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

13
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23
24 **Abstract**

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

44
45 **Keywords** geometric morphometry, distance-based morphometry, fish, population,
46 differentiation

49 **Introduction**

50 Morphological methods are generally used in ichthyology for various purposes, for example,
51 to differentiate species (Creech 1992, Mustafić et al. 2017) or to describe intraspecific
52 differences, such as sexual dimorphism (Kitano et al. 2007) and/or population-level
53 differentiation (Herler et al. 2010). Although data derived from specific body parts have
54 proven to be usable (Ponton 2006, Ibáñez et al. 2017), these surveys are mostly applied on
55 datasets derived from the entire body, which is placed in a lateral position. With few
56 exceptions (e.g., Doadrio et al. 2002), authors usually indicate which side of the studied
57 specimens is analysed. There are very few studies dealing with mixed data (Buitrago-Suarez
58 & Burr 2007) or that use data derived from different sides of the studied specimens (Dorado
59 et al. 2012, Ramler et al. 2017). However, in most of the cases, morphometric data are
60 recorded solely from the left (Burke et al. 2008, Clabaut et al. 2002, Kassam et al. 2003,
61 Kitano et al. 2007, Leionen et al. 2006), or from the right side (Haas et al. 2010, Loy et al.
62 2000, Turan et al. 2004, Valentin et al. 2008) of the fish's body. Despite this, we have thus far
63 neither found any literature explaining why a given bodyside was chosen by the authors, nor
64 why they generally analyse only one side of the studied specimens.

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

76 Whatever the reason, no relevant information exists on the significance of bodyside-specific
77 data processing of the results of populations, or species-level multivariate analyses. Thus,
78 knowing the relevance of this feature could be crucial if databases or results of morphometric
79 studies using different bodyside data are to be compared. Although applicability and usability
80 of morphometric methods have been analysed (Arnqvist & Martensson 1998; Petrýl et al.
81 2014, Takács et al. 2016), some trivial and basic issues have still not been clarified in detail.
82 For example, whether there are any “systematic” differences in the separation power of the
83 datasets derived from the opposite bodyside. Additionally, information has neither been
84 published on how the origin of the data (whether recorded on the left or the right bodyside)
85 influences the comparability of the results, nor if different (traditional or geometric)
86 morphometric methods (Adams et al. 2004, Szlachciak & Nowak 2015) are employed, nor on
87 how this feature changes if different fish species are studied.

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

91

92 **Materials and methods**

93 Four common cyprinid and perciform species were used as model objects. Twenty-five
94 specimens of bleak *Alburnus alburnus* (Linnaeus, 1758), roach *Rutilus rutilus* (Linnaeus,
95 1758), perch *Perca fluviatilis* (Linnaeus, 1758), and pumpkinseed-sunfish *Lepomis gibbosus*
96 (Linnaeus, 1758) —abbreviated as sunfish here— were collected from the same three
97 sampling sites designated in the catchment area of Lake Balaton (Hungary). As this region is
98 uncontaminated by heavy metals (Nguyen et al. 2005), its fish populations are supposedly

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

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

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

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

140 **Results**

141 The standard length (SL) of the studied individuals ranged between 69.9 and 221.9 mm (mean
142 \pm standard deviation (sd): 118.75 \pm 35.8 mm) in roach, from 54.6 to 112.5 mm (mean \pm sd:
143 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
144 68.8 to 221.9 mm (mean \pm sd: 118.76 \pm 35.8 mm) in sunfish. Since none of the TM variables
145 showed any significant correlation with SL data, after standardisation, they were all used for
146 further statistical analyses. All the analysed distance data and the regression residuals of the
147 geometric morphometric datasets are available in the Supplementary Material (S- Tables 1–
148 2).

149 For the GM datasets, the left bodyside in the cases of bleak, roach, and sunfish populations,
150 and the right bodyside in the case of perch, show higher levels of morphometric differences
151 (Table 1). For the TM datasets, the left side in the cases of bleak and perch, and the right side
152 in the cases of roach and sunfish assemblages, show a higher separative power. The pairwise
153 comparisons of the datasets of the opposite side of the same specimens show significant ($p <$
154 0.05) differences in the case of the perch and sunfish stocks collected from the S1 site (Table
155 1). The CVA scatter plots of opposite-side population-level datasets are presented separately
156 for each species and for both methods in Fig. 2.

157 The results of the two-way PERMANOVA analysis show that the sampling site has a
158 fundamental role in the formulation of group differences, while the sampled bodyside
159 generally has only a slight influence on the results (Table 2). The explained variance by the
160 sampling site varies between 7.25% and 21.52% for the GM method and between 4.81% and
161 9.72% for the TM method. While the bodyside-explained variance ranges between 0.31% and
162 2.62%, and between 0.6% and 2.63% for the GM and TM methods, respectively, only a
163 significant ($p < 0.05$) effect of bodyside is detected in the case of the sunfish, for both
164 morphometric methods (Table 2).

165 **Discussion**

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

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

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

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

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

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

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236 **Author contributions**

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

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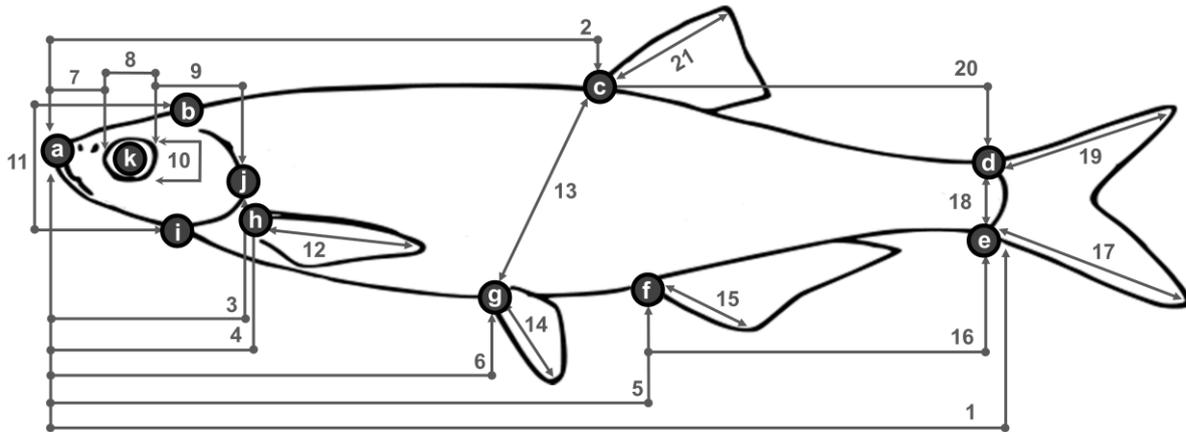
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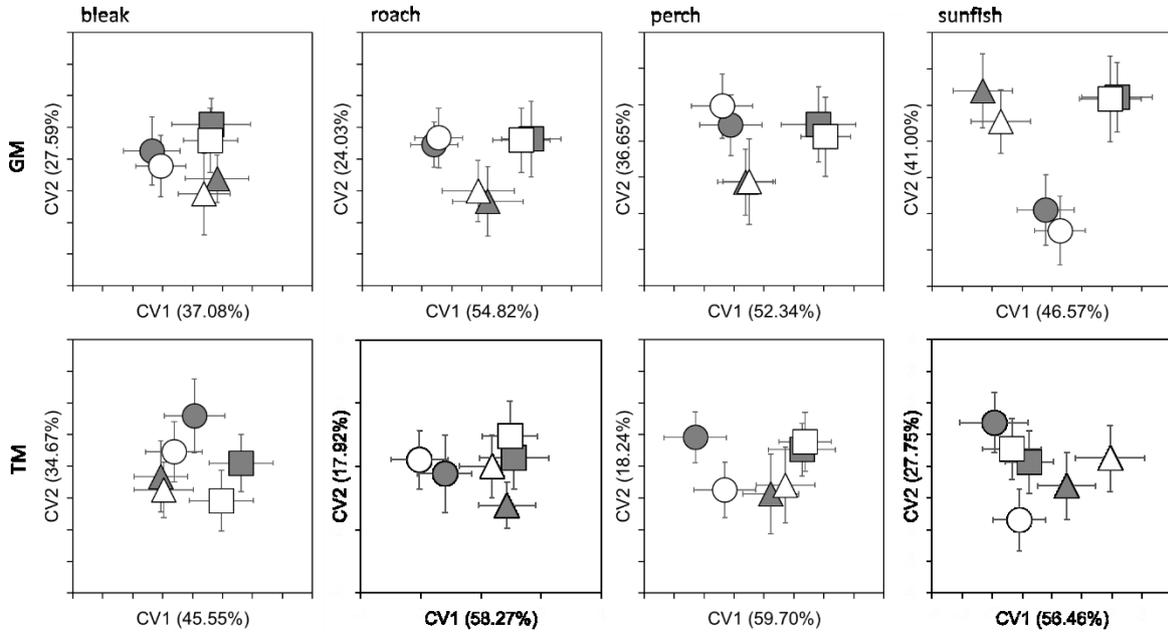
378 Figure 1

379 **Morphometric landmarks and distances recorded on the studied species.** Definitions of
380 the 11 landmarks: a: tip of snout; b: supraoccipital; c: origin of (first) dorsal fin; d: upper
381 origin of caudal fin; e: lower origin of caudal fin; f: origin of anal fin; g: origin of pelvic fin;
382 h: origin of pectoral fin; i: ventral end of opercle; j: posterior point of opercle; k: middle point
383 of eye. The recorded distance data (grey lines, and numbers): 1: standard length; 2: distance
384 between the tip of snout and the origin of (first) dorsal fin ray/spine; 3: head length; 4:
385 prepectoral distance; 5: preanal distance; 6: prepelvic distance; 7: preorbital distance; 8: eye
386 width; 9: postorbital distance; 10: eye height; 11: depth of head; 12: length of the first pectoral
387 fin ray; 13: distance between the origins of the (first) dorsal and pelvic fin rays/spines; 14:
388 length of the first pelvic fin ray; 15: length of the first anal fin ray; 16: distance between the
389 origin of anal fin and the lower origin of caudal fin rays; 17: length of lower lobe of caudal fin
390 rays; 18: distance between the upper and lower origins of caudal fin rays; 19: length of upper
391 lobe of caudal fin rays, 20: distance between the origin of (first) dorsal fin and the upper
392 origin of caudal fin rays, 21: length of (first) dorsal fin ray/spine
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395 Figure 2
 396 Canonical-variate-analysis scatterplots of standardised morphometric data derived from the
 397 right (white) and left (grey) side of the studied species using geometric (GM) and traditional
 398 morphometric (TM) methods. For clarity, only the group centroids are indicated, with vertical
 399 and horizontal whiskers indicating the standard deviation of data. Each population is
 400 represented by a different shape: ● site 1, ▲ site 2, and ■ site 3. The explained variances in
 401 each axis are indicated in parentheses.
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407 **Table 1**
 408 Squared-Mahalanobis distances of right- and left-side-derived datasets ($*= p < 0.05$) S1, S2, S3:
 409 sample sites; L, R: left and right bodysides. The pairwise distances of the opposite bodyside-derived
 410 datasets of the same populations are highlighted in bold.
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 412

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	2.025	-					S1_R	3.431	-				
	S2_L	7.078*	4.896	-				S2_L	5.656*	2.982*	-			
	S2_R	7.293*	5.657	5.687	-			S2_R	6.732*	3.598	1.190	-		
	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	2.244	-	S3_R	8.403*	5.471*	6.020*	4.797	3.144	-
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	1.811	-					S1_R	3.284	-				
	S2_L	7.814*	7.704*	-				S2_L	6.471*	10.784*	-			
	S2_R	4.906	6.034	1.416	-			S2_R	4.088	7.666*	3.186	-		
	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*	4.682	-	S3_R	6.805*	10.607*	5.040	2.575	2.245	-
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	1.941	-					S1_R	6.386*	-				
	S2_L	5.262	6.098*	-				S2_L	9.947*	6.895*	-			
	S2_R	5.542	6.937*	2.292	-			S2_R	11.739*	6.333*	2.060	-		
	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*	1.800	-	S3_R	14.46	10.430*	6.261*	3.090	1.705	-
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	4.356	-					S1_R	10.364*	-				
	S2_L	21.942*	26.841*	-				S2_L	12.522*	11.816*	-			
	S2_R	14.388*	15.173*	5.060	-			S2_R	16.895*	13.675*	3.419	-		
	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*	3.126	-	S3_R	10.604*	5.134	3.565	5.067	3.712	-

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419 Table 2
 420 Results of two-way PERMANOVA analysis (9,999 permutations) for the effect of sampling
 421 site and bodyside on the data obtained by geometric morphometrics (GM) and the traditional
 422 morphometric method (TM). The highest F value is indicated in bold, and significant values
 423 ($* = p < 0.05$; $** = p < 0.01$) 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	sample site	0.014	7.25%	2	0.007	5.766	<i>0.0001**</i>	122.33	4.81%	2	61.167	3.718	<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	sample site	0.016	10.32%	2	0.008	8.727	<i>0.0001**</i>	288.64	5.42%	2	144.32	4.254	<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	sample site	0.035	10.84%	2	0.017	8.868	<i>0.0001**</i>	376.23	6.07%	2	188.12	4.760	<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	sample site	0.082	21.52%	2	0.041	20.672	<i>0.0001**</i>	580.75	9.72%	2	290.38	8.149	<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			

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