy of marine fish in estuaries. This helps to enhance knowledge on how different fish species with related trophic requirements co-exist and contribute to the resilience of estuarine food webs.

Numerous studies have reported that most estuarine fishes demonstrate omnivorous and opportunistic feeding strategies at all sizes; a characteristic which enables them to consume the variable food resources in a dynamic environment (Cabral, 2000; Lobry *et al.,* 2008). However, there is limited knowledge on the diet and feeding strategies of marine fish at various life history stages in Tanzania estuarine systems. Studies on the diet and feeding strategies of marine fish species in estuaries are essential, especially when methods are used reveal their long term feeding history. Lança *et al.* (2013) was able to better explain the long term feeding strategy of sea lampreys on the Iberian coast of the Atlantic Ocean by using fatty acid (FA) biomarkers. Studying the diet and feeding strategy of fish by solely relying on stomach content analysis not only represents the static situation, but also neglects temporal and spatial estuarine diet dynamics (Antonio *et al.,* 2012). Fatty acid trophic markers are frequently used in studying the feeding ecology of fish because they tend to accumulate in adipose tissues over a long period through dietary intake (Maranto *et al*., 2011). These FA biomarkers produced by the primary producers are usually transferred unchanged higher up the food chain (Dalsgaard *et al.,* 2003; Parrish, 2013). In the fresh water environment, where the fish species cannot readily accumulate some FA biomarkers such as C20:*5*(n-3) and C22:6(n-3), they biosynthesize them by modifying other dietary FAs, mainly C18:2(n-6) and C18:3(n-3). Similarly, the FA biomarkers are used to infer habitat boundary connectivity since some FA are produced by habitat-specific primary producers. Among others, these include terrestrial plant FA biomarkers, mainly C18:2(n-6) and C18:3(n-3) (Meziane *et al.,* 2007), and marine micro-algae derived FA biomarkers such as C20:*5*(n-3) and C22:6(n-3) (Dalsgaard *et al.,* 2003). These attributes justify the role of FA trophic markers in understanding the quality of each diet item and the mechanisms employed by the species to acquire such food sources.

The filter feeder *Hilsa kelee* (clupeidae) and phytodetritivorous *Valamugil buchanani* (mugilidae) are among the most abundant marine fish caught for the local market and consumption by the local fishing community in the Pangani estuary. These two marine fish species use estuaries as nursery and feeding grounds during the juvenile stages. The abundance of *H. kelee* in estuaries increases during spawning periods (September-February and June) (Gjosaeter and Sousa, 1983). *Valamugil buchanani* spends most of its life in estuaries and inshore waters, and migrate to marine waters at sexual maturity for spawning (Rajesh *et al*., 2014). The trophic resources of both species include micro-algae, small fauna and other organic particles (Reintjes, 1974; Wijeyaratne and Costa, 1990). The two species are likely to share basal trophic resources, in particular in shallow and macro-tidal estuaries like the Pangani, where the mixing of trophic resources within the water column is common (Sotthewes, 2008; Pamba *et al*., 2016). There is a need to further explore how and to what extent the two species partition the trophic resource base, and their level of opportunism in the utilization of estuarine trophic resources.

The present paper reports on the diet, dietary niche overlap, and feeding strategies of *H. kelee* and *V. buchanani* from the Pangani estuary, studied by using stomach content analysis and FA biomarkers. Specifically, the objectives of this study were to: (a) describe the diets and feeding niches of *H. kelee* and *V. buchanani* in the Pangani estuary; and (b) analyze fish feeding strategies by using their dietary and FA compositions. It was hypothesized that the dietary and FA biomarkers between the two species, and during different ontogenic stages were significantly different across the longitudinal gradient of the estuary. Due to the differences in foraging mode of these species, it was expected that *V. buchanani* would exhibit higher opportunistic omnivory indices and feeding niche areas than *H. kelee* in the Pangani estuary. The findings of the present study provide baseline information that will be useful for improving the management strategies for the rapidly changing Pangani estuarine system.

Materials and methods

Study area

The present study was carried out in the funnel-shaped and permanently open Pangani estuary (38° 50˝E, 5° 20˝S and 39°E, 5° 26˝S) in the northern part of the Tanzanian coast (Mwanukuzi, 1993). It is a macro-tidal estuary of semi-diurnal type with a mean amplitude of about 3.5 m at spring tides and 3.0 during neap tides (Pamba *et al*., 2016). Average depth is about 5 m (Sotthewes, 2008). The present study was conducted within 16 km of the Pangani estuary mouth.

The estuary is fringed with mangrove forest stretching to about 11 km from the river mouth (PWBO/IUCN, 2008). Sampling was based within three predetermined zones designated as upper, middle and lower estuarine zones (Fig. 1), located in the longitudinal salinity gradient. The lower estuarine zone was located 3 km inland from the estuary mouth (salinity range 20-35 ppt), the middle zone was located 10 km from the estuary mouth (salinity range 7 – 16 ppt), and the upper portion of the estuary was situated at about 16 km from the estuary mouth (salinity range 1 - 6 ppt).

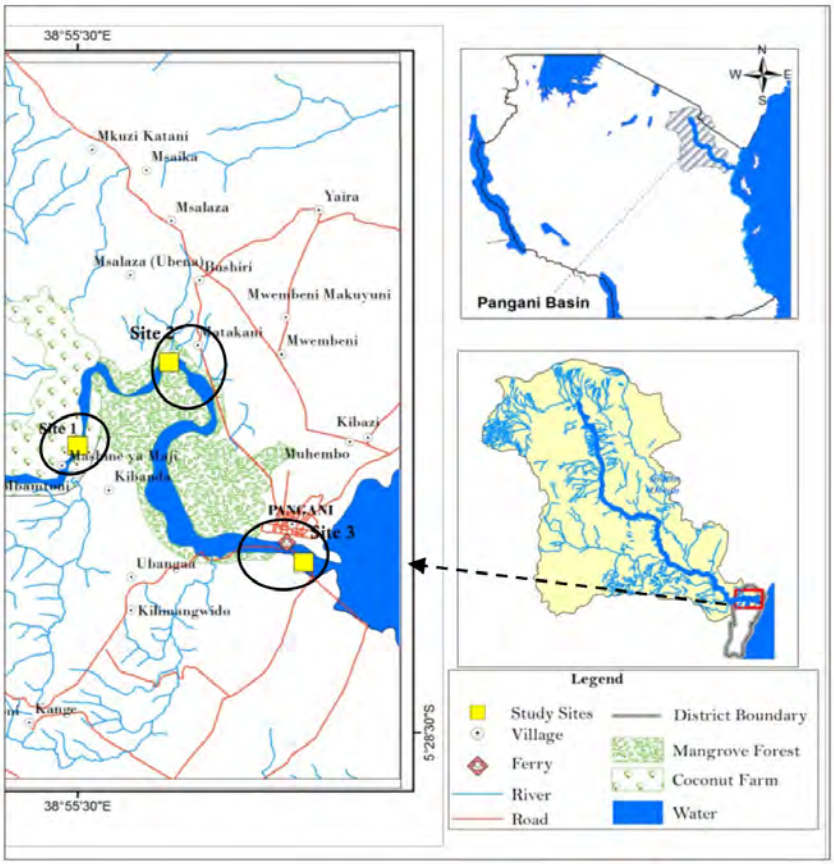

Figure 1. Map of the Pangani estuary indicating upper (site 1), middle (site 2) and lower (site 3) sampling zones (in oval shapes) along the longitudinal salinity gradient.

Collection of fish samples and analysis of diet composition

The fish species *H. kelee* and *V. buchanani* were sampled in October, 2014, and January, March, May and July, 2015 in the estuary. For each sampling period, fish were collected in daylight using seine nets with the dimension of 15m length, 1.5m width and mesh size of 0.5mm. After retrieval of the nets, all the species of interest were selected and thereafter stored in a cool box with dry ice. Later on during the evening, wet weight (nearest 0.1g) and total length (nearest 0.1cm) were recorded, and fish stomachs were removed and frozen at -20°C. At the end of each sampling period, frozen samples were transported to the laboratory at the University of Dar es Salaam, College of Agricultural Sciences and Fisheries Technology. In the laboratory, the stomach contents were examined at 200x and 400x magnification using an Olympus inverted microscope. Benthic and planktonic organic food particles were identified using available keys (Crosby and Wood, 1958; Utermohl, 1958; Mwaluma *et al.*, 2014). The frequency of occurrence (%F) and percentage volumetric contributions of food item to the total stomach volume (%V) were then determined by using the point method described by Hyslop (1980). This point semi-quantitative method was selected due to the nature of food items found in the stomachs of both species. The scale points representing the volume occupied by each dietary category used included 1, 2, 4, 8 and 16, with 1 representing the minimum amount and 16 representing the maximum amount. All food items in the stomachs were identified to the lowest taxon possible and then grouped into broader taxonomic categories

for further data analysis. Non-food items like sand, gill epithelia and unidentified materials were excluded in further steps of data analysis.

Feeding strategy and estimation of dietary indices

The feeding strategy at individual and population levels for each species was evaluated by adopting the graphical method described by Amundsen *et al*. (1996). This two-dimensional graph is defined by the percentage frequency of occurrence (%F) of diet categories on the x-axis, and the percentage of prey-specific abundance, biomass, or volume of prey i ($\%P_i$), on the y-axis. The %F was obtained using the formula Ni/N x 100 where: Ni = number of stomachs containing food item *i*, and N = total number of stomachs with food. The percentage of prey (any food item found in the stomach) specific volume ($\%P_i$) was expressed as:

$$\%Pi = \left(\frac{\sum Bi}{\sum Bti}\right) \text{ x } 100$$

Where %P*i* is the percentage of prey-specific volume of prey *i*, ∑B*i* is the volume of food item *i* in all stomachs of the species, and B*ti* is the total volume of all food items only in stomachs with food item *i*. As described by Amundsen *et al.* (1996), the prey importance, feeding strategy, and niche width were obtained by examining the distribution of data points of food items along the diagonals and the axes of the graph.

The Levin's standardized equation for food niche as described by Krebs (1999) was used to estimate the dietary niche breadth of each species in each estuarine zone. The dietary volume data were used to calculate the trophic niche breadth of each species. The Levin's standardized equation is expressed as:

$$B_A = \frac{(\sum_j P_{ij}^2)^{-1} - 1)}{(n-1)^{-1}}$$

Where: B_A is the standardized trophic niche breadth, P_{ij} is the proportion of food category *j* in the diet of the species *i* and, *n* = total number of food items in the diet of species i. The B_A values range between 0 and +1. Values closer to 0 indicate a narrow food niche (specialist), while values closer to +1 imply a broad diet niche (generalist).

Species dietary overlap (*O*) for the two species in each estuarine zone was calculated by using Pianka's index of niche overlap (Pianka, 1973). It was assumed that the different dietary resources were equally accessible to both species. The following dietary overlap formula used was:

$$O = \frac{\sum P_{ij}\, P_{ik}}{\sqrt{\sum P_{ij}^2 \sum P_{ik}^2}}$$

Where P_{ij} and P_{ik} are the proportions of diet category *i* comprised in the diets of *j* (*H. kelee*) and *k* (*V. buchanani*), respectively. According to Sá-oliveira *et al*. (2014), the diet overlap between species value is low when the value falls within the 0 - 0.39 range, intermediate when it is within the 0.4- 0.6, and high when its range is in between 0.61 - 1.

Lipid sample preparation, extraction, and fatty acid analysis

Fish samples for lipid extraction and FA analysis were limited to the upper and lower sampling zones, as well as to two sampling periods conducted in March and July 2015. This took into consideration the longitudinal movement of the species, the efficiency of the method which reveals long-term dietary dynamics, and also the budget. Fish FA data for the two sampling periods were pooled together due to less pronounced freshwater flow occurring in March and July in the estuary (Selemani *et al*., 2017). These samples were selected after their weights and lengths had been recorded and stomachs removed for stomach content analysis. Seventy fish samples of both species (34 samples from upper zone and 36 samples from lower zone) were subjected to FA analysis. The flesh from the dorsal white muscle of the individual fish was cut, skin removed, washed with distilled water, freeze-dried and kept in dry glass vials in desiccators until FA analysis. The samples were later transported to the State Key Laboratory of Estuarine and Coastal Research (SKLEC) of the East China Normal University (ECNU) in China, for FA analysis.

Lipid extraction was performed following the reduced-solvent method described by Folch *et al*. (1957). 100 mg of each freeze-dried sample was mixed with 2 µg of C21:0 FA (Fluka) as the recovery of internal standard. This was followed by the addition of 15 ml of a mixed solution of dichloromethane and methanol (v:v = 2:1), and the antioxidant BHT (butylhydroxytoluene; 0.01%). The mixture was then extracted in the CEM – Mars microwave (USA), cooled and centrifuged at 3000 rpm at 4 °C for 10 minutes. The upper phase was collected in clean glass tubes and the residue re-extracted twice. All extracts were pooled together and evaporated to 0.5 ml before drying under a stream of nitrogen to obtain the total lipid.

The FA methyl esters (FAMEs) were prepared from total lipid extracts. The total lipid was treated with 2 ml n-hexane and 4 ml methanol (containing 5% HCl) in 20 ml glass tubes, and heated at 50°C for 12 hours. After cooling in water, 1 ml Milli-Q water was added and the resulting FA methyl esters (FAMEs) were extracted three times by using 4 ml of n-hexane. The total FAME extracted were analyzed and quantified by using a gas chromatograph system equipped with a flame ionization detector and an autosampler (Agilent 6890A series GC-FID), and a silica capillary column (30 m length, 0.32 mm inner diameter, 0.25 µm film thickness; Supelco). Helium was used as the carrier gas. The programmable temperature vaporizer injector was applied for the standards (FAME Mix, Supelco, USA) and for the sample injection exercise. For each sample, the different FAMEs composition were identified by comparing their specific retention times with those from standards of known composition. The retention times and the peak area were recorded and used to quantify the relative percentage of each FA to the total fatty acids (FAs) identified in a sample. All FAs were expressed as a percentage of total FAs. The FA biomarkers and feeding strategy indices applied were referred from Graeve *et al.* (1997), Dalsgaard *et al.* (2003), Gatune *et al.* (2012), Kelly and Scheibling (2012), Lança *et al*. (2013), and Parrish (2013).

Statistical analysis of diet and fatty acid composition data

Non-parametric multivariate and parametric univariate analyses were used to examine the level of similarity and to test for intra- and inter-specific variations in dietary and FA composition among estuarine zones. The multivariate analyses of dietary and FA data were performed by using PRIMER6 (Clarke and Gorley, 2006) and its PERMANOVA+ software packages (Anderson *et al.,* 2008). By using a two factorial design (fish species and estuarine zone factors) permutational multivariate analysis of variance (PERMANOVA), the study revealed the degree of variations in dietary and FA composition for the fish species. Square roots of estimated variance components obtained when running PERMANOVA were used to assess the contribution of each factor to overall variations in the tests. The non-metric multidimensional scaling ordinations (nMDS) were used to visually assess the feeding patterns of the species as well as the variations in FA composition of each species between the estuarine zones.

The pair-wise PERMANOVA tests were run in order to assess the variations in diet and FA profiles of individual species across the three estuarine zones. In addition to that, one-way PERMANOVA was performed to evaluate the level of variation in diet and FA composition by the size of each fish species in each sampling zone of the estuary. Different length groups with the size-class interval of 4 cm total length were used to determine diet differences by fish size, depending on the availability of enough samples for stomach content analyses. Due to the limited FA data in all length groups, two size classes (12 -15 cm and 20-23 cm total length for *V. buchanani*; and 12-15 cm and 16-19 cm total length for *H. kelee*) were used to explore the pattern of FA compositions by fish sizes. All PERMANOVA tests and nMDS were run based on the Bray-Curtis similarity matrices of square root transformed dietary, and untransformed FA composition data. Similarity percentage (SIMPER) was run based on the dietary and FA composition data in order to identify the main food items and FAs responsible for differences detected by PERMANONA.

Stable Isotope Bayesian Ellipses in R (SIBER) package was applied to assess the feeding niches by using FAs. That is, the FA profiles of each fish species were used in the SIBER package to examine the species trophic niches in space, as a proxy of isotopic niche space. The x and y coordinates of the nMDS analysis of untransformed FA data for each fish species were used. The data points identified as outliers in the FA profile of each species were put aside when developing the nMDS plot. The nMDS analysis in each zone was done using the vegan package under the function "monoMDS" in the R statistical working environment. Then, according to the concept of the SIBER package (Jackson *et al*., 2011; Layman *et al*., 2011), FA niche widths were presented by the standard ellipse areas comprising the maximum percentage of possible datasets along the convex hulls. Thereafter, the differences in areas of the ellipses (FA niche width) between the species were tested using one-way ANOVA.

Results

Diet compositions

In total, 185 stomachs of *H. kelee* and 196 stomachs of *V. buchanani* were found to contain food, while 49 *H. kelee* and 37 *V. buchanani* stomachs were empty. Among stomachs containing food, 25.4% for *H. kelee* and 13.8% for *V. buchanani* comprised unidentifiable food items (Table 1). The size of *H. kelee* analyzed ranged from 8 to 21 cm (total length) whereas that of *V. buchanani* ranged from 8.5 to 35.2 cm (total length). The diet of *H. kelee* was largely dominated by diatoms

Table 1. Percentage frequency of occurrence (% F) and mean percentage volumetric contribution (% V) of diet categories found in stomachs of *Hilsa kelee* and *Valamugil buchanani* from Pangani estuary.

Major dietary categories	Upper estuary				Middle Estuary				Lower Estuary			
	H. kelee		*V. buchanani*		*H. kelee*		*V. buchanani*		*H. kelee*		*V. buchanani*	
	%F	%V	%F	%V	%F	%V	%F	%V	%F	%V	%F	%V
Diatoms	96.77	42.43	97.37	27.30	96.91	42.51	97.01	26.89	96.49	46.64	98.90	30.47
Dinoflagellates	22.58	1.92	18.42	0.66	16.49	1.28	17.91	0.76	14.04	1.83	18.68	0.82
Filamentous and other green algae	35.48	5.05	15.79	0.76	22.68	2.07	16.42	1.01	8.77	1	41.76	3.83
Cyanobacteria	11.18	0.48	10.53	0.72	10.31	0.91	25.37	2.31	3.51	0.27	62.64	3.36
Copepods	61.29	13.24	10.53	0.82	37.11	4.96	10.45	0.94	35.09	5.75	4.40	0.18
Fish larvae	3.23	0.97	-	-	5.15	1.24	-	-	8.77	1.67	-	-
Nauplii copepod larvae	12.90	1.5	10.53	0.84	14.43	1.44	7.46	0.39	14.04	1.57	-	-
Other crustacean larvae	22.58	4.18	15.79	1.53	12.37	2.45	2.99	0.22	7.02	0.93	1.10	0.06
Fish eggs and other unidentified eggs	16.13	1.55	-	-	15.46	1.63	-	-	14.04	1.7	-	-
Plant vascular tissues	-	-	34.21	7.24	-	-	31.34	6.63	-	-	60.44	7.40
Detritus	48.39	12.27	86.84	31.82	82.47	26.18	83.58	30.97	77.19	28.07	91.21	28.81
Gill epithelia	-	-	21.05	1.34	-	-	13.43	1.36	-	-	35.16	4.03
Sand/sediment	51.61	15.29	94.74	26.97	42.27	12.23	94.03	27.96	29.82	6.54	73.63	19.21
Unidentified materials	9.68	1.13	0	0	24.74	3.09	5.97	0.55	29.82	4.03	25.27	1.63
Total - empty stomach (%)	6 (16.22)		5 (11.63)		9 (8.49)		21 (23.86)		34 (37.36)		11 (10.78)	
Total - stomach with food (%)	31 (83.78)		38 (88.37)		97 (91.51)		67 (76.14)		57 (62.64)		91 (89.22)	

(percentage volume (%V) 42.4 - 46.6 %V), detritus (12.3 – 28.1%V) and copepods (5.0 to 13.2 %V) in all three estuarine zones (Table 1). The dominant food items in the diet of *V. buchanani* were detritus (28.8 – 31.8%V), diatoms (26.8 - 30.5 %V) and decaying vascular plant tissue (6.6 - 7.4 %V) in all estuarine zones (Table 1).

The two way-PERMANOVA test revealed significant differences in dietary composition between species and estuarine zones (two factors, species and zones), and the interaction between the main factors (pseudo-$F \geq 4.15$; $p = 0.001$) (Table 2). However, the non-metric MDS ordination depicted the marginal differences in dietary compositions of the two fish species in all three estuarine zones (Fig. 2). This was indicated by the unclear pattern and overlaps of some dietary data points for both species in the ordination space. According to the similarity percentage (SIMPER) routine, the average dissimilarity in diet of the two species were about 50.3% in the upper zone, 39.9% in the middle zone, and 41.7% in lower zone of the estuary. Such dissimilarity mainly resulted from different amount of detritus, diatoms, copepods, and decomposing vascular tissue present in the stomachs of both *H. kelee* and *V. buchanani*. The PERMANOVA test also showed that the factor of fish species explained twice as much of the variations relative to the interactions between the species and estuarine zone factors. The estuarine zone factor contributed the lowest variability in diet when compared with other factors (Table 2).

Table 2. Two-ways PERMANOVA of dietary composition in *Hilsa kelee* and *Valamugil buchanani* in upper, middle and lower zones of Pangani estuary. Note: df = degrees of freedom, pseudo –F = pseudo-F ratios, p = significance level and CoV = square root component of variation.

Source of variation	df	Mean square	Pseudo-F	*p*	CoV
Main factor					
Fish species	1	43748.00	57.29	0.001	16.38
Estuarine zone	2	3167.80	4.15	0.001	4.55
Two-way interactions					
Fish species Vs zone	2	5303.30	6.95	0.001	8.84
Residual	375	763.65			27.63
Total	380				

The pair-wise PERMANOVA tests and non-metric MDS ordination further confirmed estuarine zone to be a weak factor. Both analyses indicated that there were slight intra-specific differences in diet across the upper, middle and lower sampling zones (Table 3, Fig. 2). The non-metric MDS ordination indicated less intra-specific dissimilarity in diet of both species among the estuarine zones as the data points were close and only slightly distinguishable. The diets by size for *H. kelee* were slightly different in the upper (one-way PERMANOVA, pseudo-$F_{2, 28}$ = 4.93, p = 0.001) and lower (pseudo-$F_{2, 54}$ = 6.44 p = 0.001) estuarine zones only. With regard to the diet of different sizes of *V. buchanani,* the variations were marginally detected in the samples caught from the upper estuarine zone (pseudo-$F_{4, 33}$ = 2.67, p = 0.01).

The Pianka's index measure of diet niche overlap between *H. kelee* and *V. buchanani* were very high and agreed with the findings portrayed in the dietary non-metric MDS ordination. The diet overlap was extremely high in the lower portion of the estuary (O = 0.95), followed by the middle zone (O = 0.94), and lastly the upper estuarine zone (O = 0.79). The overall dietary niche breadth of *H. kelee* was relatively low compared to that of *V. buchanani* in the three zones of the estuary. The dietary niche breadth of *H. kelee* was slightly higher in the upper estuary (B_A = 0.40) and decreased gradually in the middle (B_A = 0.33) and lower (B_A = 0.26) estuarine zones. In contrast, the dietary niche breadth of *V. buchanani* progressively increased from the upper zone (B_A = 0.56) via the middle zone (B_A = 0.60), and eventually to the lower estuarine zone (B_A = 0.63).

Feeding strategies in relation to fish diet

The results of the study on feeding strategy of fish species indicated that diatoms had a food item-specific volume above 50% and the highest frequency of occurrence in the diet of *H. kelee* (Fig. 3a-c). Detritus

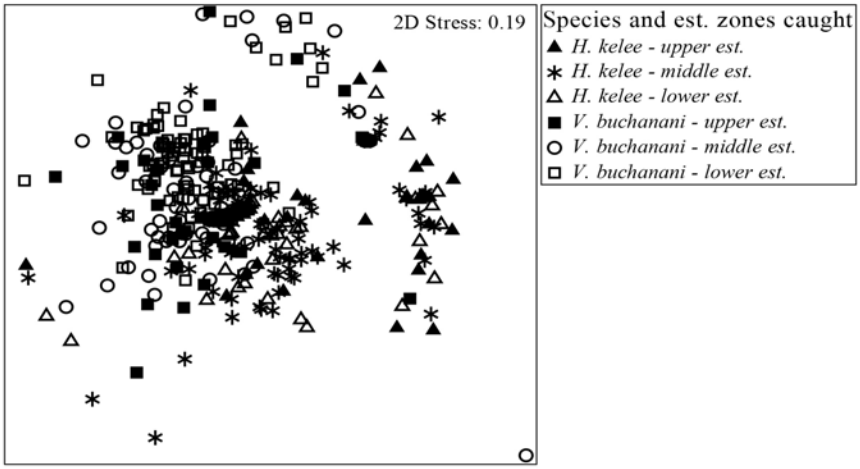

Figure 2. Non-metric multi dimension scaling ordination (nMDS) plot showing the dietary composition pattern of *H. kelee* and *V. buchanani* in upper, middle and lower zones of Pangani Estuary

Table 3. Pair-wise PERMANOVA tests comparing the feeding patterns of *H. kelee* and *V. buchanani* at estuarine zonal scale in Pangani estuary.

	H. kelee			*V. buchanani*		
Estuarine zone comparisons	**Den. df**	**t**	***p*(perm)**	**Den. df**	**t**	***p*(perm)**
Upper and middle zone	126	2.99	0.001	103	0.61	0.772
Upper and lower zone	86	2.83	0.001	127	2.92	0.001
Middle and lower zone	152	1.09	0.33	156	2.77	0.001

Significance threshold at p (perm) = 0.05; Den. df = denominator degrees of freedom.

and copepod dietary categories of *H. kelee* indicated moderate to high frequency of occurrence, and below 50% food item-specific volume. This verified the generalist nature of the population with moderate diet niche width contributed by mostly generalist individuals (high within-phenotype component). *V. buchanani* showed an even more generalized feeding strategy and a broader diet niche width contributed to by most individuals in the population feeding on diatoms and detritus (higher within-phenotype component) (Fig. 3a-c). That is, the diet of *V. buchanani* comprised all dietary categories with food item-specific volume ≤ 50% and some, such as diatoms and detritus, with highest frequency of occurrence in all three estuarine zones. Furthermore, the population of *V. buchanani* displayed a generalist feeding strategy with about half of the individuals utilizing decaying vascular tissues, cyanobacteria and green algae in the lower estuarine zone.

Fatty acid composition in *H. kelee* and *V. buchanani*

The total length of the fish specimens utilised in FA analysis ranged from 12-19 cm for *H. kelee*, and 12-23 cm for *V. buchanani*. Twenty eight FAs were detected in these two species from the upper and lower estuarine zones. The FA profile of *H. kelee* comprised about 48.2%, 49.4% saturated FAs (SFAs), 21.7%, 22.2% monounsaturated FAs (MUFAs), and 29.7%, 28.2% polyunsaturated FAs (PUFAs) in the upper and lower estuarine zones respectively. In contrast, *V. buchanani* showed lower levels of SFAs (45.0%, 45.5%), higher levels of MUFAs (24.7%, 25.8%) and PUFAs (30.2%, 28.6%) in the upper and lower portions of the estuary (Table 4). The PERMANOVA tests showed the differences in the FA present in the two species (pseudo- $F_{1, 66}$ = 36.42; p = 0.001) and between the sampling zones (pseudo-$F_{1, 66}$ = 4.61, p = 0.002). However, the same test suggested an absence of interaction between the factors, species and estuarine zones (pseudo- $F_{1, 66}$ = 1.54; p > 0.05). The nMDS support the PERMANOVA results to some extent because the data points of FA for the two species were at least distinguishable in both sampling zones (Fig. 4). Similarly, this PERMANONA test showed that the FA profiles of the individual species were the main determinant of variation (square root component of variation = 8.53), followed by estuarine zone factor (2.72), and lastly the interaction between the species and estuarine zone (1.49). According to SIMPER, DHA, C16:0, EPA, C16:1, C18:1(n-9), ARA and C18:0 were the major FAs responsible for the distinction of the FA profile of *H. kelee* and that of *V. buchanani*.

The results of the FA biomarker analysis indicated that *V. buchanani* exhibited elevated levels of the diatom FA marker, EPA, in the upper (11.2%) and lower (12.2%) estuarine zones when compared to the level of EPA shown by *H. kelee* in the respective zones (9.3%, 9.1%) (Table 4). The proportion of dinoflagellate FA biomarker, DHA, in *H. kelee* (11.6%, 11.7%) was about twice as much as that found in *V. buchanani* (6.13%, 6.7 %) in the upper and lower zones respectively. Due to this, the DHA:EPA ratio was higher in *H. kelee* (1.3) than that in *V. buchanani* (0.6) in both sampling zones (Table 4). The terrestrial input FA biomarkers, linoleic acid (LIN) plus alpha linolenic acid (ALA) were more elevated in *V. buchanani* in comparison to that of *H. kelee* from both estuarine zones (Table 4). Also, the levels of LIN plus ALA were more elevated in *V. buchanani* caught from the upper portion of the estuary (3.8%) than those collected in the lower estuarine zones (1.7%) (Table 4). The n-3: n-6 PUFA ratio of *H. kelee* (6.8, 8.8) and *V. buchanani* (3.5, 6.2) in corresponding sampling zones were also different (Table 4). Similarly, the FA index markers of foraging mode, and thus feeding strategy, C18:1(n-9):C18:1(n-7), was relatively lower in *H. kelee* (4.50, 4.25) compared to that of *V. buchanani* (7.0, 5.2) from the upper and lower sampling zones, respectively. The levels of bacterial FA biomarkers between the two species were more or less similar (Table 4). Additionally, the copepod marker, C20:1(n-9), was relatively higher in *H. kelee* when compared to that of *V. buchanani*.

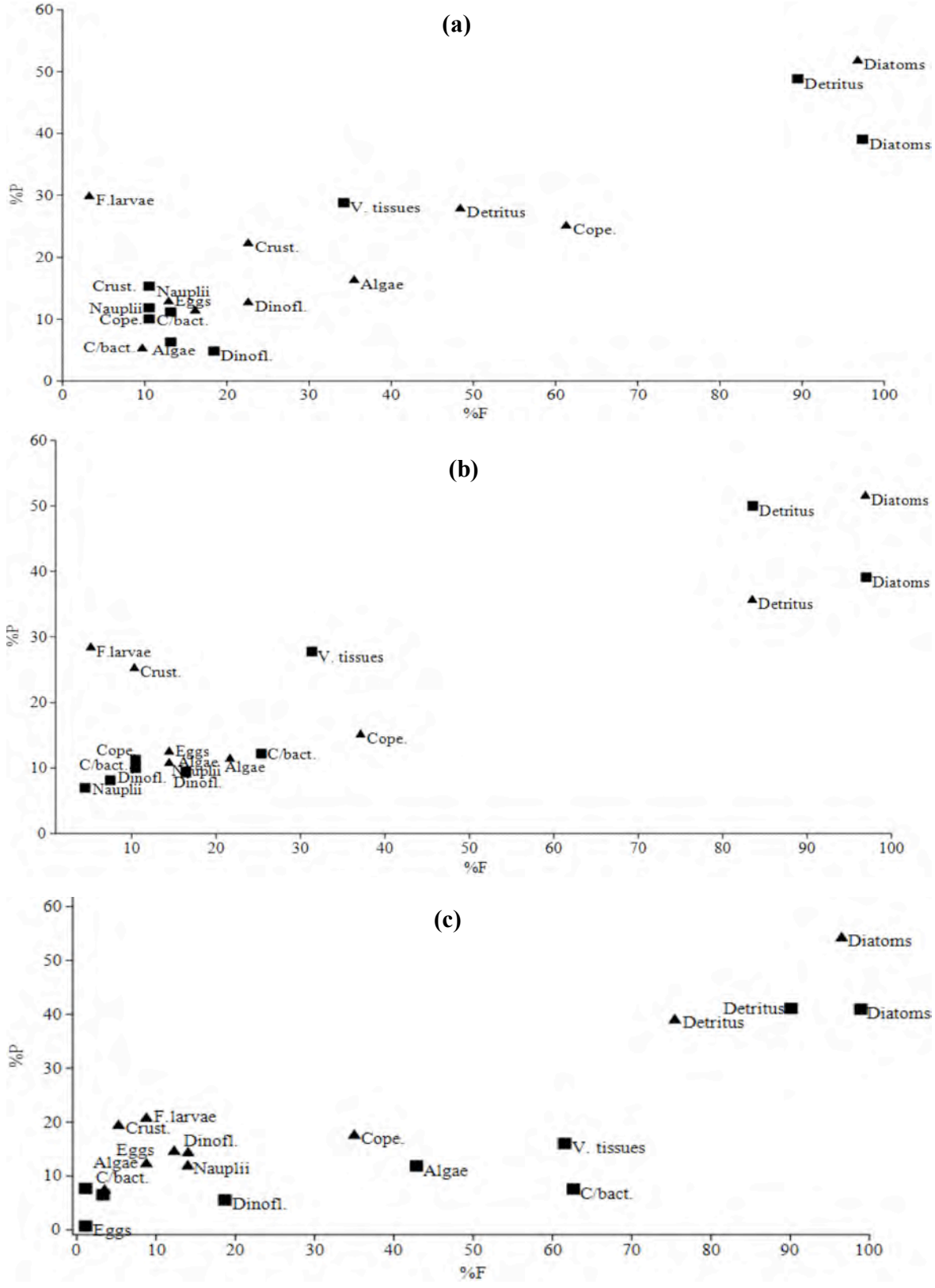

Figure 3. Feeding strategy diagrams for *H. kelee* (filled triangle data points) and *V. buchanani* (filled square data points) in (a) upper (b) middle and (c) lower zones of Pangani estuary. The percentage of food item-specific volume (%P) plotted against frequency of occurrence (%F) of dietary categories found in the stomachs of fish. Legend: Dinofl = dinoflagellates; Algae = green filamentous algae; c/bacteria = cyanobacteria and other bacteria; V. tissue = decaying vascular plant tissues; Cope. = copepod; F.larvae = fish larvae; Crust. = other crustacean larvae; Eggs = fish eggs and other unidentified eggs.

Table 4. The composition of fatty acids (mean % of total FAs ± standard deviation) in *Hilsa kelee* and *Valamugil buchanani* from Pangani estuary.

Fatty acid	Upper estuary		Lower estuary	
	H. kelee (n = 17)	*V. buchanani* (n = 17)	*H. kelee* (n = 18)	*V. buchanani* (n = 18)
C12:0	0.21 ± 0.13	0.18 ± 0.24	0.14 ± 0.09	0.08 ± 0.06
C14:0	6.41 ± 0.99	6.55 ± 0.85	6.32 ± 1.53	6.77 ± 1.30
C15:0[b]	1.49 ± 0.79	1.40 ± 0.83	1.18 ± 0.40	1.86 ± 1.05
C16:0	30.27 ± 2.28	27.0 ± 2.09	31.87 ± 2.63	27.38 ± 2.73
C17:0[b]	0.79 ± 0.20	0.96 ± 0.55	0.66 ± 0.15	0.95 ± 0.70
C18:0	8.24 ± 1.35	8.17± 1.22	8.35 ± 1.51	7.63 ± 1.36
C20:0	0.29 ± 0.17	0.32 ± 0.24	0.34 ± 0.14	0.23 ± 0.17
C22:0	0.24 ± 0.10	0.34 ± 0.19	0.29 ± 0.09	0.34 ± 0.16
C24:0	0.31 ± 0.15	0.13 ± 0.14	0.28 ± 0.14	0.23 ± 0.22
C14:1	0.09 ± 0.15	0.09 ± 0.06	0.02 ± 0.03	0.10 ± 0.12
C16:1	5.27 ± 0.86	7.78 ± 1.09	5.62 ± 1.90	7.87 ± 1.86
C17:1	1.36 ± 0.68	2.03 ± 1.10	1.71 ± 0.86	2.27 ± 0.89
C16:1(n-7)	0.17 ± 0.12	1.10 ± 0.35	0.05 ± 0.19	0.03 ± 0.05
C18:1(n-7) (VA)[b]	2.46 ± 0.86	1.87 ± 0.95	2.90 ± 0.97	2.73 ± 1.03
C18:1(n-9) (OA)[ci]	9.55 ± 0.83	10.74 ± 1.36	9.38 ± 1.11	11.19± 2.39
C20:1(n-9)[c]	2.19 ± 1.09	0.41 ± 0.40	1.97 ± 0.98	0.88 ± 0.56
C22:1(n-9)	0.28 ± 0.21	0.33 ± 0.19	0.30 ± 0.11	0.36 ± 0.22
C24:1(n-9)	0.35 ± 0.23	0.30 ± 0.26	0.20 ± 0.10	0.35 ± 0.17
C20:2	0.29 ± 0.17	0.30 ± 0.22	0.18 ± 0.15	0.27 ± 0.17
C18:2(n-6) (LIN)[t]	1.61 ± 0.77	2.02 ± 0.87	1.07 ± 0.55	0.76 ± 0.27
C18:3(n-3) (ALA)[t]	0.98 ± 0.53	1.74 ± 0.92	0.76 ± 0.60	0.90 ± 0.85
C18:3(n-6)	0.14 ± 0.16	0.55 ± 0.24	0.08 ± 0.01	0.18 ± 0.18
C20:3(n-6)	0.40 ± 0.22	0.57 ± 0.30	0.25 ± 0.16	0.23 ± 0.18
C18:4(n-3)	0.49 ± 0.30	0.76 ± 0.39	0.52 ± 0.26	1.74 ± 1.03
C20:4(n-6) (ARA)[be]	2.08 ± 1.02	3.77 ± 1.00	1.67 ± 0.89	2.29 ± 0.95
C20:5(n-3) (EPA)[d]	9.29 ± 1.47	11.18 ± 1.63	9.09 ± 1.61	12.19 ± 1.91
C22:5(n-3)	2.84 ± 1.06	3.18 ± 0.81	2.75 ± 1.34	2.83 ± 0.96
C22:6(n-3) (DHA)[f]	11.56 ± 1.48	6.13 ± 1.16	11.86 ± 1.83	6.56 ± 1.45
∑SFA	48.18 ± 2.92	45.04 ± 3.06	49.44 ± 3.04	45.47 ± 3.68
∑MUFA	21.72 ± 2.43	24.65 ± 1.96	22.15 ± 1.98	25.78 ± 1.76
∑PUFA	29.68 ± 3.40	30.20 ± 3.08	28.22 ± 2.78	28.64 ± 3.69
DHA:EPA[hi]	1.28 ± 0.28	0.56 ± 0.13	1.33 ± 0.29	0.55 ± 0.15
ALA+ LIN[t]	2.59 ± 1.01	3.76 ± 1.33	1.82 ± 0.79	1.66 ± 0.8
OA:VA[om]	4.50 ± 1.96	7.00 ± 2.92	4.25 ± 4.25	5.19 ± 4.20
BAFAs	4.66 ± 1.02	4.22 ± 1.41	4.75 ± 0.87	5.54 ± 1.99
n-3PUFA/n-6PUFA	6.75 ± 2.46	3.54 ± 1.02	8.77 ± 2.93	6.18 ± 1.61

The lowercase superscripts indicate FA biomarkers: b = bacteria; c = copepods; d = diatoms; f = dinoflagellates; t = terrestrial vascular plant; be = benthic feeding mode and macro-algae indicator; ci = carnivory index; hi = herbivory index ratio; and om = omnivory index ratio. n-3PUFA and n-6PUFA = sum of the levels of all PUFA of which the first double bound counted from the terminal methyl group occurs at the third and sixth positions respectively. DHA = docosahexaenoic acid; EPA = eicosapentaenoic acid; ALA = alpha linolenic acid; LIN = linoleic acid; OA = oleic acid; VA = vaccenic acid; BAFAs = total bacterial fatty acids; SFA = saturated FAs; MUFA = monounsaturated FAs; PUFA = polyunsaturated FAs.

Spatially, the FA profiles of *V. buchanani* from the two estuarine zones were significant different (pair-wise PERMANOVA, t_{33} = 2.03, p(perm) = 0.002). This correlated with the nMDS which indicated spatial dissimilarity for the FA profiles of *V. buchanani* from the upper and lower estuarine zones (Fig. 4). The SIMPER highlighted that such spatial dissimilarities in the FA profile were mostly related to C16:0, C18:1(n-9), C20:5(n-3), C16:1, C18:0, C22:6(n-3), C18:1(n-7) and C18:2(n-6). In contrast, the FA profiles of *H. kelee* from both upper and lower parts of the estuary were relatively similar (pair-wise PERMANOVA, t_{33} = 1.32, p (perm) > 0.05; Fig. 4). Furthermore, the one-way PERMANOVA indicated low levels of variation in FA profiles for the larger size classes of *V. buchanani* collected from the lower part of the estuary (Pseudo-$F_{1, 16}$ = 3.21, p = 0.01). The FA compositions by size for *V. buchanani* from the upper estuarine zone were very similar (Pseudo- $F_{1,15}$ = 1.46, p > 0.05). The FA composition in *H. kelee* changed significantly with increasing fish length in both the upper and lower estuarine zones (Pseudo-$F_{1, 15} \geq 3.5$, $p < 0.05$). The SIMPER highlighted that the differences in size-classes of both species were largely contributed to by C16:0, DHA, EPA, C18:0, and C14:0.

Fatty acid trophic niche width

The FA niche areas (niche width) between fish species overlapped to a greater extent in the upper estuarine zone as compared to the lower portion of the estuary. The FA niche widths measured by standard ellipse area (SEAc) and total area of the convex hulls (TA) between *H. kelee* and *V. buchanani* were marginally different (Fig. 5a, b). The SEAc and TA of *H. kelee* were wider in the lower estuarine zone than that of *V. buchanani* (Fig. 5b), and the opposite situation was found in the upper estuarine zones (Fig. 5a) (ANOVA, $F = 0.55$, $p > 0.05$ in upper zone, ANOVA, F = 7.39, p = 0.02 in lower zone). These results highlighted that the two species had different trophic niches comprising different proportions of dietary FA across the estuary salinity gradient.

Discussion

Diet composition and feeding strategies

As previously predicted, substantial differences in diet and feeding strategy were found that together, contributed to dietary resource partitioning of *H. kelee* and *V. buchanani* in the Pangani estuary. The ecological index revealed strong dietary overlap for the two species, but the multivariate analyses revealed significant differences in the dietary and FA compositions of the two species. This is presumably explained by the weakness of overlap ecological index, and the power of statistical multivariate analyses for dietary and FA related data. The observed variations in dietary data for *H. kelee* and *V. buchanani* is most likely due to spatial segregation in foraging area within the water column, as well as differences in foraging mode. It is known that *H. kelee* is a filter feeder in the pelagic habitat (Blaber, 1979; Debasis *et al.,* 2013) and *V. buchanani* is a benthic feeder (Wijeyaratne and Costa, 1990; Rao and Babu, 2013). Thus, *H. kelee* and *V. buchanani* do exhibit partitioning in their feeding habitats, but because of macro-tidal activity that mixes the trophic resources throughout the water column, dietary overlap was strongly noticed in this study.

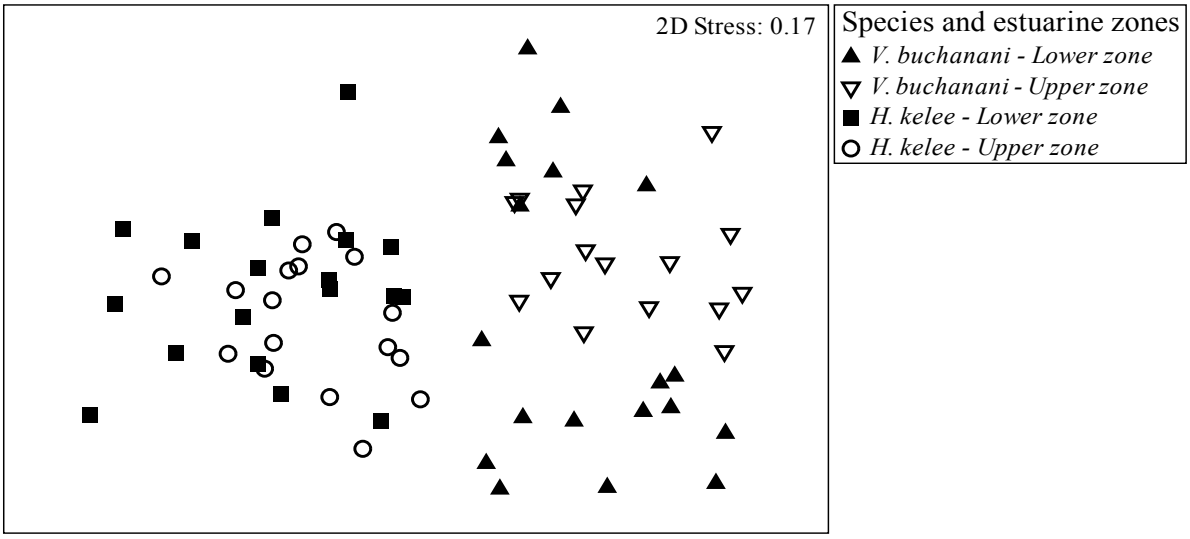

Figure 4. Non-metric MDS plot showing the FA compositions in *H. kelee* and *V. buchanani* from upper and lower zones of the Pangani Estuary.

Both species showed a dominance of micro-algae and detritus in their diets, in addition to different volumes of animal input, indicative of a detritivorous and omnivorous feeding mode. The two species differ in the extent to which these feeding modes adhered to. This can be explained by the differences in dietary niche breadth of the species. The analysis of dietary niche breadth, together with the feeding strategy, suggests that the species differ in their ability to access, and ultimately assimilate, the different dietary resources available in the estuary. Such variation in feeding strategy is presumably correlated to the morphological limitations of the two species, restricting them to certain prey items. For instance, the morphological features of *V. buchanani* facilitate easy digestion of vascular plant tissues (Mann, 1988; Cardona, 2016) when compared to *H. kelee*. Similarly, these two analyses indicated that the individuals and population of *H. kelee* exhibit a mixed feeding strategy incorporating both specialization and generalization, and that this contributes to

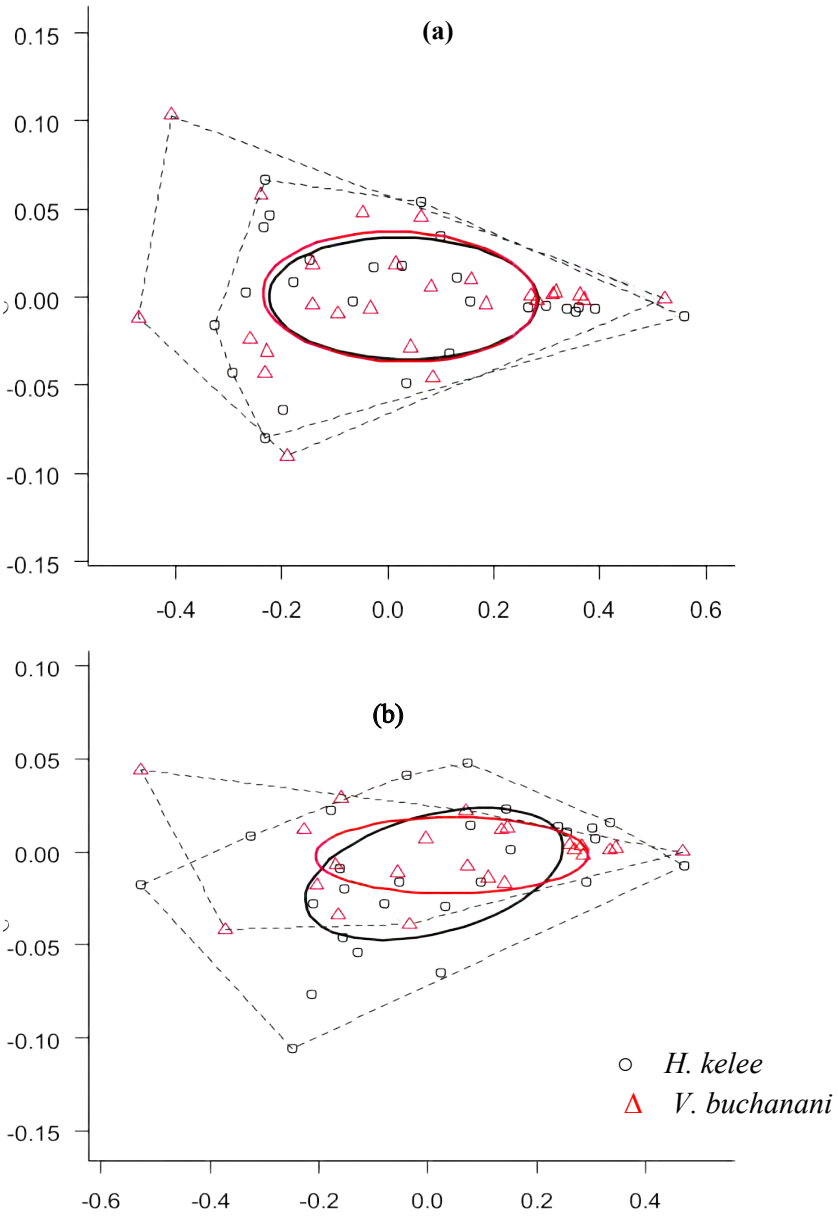

Figure 5. Fatty acid (FA) trophic niches of *H. kelee* and *V. buchanani* presented in x and y ordinates of non-metric multi-dimensional scaling (nMDS) for FA profiles of the species (n = 28 FA; Euclidean distance) in (a) upper and (b) lower zones of the Pangani Estuary. The dotted polygons are the convex hulls (TA) and the solid lines are standard ellipse areas (FA SEAc), both indicating FA feeding niche widths of the species.

intermediate niche width of high within-phenotype. This implies that part of the population of *H. kelee* appears to specialize on diatoms, contributing to the narrow diet niche breadth, and part of the population are generalists, comprising moderate generalist individuals that feed on detritus and copepods. In contrast, most of the population of *V. buchanani* displayed a generalist feeding strategy, contributing to greater trophic niche width and diet niche breadth. The generalist feeding strategy and omnivorous foraging mode displayed by both species agrees with previous studies which show that most estuarine fish are generalists (e.g. Pasquaud *et al.,* 2010). Moreover, the present findings emphasize that the degree of generalization and opportunistic omnivorous feeding behaviour tends to differ among marine fish found in estuaries.

Fatty acid composition and feeding strategy

The FA composition in *H. kelee* and *V. buchanani* follows the normal trend found in marine fish that are commonly dominated by, among others, C16:0, C18:1(n-9), EPA and DHA. Moreover, the significant variations in FA composition between the species were contributed to by the type of food assimilated by the species. The elevated DHA:EPA ratio in *H. kelee* is an indication of the pelagic feeding mode, with pelagic species tending to accumulate and retain higher level of DHA in their tissues *(*Dalsgaard *et al*., 2003; Pethybridge *et al*., 2014). This is possibly due to high abundance of dinoflgellates, the main source of DHA in pelagic habitats (Dalsgaard *et al*., 2003). It is also important to note that DHA is essential, and most elevated, in fish during reproductive periods, as it enhances reproductive performance (Luo *et al*., 2015). Because the high abundance of *H. kelee* in estuaries is related to spawning (Gjosaeter and Sousa, 1983), this could explain why large individuals of the species displayed such high levels of DHA in this study. However, because small immature size classes of *H. kelee* also displayed an elevated level of DHA in comparison to that of *V. buchanani*, it remains relevant for DHA to be used as an indicator of the nature of trophic resources consumed by both species.

The low DHA:EPA ratio in *V. buchanani* suggests an herbivorous feeding mode with the species intensively grazing on diatoms. This also implies that the efficiency of *V. buchanani* to retain and accumulate DHA is lower than *H. kelee*. In contrast, *H. kelee* showed lower herbivorous and higher carnivorous feeding modes as indicated by the higher level of DHA. A high proportion of DHA in animals suggests a carnivorous foraging mode since this FA concentrates higher up the food chain. The FA copepod marker, C20:1(n-9) in *H. kelee*, further confirmed its carnivorous feeding habits. These findings concurred with the dietary composition in *H. kelee* which showed that the species consumes microalgae, copepods and other zooplankton simultaneously. In addition to that, previous studies confirmed that carnivorous fish species tend to accumulate and retain DHA to a greater extent than herbivorous species (Dalsgaard *et al*., 2003; Copeman *et al*., 2009).

The C18:1(n-9) FA carnivory index was found to be higher in *V. buchanani* than in *H. kelee*. This is probably linked the different types of food chains that the two species are part of. It is possible that *V. buchanani* has more diverse mechanisms of accumulating C18:1(n-9) than *H. kelee*. *V. buchanani* is likely to obtain C18:1(n-9) through feeding on carnivorous detrital organic matter, through the consumption of benthic macro-algae and meiobenthos rich in C18:1(n-9). In contrast, *H. kelee* accumulates C18:1(n-9) through consumption of pelagic carnivorous zooplankton. Furthermore, vascular plants were part of the sources of nutrition for both species, bearing in mind that this food source is assimilated by most fish species to a limited extent (Mann 1988). The high level of LIN plus ALA in *V. buchanani* emphasizes that the species obtains these dietary FAs by directly browsing on decaying terrestrial plants and through the consumption of bacteria. Bacteria extract and use LIN and ALA from terrestrial plants. Likewise, the proportion of LIN, ALA and bacteria FA in *H. kelee* is an indication that the species extracts these FAs from bacteria. This also depicts different levels of niche breadth and generalization of feeding strategies in the species examined.

The FA trophic niches (measured by SEAc), and the omnivory index, C18:1(n-9)/C18:1(n-7) ratio, suggest that both species are opportunistic omnivores, but the degree of omnivory was found not similar in all estuarine zones. The high FA omnivory index, C18:1(n-9)/C18:1(n-7) ratio, and large FA niche area (SEAc) of *V. buchanani* implies a more generalized diet and opportunistic feeding strategy. Specifically, the wider FA trophic niche width found in *V. buchanani* in the upper estuarine zones could be explained by the presence of a higher diversity of dietary resources and associated dietary FA in this zone. This suggests that the upper part of the estuary has a high level of decomposing vascular plant and algal material, and accompanying bacteria, in the surface sediments, possible due to reduced tidal influences when compared to the lower estuarine

zone. To some extent, this disagrees with the patterns of dietary niche breadth showed by *V. buchanani*, which was wider in the lower zone and narrow in the upper estuarine zone. Such contradictory results could reflect the long-term and the instantaneous nature of feeding mode information revealed by FA and gut content analyses, respectively. However, the findings from both methods emphasize that *V. buchanani* has a greater dietary niche width and consequently a more opportunistic omnivore than *H. kelee*.

Intra-specific trophic resource use variations

The slight spatial differences in diets exhibited by *H. kelee* and *V. buchanani* could primarily be a result of their feeding strategies which involved feeding on the readily available food items in the estuarine system. Most probably, this was influenced by heterogeneity in distribution and abundance of potential diet categories in the longitudinal salinity gradient. The long term spatial intra-specific feeding patterns of both species revealed by their FA profiles justifies the findings of the gut content analyses. The variation in FAs showed by *V. buchanani* collected in different estuarine zones could be linked to periodic migration across the zones. This implies that *V. buchanani* might have spent at least two weeks at a time feeding in one estuarine zone before migrating to other zones. Chang and Iizuka (2012) and Loc'h *et al*. (2015) also reported that mugilidae can experience periodic movement in the estuary. The study by Odom (2012) also revealed that the FA signatures of white mullet were significantly variable within the spatial fine scale sampling zones in the Indian River lagoon, Florida. In contrast, the nature of the movement (moving with the tidal currents) and high swimming speed of the *H. kelee,* presumably explains the spatial intra-specific differences in FA composition. More studies on the movement and foraging pattern of different fish species that differ in mobility and swimming speed in the Pangani estuary are required to better understand these dynamics. Changes in FA composition with fish size were much more evident in *H. kelee* than in *V. buchanani*. This is likely connected to species differences in sexual maturity and spawning period (Taşbozan and Gökçe, 2017). DHA and EPA, which are the most important PUFA during reproduction in fish, were found in the highest proportion in the large sexually mature size classes of *H. kelee*.

Conclusion

The present study applied the FA trophic marker and stomach content methods to explore the feeding patterns and feeding strategies of *H. kelee* and *V. buchanani* from the Pangani estuary. The non-metric multivariate analyses revealed significant differences in dietary and FA compositions between the species across the estuarine zones. Despite the extreme inter-specific diet overlap indicated by the ecological index, a segregation in trophic resource use pattern (feeding in the benthic habitats versus feeding within the water column) was noticed. The dietary FA signatures and FA trophic niches, the dietary niche breadth ecological index and feeding strategy diagrams, strongly suggest that *V. buchanani* exhibits a generalist feeding strategy, contributed to by most individuals in the population. Contrary to this, *H. kelee* uses a mixed feeding strategy (generalist and specialist) that is more skewed towards a generalist feeding strategy. Because of the presence of a greater dietary and FA niche width, *V. buchanani* is considered a more efficient opportunistic omnivore than *H. kelee* throughout the Pangani estuary. Thus, *V. buchanani* is likely to show a higher degree of resilience to anthropogenic disturbances compared to *H. kelee* in the feeding and nursery grounds of the Pangani estuarine system.

Acknowledgements

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