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Full title: Effect of bodyside-specific data processing on the results of fish morphometric studies.

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## **Abstract**

Morphometric measurements on bilateral symmetric fish are usually made on one bodyside of the studied specimens. Since there is no consensus about which side is more appropriate for morphometric studies, one finds notes originating from datasets of both the right and left sides. Moreover, no information has been published about how the bodyside-specific data processing influences the comparability of population-level morphometric studies, and how this feature changes if different morphometric methods are used. Our aims were, therefore, 1) to reveal how the degree of separation varies for data obtained from opposite bodysides, and 2) to examine the significance of bodyside-specific data processing for 3) the morphometric (traditional or geometric) method used, and 4) the species analysed. To facilitate the analyses, data of four common fish species (bleak, roach, perch, pumpkinseed sunfish) collected from three closely related sites were used. The separative powers of the datasets derived from opposite bodysides do not show systematic differences in any of the studied species. The bodyside “per se”, therefore, does not affect the results of the morphometric studies. Results show that the population origin is of significantly (up to 35 times) greater importance than the bodyside if the geometric method is used. While the traditional method demonstrates a similar trend, due to the general uncertainties of this method, the bodyside origin of the data must be taken into consideration. Our findings are significant for datasets containing different aggregated or merged bodyside-originated data, or if the results of different investigations are to be compared.

**Keywords** geometric morphometry, distance-based morphometry, fish, population, differentiation

## Introduction

Morphological methods are generally used in ichthyology for various purposes, for example, to differentiate species (Creech 1992, Mustafić et al. 2017) or to describe intraspecific differences, such as sexual dimorphism (Kitano et al. 2007) and/or population-level differentiation (Herler et al. 2010). Although data derived from specific body parts have proven to be usable (Ponton 2006, Ibáñez et al. 2017), these surveys are mostly applied on datasets derived from the entire body, which is placed in a lateral position. With few exceptions (e.g., Doadrio et al. 2002), authors usually indicate which side of the studied specimens is analysed. There are very few studies dealing with mixed data (Buitrago-Suarez & Burr 2007) or that use data derived from different sides of the studied specimens (Dorado et al. 2012, Ramler et al. 2017). However, in most of the cases, morphometric data are recorded solely from the left (Burke et al. 2008, Clabaut et al. 2002, Kassam et al. 2003, Kitano et al. 2007, Leionen et al. 2006), or from the right side (Haas et al. 2010, Loy et al. 2000, Turan et al. 2004, Valentin et al. 2008) of the fish's body. Despite this, we have thus far neither found any literature explaining why a given bodyside was chosen by the authors, nor why they generally analyse only one side of the studied specimens.

Use of a single side may have two plausible reasons. The first is that side-specific data management is employed to eliminate the effect of fluctuating asymmetry (Van Vallen 1962, Parsons 1990, Klingenberg 2015). Thus, it has long been known that the symmetry of bilaterally-symmetric animals more or less deviate during the ontogeny. Moreover, the degree of deviation from the original bilateral symmetry is related to (negative) environmental effects (Ames et al. 1979, Palmer & Strobeck 1986, Wiener & Rago 1987) and/or genetic reasons (Parsons 1992). This feature is considered to be responsible for the  $\leq 1\text{--}5\%$  of the total variance in a given morphometric trait for mammals and birds (Merilä & Björklund 1995). The other potential reason for the usage of a given bodyside-derived dataset is simply practical: if all the studied specimens are laid in the same direction, there is a lower chance for measurement failures.

Whatever the reason, no relevant information exists on the significance of bodyside-specific data processing of the results of populations, or species-level multivariate analyses. Thus, knowing the relevance of this feature could be crucial if databases or results of morphometric studies using different bodyside data are to be compared. Although applicability and usability of morphometric methods have been analysed (Arnqvist & Martensson 1998; Petrýl et al. 2014, Takács et al. 2016), some trivial and basic issues have still not been clarified in detail. For example, whether there are any “systematic” differences in the separation power of the datasets derived from the opposite bodyside. Additionally, information has neither been published on how the origin of the data (whether recorded on the left or the right bodyside) influences the comparability of the results, nor if different (traditional or geometric) morphometric methods (Adams et al. 2004, Szlachciak & Nowak 2015) are employed, nor on how this feature changes if different fish species are studied.

The aims of this study are, therefore, 1) to assess the effect of bodyside on the results of morphometric analyses, and to specify its significance if 2) different morphometric methods are applied, 3) and/or different species are analysed.

## Materials and methods

Four common cyprinid and perciform species were used as model objects. Twenty-five specimens of bleak *Alburnus alburnus* (Linnaeus, 1758), roach *Rutilus rutilus* (Linnaeus, 1758), perch *Perca fluviatilis* (Linnaeus, 1758), and pumpkinseed-sunfish *Lepomis gibbosus* (Linnaeus, 1758) —abbreviated as sunfish here— were collected from the same three sampling sites designated in the catchment area of Lake Balaton (Hungary). As this region is uncontaminated by heavy metals (Nguyen et al. 2005), its fish populations are supposedly

minimally exposed to developmental disorders, which may increase the asymmetry of fish (Jezierska et al. 2009).

Sites 1 and 2 are situated at the mouth of inflowing streams (coordinates: N46.80347 E17.40449 and N46.75330 E17.56730 respectively), while site 3 is located at a near shoreline area of the lake (coordinates: N46.91441 E17.89304). The first two sampling sites are also lentic habitats, providing highly-similar environmental conditions to the lake. Moreover, no physical barriers restrict the connection between the sampled sites. Specimens were collected by electrofishing (permission number of the Ministry of Agriculture: HHgF/230-4/2016) in the late summer of 2016. To minimize suffering, specimens collected for this study were immediately euthanized with an overdose of clove oil (Anderson et al. 1997). In the laboratory, they were then placed flat on a table surface and both sides were photographed from a perpendicular angle using a tripod-mounted Nikon D80 digital camera with a fixed zoom range. To eliminate intermeasurer variability (Takács et al. 2016), all measurements were made by the same person (ZV). Moreover, in order to reduce the risk of measurement bias, all the photos taken from the right side of the studied specimens were reflected horizontally. To our knowledge, none of the studied species show obvious sexual dimorphism beyond the spring-breeding period; therefore, we did not differentiate the data for males and females during the analysis, and supposed a 1:1 sex ratio in our samples. The digital images were further analysed with two different methods: the landmark-based geometric morphometrics (GM) (Adams et al. 2004), and the traditional, distance-based morphometric method (TM) (Cadrin 2000). For the GM method, 11 landmarks were recorded on each image (Fig. 1) using tpsUtil and tpsDig2 digital-imaging software (Rohlf 2010a, 2010b). For the TM method, 22 distances were measured between homologous points of the fish body (Fig. 1) using imageJ software (Schneider et al. 2012). To eliminate any size effect in the datasets measured for the TM analyses, the allometric formula of Elliott et al. (1995) was used. To check the efficiency of data standardization, all standardised TM variables were rechecked against the standard length (SL) values. For GM coordinates, a full Procrustes fit was undertaken on the landmark data, followed by multivariate-regression analysis on the logarithm of the centroid size (Klingenberg 2011). Additional statistical analyses were performed on the residuals of the regression analyses.

Multivariate analysis of variance (MANOVA) (Alvin 2002) and canonical variate analyses (CVA) were used for testing and visualizing the between-side and among-population differences for all the studied species in both methods.

To compare the separative power of the datasets derived from the opposite bodyside, used here are the CVA group-centroid differences quantified by their squared Mahalanobis distances, as well as Bonferroni-corrected pairwise Hotelling  $p$  values. To characterise the importance of the sample site and the side-specific data management on the results, a two-way permutational analysis of variance (PERMANOVA) (Anderson 2001) was conducted (Giordani et al. 2013) using the Euclidean-distance measure with 9'999 permutations. The analysis was performed independently for each method and for each species. All statistical analyses were carried out using PAST v.2.17c software (Hammer et al. 2001).

## Results

The standard length (SL) of the studied individuals ranged between 69.9 and 221.9 mm (mean  $\pm$  standard deviation (sd): 118.75 $\pm$ 35.8 mm) in roach, from 54.6 to 112.5 mm (mean $\pm$ sd: 81.87 $\pm$ 12.1 mm) in bleak, 44.8 to 173.2 mm (mean $\pm$ sd: 103.04 $\pm$ 24.9 mm) in perch, and from 68.8 to 221.9 mm (mean $\pm$ sd: 118.76 $\pm$ 35.8 mm) in sunfish. Since none of the TM variables showed any significant correlation with SL data, after standardisation, they were all used for further statistical analyses. All the analysed distance data and the regression residuals of the geometric morphometric datasets are available in the Supplementary Material (S- Tables 1–2).

For the GM datasets, the left bodyside in the cases of bleak, roach, and sunfish populations, and the right bodyside in the case of perch, show higher levels of morphometric differences (Table 1). For the TM datasets, the left side in the cases of bleak and perch, and the right side in the cases of roach and sunfish assemblages, show a higher separative power. The pairwise comparisons of the datasets of the opposite side of the same specimens show significant ( $p < 0.05$ ) differences in the case of the perch and sunfish stocks collected from the S1 site (Table 1). The CVA scatter plots of opposite-side population-level datasets are presented separately for each species and for both methods in Fig. 2.

The results of the two-way PERMANOVA analysis show that the sampling site has a fundamental role in the formulation of group differences, while the sampled bodyside generally has only a slight influence on the results (Table 2). The explained variance by the sampling site varies between 7.25% and 21.52% for the GM method and between 4.81% and 9.72% for the TM method. While the bodyside-explained variance ranges between 0.31% and 2.62%, and between 0.6% and 2.63% for the GM and TM methods, respectively, only a significant ( $p < 0.05$ ) effect of bodyside is detected in the case of the sunfish, for both morphometric methods (Table 2).

## Discussion

The results of squared-Mahalanobis-distance comparisons do not show clear and systematic (trend-like) differences in the separative power of the various bodyside-derived datasets in either of the methods tested. Therefore, there is no evidence that the analysis of datasets derived from the right or left side would produce differences of a higher level in the case of population-level comparisons for all the four studied species.

Although all the studied species were collected from the same three sampling sites, the CVA scatterplots of the GM datasets reveal different levels of population segregation. The populations of the pelagic, “obligate” schooling (Haberlehner 1988) bleak differentiate the least, while the assemblages of sunfish, which is a benthic and territorial (Miller 1963, Beacham 1988, Colgan et al. 1981) species, showed the most robust population-level segregation (Table 1, Fig. 2). The differences in the dataset derived from the opposite bodysides are in accordance with this finding (Table 1). Thus, the highest differences are found in the case of sunfish. While the reasons of this congruence need to be clarified by detailed studies, we also assume genetic reasons in this case. Namely, the benthic and territorial sunfish may have a more pronounced population genetic isolation than the pelagic, “obligate” schooling bleak. Furthermore, we have to consider the fact that the sunfish is the only non-indigenous species out of the studied four to have been introduced into Lake Balaton more than a century ago (Takács et al. 2017). Therefore, some specific genetic features, e.g., the founder effect (Dlugosch & Parker 2008), and the higher level of inbreeding caused by the restricted gene flow among populations may manifest in increased asymmetry of the studied sunfish individuals as well. Here, we have to note that while the (fluctuating) asymmetry may be the most important factor, it is not the only reason for the indicated bodyside differences. In our case, the measurement error may play an important role as well, resulting from the imaging process. After taking a photo of the first bodyside, the measurer may not only change the side, but slightly the shape characteristics of the fish as well. While the measurer tried to not change any characteristics of the fish during changing the bodyside of the fish, minimal changes in, e.g., the dorso-ventral curvature of the body, and therefore the position of fin stems, may also change and effect the landmark positions. This phenomenon may increase the probability of optical and/or digital distortion and misinterpretation errors (Arnqvist & Martensson, 1998). Therefore, besides the “true” asymmetry, the measurement error may also be responsible for the indicated differences. Unfortunately, as our data structure is not suitable to divide these components, the determination of the significance of asymmetry and measurement error is beyond the scope.

The results of PERMANOVA analyses demonstrate that the proportion of sampling-site-explained variance is notably lower for the TM method than for the GM method (6.51% versus 12.4% on average). At the same time, the proportion of bodyside-explained variance is similar as revealed by the GM method, 1.03% and 1.28% on average for the TM and GM methods, respectively (Table 2) (overall ranging from 0.31–2.62%). Moreover, our results show that the side-specific data processing has a considerably (up to 35 times for the GM method, and up to nine times for the TM method) less influence on the results than the population origin (Table 2).

The results of the CVA/MANOVA analyses show notable differences between the two methods (Fig. 2) as well. In the case of the GM method, the three studied populations differentiate on the CVA plots in a similar way in all the four studied species, regardless of which side-derived dataset was used for the analyses. However, the relative positions of the side-specific group centroids slightly shift along the  $y$ - and/or  $x$ -axes in every case. The CVA plot of TM datasets shows a similar pattern, but a higher level between group-centroid differences are detected. These findings are supported by the results of the MANOVA analyses, because the Bonferroni-corrected Hotelling  $p$  values are not significant in any

pairwise comparison at the right and left GM datasets of the same populations. In contrast to this, we find significant differences between the TM datasets derived from the opposite side of some perch and sunfish populations (Table 2). This feature may be attributed to the restricted usability of the traditional morphometric methods when closely related entities are analysed (Maderbacher et al. 2008, Takács et al. 2016).

In conclusion, the results of our methodological study using multispecies data show slight but detectable differences in each case if datasets derived from opposite bodysides are compared. However, the relevance of this feature depends on the species examined and on the methods applied. Therefore, the bodyside origin may only have relevance for population-level investigations. Moreover, when the data from opposite sides are not congruent, both sides of the fish should be taken into account during these analyses. Our findings should be taken into consideration if datasets containing different bodyside-originated data are aggregated or merged, or if the results of different investigations are to be compared.

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### **Author contributions**

P.T conceived the study, collected samples, performed statistical analyses, and wrote the manuscript. Á.F and Á.S. collected samples, provided discussion, and edited the manuscript. Z.V. collected samples, made the morphometric measurements and edited the manuscript.

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Figure 1

**Morphometric landmarks and distances recorded on the studied species.** Definitions of the 11 landmarks: a: tip of snout; b: supraoccipital; c: origin of (first) dorsal fin; d: upper origin of caudal fin; e: lower origin of caudal fin; f: origin of anal fin; g: origin of pelvic fin; h: origin of pectoral fin; i: ventral end of opercle; j: posterior point of opercle; k: middle point of eye. The recorded distance data (grey lines, and numbers): 1: standard length; 2: distance between the tip of snout and the origin of (first) dorsal fin ray/spine; 3: head length; 4: prepectoral distance; 5: preanal distance; 6: prepelvic distance; 7: preorbital distance; 8: eye width; 9: postorbital distance; 10: eye height; 11: depth of head; 12: length of the first pectoral fin ray; 13: distance between the origins of the (first) dorsal and pelvic fin rays/spines; 14: length of the first pelvic fin ray; 15: length of the first anal fin ray; 16: distance between the origin of anal fin and the lower origin of caudal fin rays; 17: length of lower lobe of caudal fin rays; 18: distance between the upper and lower origins of caudal fin rays; 19: length of upper lobe of caudal fin rays, 20: distance between the origin of (first) dorsal fin and the upper origin of caudal fin rays, 21: length of (first) dorsal fin ray/spine

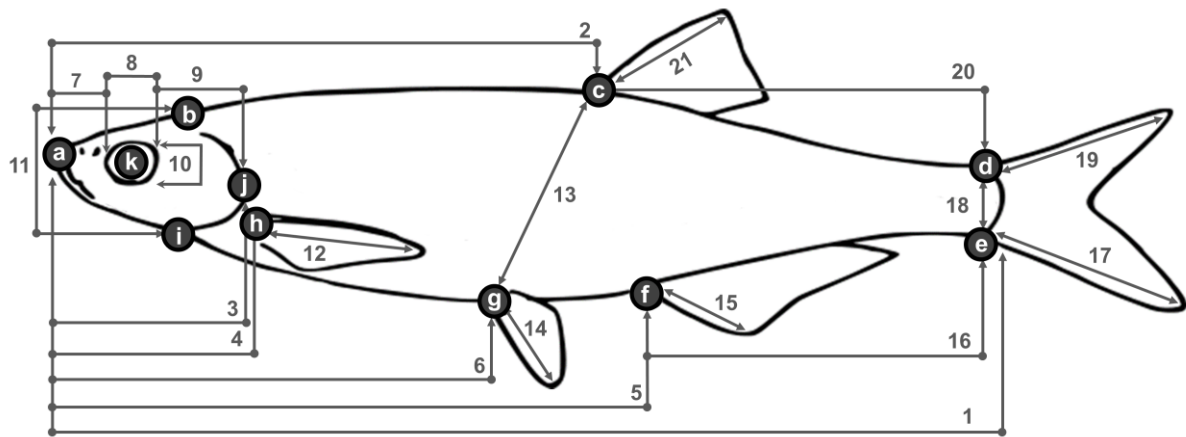
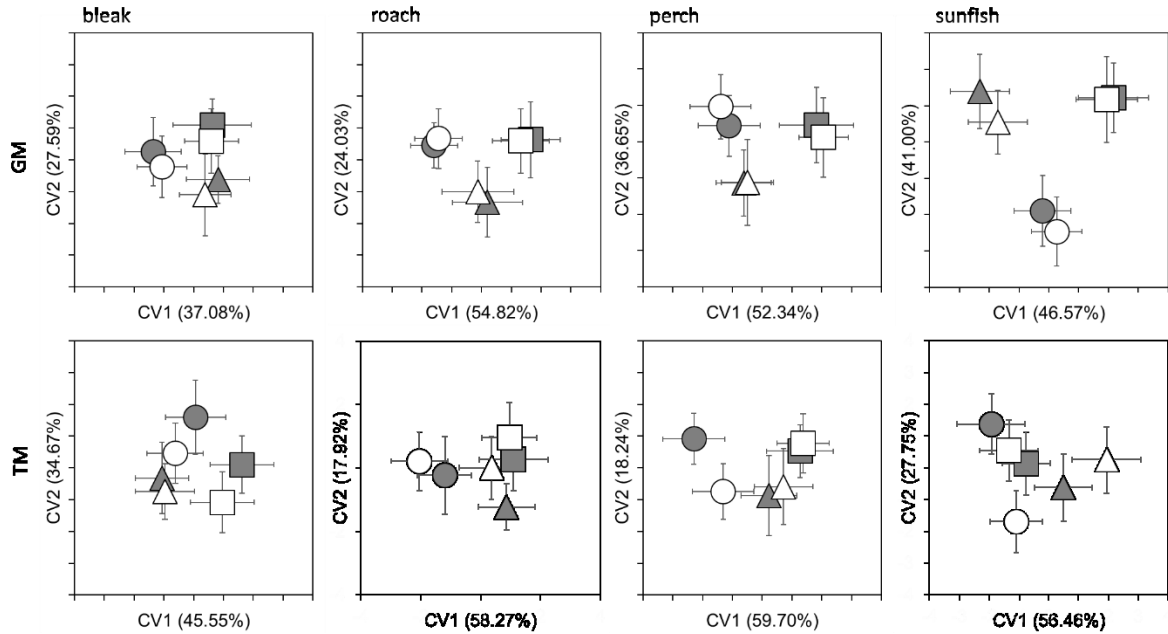


Figure 2  
 Canonical-variate-analysis scatterplots of standardised morphometric data derived from the right (white) and left (grey) side of the studied species using geometric (GM) and traditional morphometric (TM) methods. For clarity, only the group centroids are indicated, with vertical and horizontal whiskers indicating the standard deviation of data. Each population is represented by a different shape: ● site 1, ▲ site 2, and ■ site 3. The explained variances in each axis are indicated in parentheses.



**Table 1**

Squared-Mahalanobis distances of right- and left-side-derived datasets (\*=  $p < 0.05$ ) S1, S2, S3: sample sites; L, R: left and right bodysides. The pairwise distances of the opposite bodyside-derived datasets of the same populations are highlighted in bold.

species	GM							TM							
Bleak		S1_L	S1_R	S2_L	S2_R	S3_L	S3_R		S1_L	S1_R	S2_L	S2_R	S3_L	S3_R	
	S1_L	-						S1_L	-						
	S1_R	<b>2.025</b>	-					S1_R	<b>3.431</b>	-					
	S2_L	7.078*	4.896	-				S2_L	5.656*	2.982*	-				
	S2_R	7.293*	5.657	<b>5.687</b>	-			S2_R	6.732*	3.598	<b>1.190</b>	-			
	S3_L	6.137	5.291	5.145	6.664	-		S3_L	5.292*	6.997*	7.680*	7.911*	-		
	S3_R	5.018	5.558	3.981	5.638	<b>2.244</b>	-	S3_R	8.403*	5.471*	6.020*	4.797	<b>3.144</b>	-	
Roach		S1_L	S1_R	S2_L	S2_R	S3_L	S3_R		S1_L	S1_R	S2_L	S2_R	S3_L	S3_R	
	S1_L	-						S1_L	-						
	S1_R	<b>1.811</b>	-					S1_R	<b>3.284</b>	-					
	S2_L	7.814*	7.704*	-				S2_L	6.471*	10.784*	-				
	S2_R	4.906	6.034	<b>1.416</b>	-			S2_R	4.088	7.666*	<b>3.186</b>	-			
	S3_L	12.173*	11.042*	7.473*	7.612*	-		S3_L	7.621*	10.427*	3.182	3.221	-		
	S3_R	11.004*	11.123*	7.724*	7.083*	<b>4.682</b>	-	S3_R	6.805*	10.607*	5.040	2.575	<b>2.245</b>	-	
Perch		S1_L	S1_R	S2_L	S2_R	S3_L	S3_R		S1_L	S1_R	S2_L	S2_R	S3_L	S3_R	
	S1_L	-						S1_L	-						
	S1_R	<b>1.941</b>	-					S1_R	<b>6.386*</b>	-					
	S2_L	5.262	6.098*	-				S2_L	9.947*	6.895*	-				
	S2_R	5.542	6.937*	<b>2.292</b>	-			S2_R	11.739*	6.333*	<b>2.060</b>	-			
	S3_L	10.447*	11.699*	9.756*	9.117*	-		S3_L	13.814*	9.668*	4.813*	3.089	-		
	S3_R	10.079*	12.912*	9.170*	7.070*	<b>1.800</b>	-	S3_R	14.46	10.430*	6.261*	3.090	<b>1.705</b>	-	
Sunfish		S1_L	S1_R	S2_L	S2_R	S3_L	S3_R		S1_L	S1_R	S2_L	S2_R	S3_L	S3_R	
	S1_L	-						S1_L	-						
	S1_R	<b>4.356</b>	-					S1_R	<b>10.364*</b>	-					
	S2_L	21.942*	26.841*	-				S2_L	12.522*	11.816*	-				
	S2_R	14.388*	15.173*	<b>5.060</b>	-			S2_R	16.895*	13.675*	<b>3.419</b>	-			
	S3_L	21.077*	20.956*	24.543*	19.584*	-		S3_L	4.039	4.425	6.189*	8.493*	-		
	S3_R	19.415*	17.747*	25.408*	16.043*	<b>3.126</b>	-	S3_R	10.604*	5.134	3.565	5.067	<b>3.712</b>	-	

Table 2

Results of two-way PERMANOVA analysis (9,999 permutations) for the effect of sampling site and bodyside on the data obtained by geometric morphometrics (GM) and the traditional morphometric method (TM). The highest  $F$  value is indicated in bold, and significant values ( $* = p < 0.05$ ;  $** = p < 0.01$ ) are italicized.

Species	Source	GM						TM					
		Sum of squares	explained variance	df	Mean square	F	p	Sum of squares	explained variance	df	Mean square	F	p
bleak	<b>sample site</b>	0.014	7.25%	2	0.007	<b>5.766</b>	<i>0.0001**</i>	122.33	4.81%	2	61.167	<b>3.718</b>	<i>0.0001**</i>
	bodyside	0.001	0.52%	1	0.001	0.903	0.4533	27.966	1.10%	1	27.966	1.700	0.0915
	Interaction	0.002	1.04%	2	0.001	0.919	0.4861	23.182	0.91%	2	11.591	0.705	0.7956
	Residual	0.175	90.67%	144	0.001			2369.2	93.18%	144	16.453		
	Total	0.193		149				2542.7		149			
roach	<b>sample site</b>	0.016	10.32%	2	0.008	<b>8.727</b>	<i>0.0001**</i>	288.64	5.42%	2	144.32	<b>4.254</b>	<i>0.0001**</i>
	bodyside	0.001	0.65%	1	0.001	1.125	0.3208	31.916	0.60%	1	31.916	0.941	0.4542
	Interaction	0.003	1.94%	2	0.001	1.496	0.0804	120.31	2.26%	2	60.154	1.773	<i>0.0328*</i>
	Residual	0.135	87.10%	144	0.001			4884.8	91.72%	144	33.922		
	Total	0.155		149				5325.7		149			
perch	<b>sample site</b>	0.035	10.84%	2	0.017	<b>8.868</b>	<i>0.0001**</i>	376.23	6.07%	2	188.12	<b>4.760</b>	<i>0.0001**</i>
	bodyside	0.001	0.31%	1	0.001	0.517	0.8571	54.413	0.88%	1	54.413	1.377	0.1941
	Interaction	0.003	0.93%	2	0.002	0.808	0.6584	74.932	1.21%	2	37.466	0.948	0.4833
	Residual	0.284	87.93%	144	0.002			5691.2	91.84%	144	39.522		
	Total	0.323		149				6196.8		149			
sunfish	<b>sample site</b>	0.082	21.52%	2	0.041	<b>20.672</b>	<i>0.0001**</i>	580.75	9.72%	2	290.38	<b>8.149</b>	<i>0.0001**</i>
	bodyside	0.010	2.62%	1	0.010	4.934	<i>0.0004*</i>	151.34	2.53%	1	151.34	4.247	<i>0.0007**</i>
	Interaction	0.004	1.05%	2	0.002	1.072	0.3645	113.96	1.91%	2	56.981	1.599	0.0706
	Residual	0.285	74.80%	144	0.002			5131.3	85.85%	144	35.634		
	Total	0.381		149				5977.4		149			