

1 **Impact of environmental and genetic factors on the scale shape of zebrafish *Danio rerio***
2 **(Hamilton 1822): a geometric morphometric study**

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18

19 **Running title:** Factors influencing zebrafish scale shape

20 **Keywords:** landmark-based geometric morphometrics; phenotypic plasticity; shape analysis

21

22 **Abstract**

23

24 Intraspecific morphological variability may reflect either genetic divergence among groups of
25 individuals or response of individuals to environmental circumstances within the frame of
26 phenotypic plasticity. Several studies were able to discriminate wild fish populations based on
27 their scale shape. Here we examine whether the variations in the scale shape in fish
28 populations could be related to genetic or environmental factors, or to both of them. In the
29 first experiment, two inbred lines of zebrafish *Danio rerio* (Hamilton 1822) reared under
30 identical environmental conditions were compared. Secondly, to find out what effect
31 environmental factors might have, offsprings were divided into two groups and reared on
32 different diets for 12 weeks. Potential recovery of scales from an environmental effect was
33 also assessed. Experimental groups could successfully be distinguished according to the shape
34 of scales in both experiments, and the results showed that both genetic and environmental
35 factors may notably influence scale shape. It was concluded that scale shape analysis might be
36 used as an explanatory tool to detect potential variability of environmental influences
37 impacting genetically homogeneous groups of fish. However, due to its sensitivity to
38 environmental heterogeneity, the applicability of this technique in identifying intraspecific
39 stock membership of fish could be limited.

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44 **Introduction**

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46 When phenotypes can clearly associate with specific genotypes then they may be used to
47 separate among genetically different populations or groups of individuals of a species.

48 However, if environmental effects can be captured safely in the formation of a specific
49 morphological character then this character may be used as a good and simple indicator for
50 distinguishing among individuals experienced different environmental circumstances or, in
51 general, monitor environmental impacts stressed the population under study.

52 In fishes, morphometric analysis is especially suitable to assess various genetic,
53 environmental and physiological effects hit the individuals (36). Besides the genetic
54 variability, effects of food availability (4, 16, 20) and type of food (5), temperature (2, 16,
55 31), or the presence of predators (3) on body shape have been reported. However, the process
56 of taking a proper morphometric image of the whole body is highly stressful for fish, and
57 therefore, the investigation of a structural component, variable enough to distinguish
58 populations and easy to collect without permanently damaging the animal is more expedient
59 (8). Assuming a strong genetic definiteness, scales, similarly to other hard structural
60 components like otolith (1, 17) and in general bony structures (33), are regularly used to
61 distinguish among species or even populations of fish (10, 11, 12, 22, 23, 24). The
62 examination of scales proved to be a practical and cheap tool to identify fish including
63 archaeological samples as well (15, 30). On the other hand, scales are also widely used to
64 evaluate individual life histories and living conditions of fish by determining their growth
65 dynamics (25) and identifying diseases (19).

66 Some researchers argue that most of intraspecific variations in shapes of scales and other hard
67 morphological structures could simply be explained by phenotypic plasticity (16), and
68 actually, the relative importance of genetic and environmental factors on scale-morphology is

69 still not exactly known. Some studies have already addressed the questions whether the
70 differences observed in the scale shape could be attributed to differences in life histories of
71 populations, and whether environmental factors, such as recovering food quantity (ie.
72 compensatory growth) (12), or cadmium treatment (26, 34) could affect the reliability of scale
73 shape based stock identifications (12). However, because of the complex effects of numerous
74 factors and the high degree of genetic diversity, it is generally difficult to evaluate the relative
75 importance of specific factors based on field samples (12). Nevertheless, no controlled
76 laboratory experiment has already been reported on the potential role of environmental factors
77 in formation of scale shape. According to the results on other morphological features (9, 20,
78 21), it is very likely however that scale shape might also vary along environmental gradients.
79 In this study laboratory experiments were carried out to investigate whether environmental
80 factors, namely the food supply, could affect scale shape during the ontogeny in fish. Two
81 genetically separated, inbred zebrafish *Danio rerio* (Hamilton 1822) stocks (Figure 1) and
82 two feeding protocols were compared in order to assess the role of genetic and environmental
83 components in scale shape variability. Zebrafish is especially suitable model organism for
84 controlled laboratory investigations, as it has well-known environmental needs (14), reaches
85 the adult size rapidly, after 12 weeks, and the optimal dietary needs are known for the whole
86 life cycle (14).

87 Specific hypotheses of this study were 1) the genetic background has detectable influence on
88 the scale shape; 2) the feeding conditions during the ontogeny affects the scale shape with
89 greater impact; and 3) with the improvement of food supply the scale shape could be
90 recovered.

91

92 **Materials and methods**

93 **Experimental stocks and design**

94 Zebrafish were maintained in a recirculated system (Tecniplast) (temperature= 25 ± 0.5 °C,
95 pH= 7.4 ± 0.2 , conductivity= 525 ± 50 μ S; mean \pm SD) in a light cycle of 14 hours light and 10
96 hours dark, in 30 individuals per 3.5 liters density.

97 To determine the genetic impact on scale shape, zebrafish specimens from a homogenous
98 registered line (AB line) and a commercial stock (LF BASKA stock) were compared (Figure
99 1). Individuals were kept under the same controlled laboratory conditions and fed according
100 to the control regimen (Table 1).

101 Two groups were created from the offspring of each of four AB line females (altogether eight
102 experimental groups) originated from a single propagation to examine the environmental
103 effect. Thus, genetic differences between these parallel groups were minimal. Groups labeled
104 with “N” were fed following the control regimen (Table 2) according to their age while
105 groups labeled with “H” were fed following the reduced regimen (Table 2). Fish were reared
106 for 12 weeks, when they normally became adults. Two H groups (H2, H3) were kept for
107 another 12 weeks and fed according to the control regimen (Table 2) (REH2, REH3) to
108 examine whether any effects of juvenile starving on scale shape may be compensated later.
109 Group descriptions are shown in Table 2.

110

111 **Sampling**

112 Scale samples were collected from 20 individuals of each experimental group. One scale was
113 removed from each specimen, from the flank anterior to the dorsal fin (Figure 2A), (8).

114 Scales were then placed between two glass slides and scanned with an HP ScanJet 5300C
115 XPA scanner at 2400dpi. Seven easily definable landmarks were recorded for each scale
116 using tpsUtil (28) and tpsDig2 (29) softwares (Figure 2B). Landmarks 1 and 2 are the ventro-
117 and dorso-lateral tips of the anterior portion of the scale, landmarks 3 and 4 are at the
118 boundary between the area covered by the other scales and the exposed area, landmark 5 is

119 positioned at the tip of the posterior portion of the scale, landmark 6 is in the center of the
120 anterior edge of the scale, and landmark 7 is the focus of the scale.

121

122 **Statistical analysis**

123 Scale shape data were processed with the MorphoJ software package (13). Group identities
124 (ID) were assigned to scales. Scale size was characterized with the scale centroid size, which
125 is the square root of the sum of squared distances between the scale centroid and each
126 landmark, and that is considered as a mathematically shape-free size variable (36).
127 Generalized least-squares Procrustes superimposition (GLS) was performed on the raw
128 landmarks data on the basis of the principal axis so the landmarks were scaled, rotated and
129 aligned into new shape variables (partial warps, PW), independent of the scale size (27). A
130 multivariate regression of shape (dependent variable: Procrustes coordinates) on size
131 (independent variable: logarithm of scale centroid size) was performed for each group.
132 Significance of the relationship (i.e. the presence of an allometric effect) was evaluated by
133 using a permutation test against the null hypothesis of independence (10 000 iterations). As
134 data being free of the allometric effects associated with growth, residuals of this regression
135 provided the basis of further analyses (7). Differentiation of groups was examined with
136 Canonical Variate Analysis (CVA) and Discriminant Function Analysis (DFA). In all cases, a
137 permutation test (10 000 iterations) was performed to test the reliability of results. In case of
138 DFA, cross-validation was also made to test the reliability of classification. For better
139 visibility of the results, averages of the groups were plotted on graphs. Group comparisons
140 from the investigation of the diet impact were classified into five types (“group type”),
141 according to the group relations tested (N vs. N, N vs. H, H vs. H, N vs. REH, and REH vs.
142 H). One way ANOVAs were performed to test the significance of distance data (T-square
143 statistics, Mahalanobis distances) of each group type, and the homogeneity of variances was

144 also tested to determine the appropriate type of post-hoc tests. Since the variances proved to
145 be equal across the compared groups, thus the Tukey HSD test was used for the post hoc
146 comparisons.

147

148 **Results**

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150 Regression of scale shape (Procrustes coordinates) on scale centroid size indicated notable
151 allometry (i.e. dependence of shape on size) in all cases. The predicted percentage of the total
152 variation in scale shape accounted for by the allometric effect was 7.7% ($p < 0.001$) in the
153 experiment comparing AB line versus LF Baska stock and 24.3% ($p < 0.001$) in the experiment
154 comparing N, H and REH treatment groups. Therefore, controlling scale shape data for the
155 scale size effect was necessary in all further analyses.

156

157 **Between stock differences**

158 The two zebrafish stock, the AB line and the LF BASKA stock, kept under the same, optimal
159 conditions, could be distinguished with medium reliability based on scale shape. The average
160 shape and the separation of the groups are shown in Figure 3. The main differences between
161 the two groups were in landmarks 3 and 4, which means that the exposed area was bigger in
162 the LF BASKA stock and bigger area covered by other scales in AB line.

163 Mahalanobis distance (D) between the two groups was 1.5 and indicated a high reliability
164 based on the permutation test ($p < 0.001$). The T-square (115.2) statistics showing average
165 distances of groups from the full sample also showed high reliability ($p < 0.001$). According to
166 the validation results of the DFA, scale shape based group classification showed 81% identity
167 with real groups on average (cross-validated rate was 78.8%) (Table 3).

168

169 **Between feeding regime differences**

170 Treatment groups reared on different diets could successfully be distinguished based on scale
171 shape. The CVA-plot (Figure 4) shows that the H and N groups separated well from each
172 other, while the REH group positioned between the two former groups.

173 Between groups Mahalanobis-distances were: 3.9 ± 1.4 (mean \pm SD) for N_x vs. H_x , 2.8 ± 1.9 for
174 N_x vs. REH_x and 3.2 ± 0.6 for H_x vs. REH_x comparisons, respectively. The mean T-square
175 statistic values between the groups were 266.1 ± 141.5 for N_x vs. H_x , 101.2 ± 59.8 for N_x vs.
176 REH_x and 168.0 ± 55.3 for N_x - REH_x comparisons, respectively.

177 Validation results (Table 3) show that the N, H and REH groups could successfully be
178 classified with an average rate of 96.9% (cross-validated rate was 90.4%). The mean scale
179 shapes of groups are shown in Figure 5. The main differences between groups were that H
180 fish had landmarks 6 and 5 closer to each other reflecting a cranio-caudal flattened scale
181 shape compared to N fish. Scale shape of REH fish proved to be intermediate between scales
182 shapes of N and H group members.

183 Mahalanobis distance test results for between group types comparisons are shown in Figure 6.

184 Distances between N and H groups were significantly greater than the distances within the N-
185 groups, H-groups and between the N and REH-groups, either by using T-square statistics
186 ($F_{4,39}=9.2$, $p<0.001$) or Mahalanobis distances ($F_{4,39}=8.4$, $p<0.001$). However, none of the
187 distances representing the above relations differed significantly from the distances
188 characterizing the H vs. REH groups relations.

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190

191 **Discussion**

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193 Based on positive field experiences, scale shape analysis has recently become a widely used
194 tool for differentiating among populations or stocks of fish species (8, 10, 11, 12, 22, 23, 24),
195 for all that the background of these differences is still not exactly understood. In this study, it
196 was shown however that both genetic and environmental factors contribute to intraspecific
197 variability in scale shape of fish and might induce comparable differences.

198 Our first experiment proved that genetically different zebrafish stocks may be separated based
199 on the shape of their scales. This result supports that intraspecific variability of scale shape of
200 fish has a strong genetic component and genetically isolated populations of fish might have
201 different scale shape patterns in the wild as well. Genetic divergence among metapopulations
202 of fish could successfully be captured earlier in body shape. For example, Marcil et al. (16)
203 documented that genetic divergence between spawning aggregations of Atlantic cod *Gadus*
204 *morhua* L. 1758 caused detectable morphological differences even at small spatial scales
205 (<100 km).

206 Our second experiment proved that the food supply, which is one of the most important
207 environmental factors effecting natural fish populations, can also notably influence the shape
208 of the scale of fish. In zebrafish, scales get flattened in the cranio-caudal direction which
209 cannot be fully recovered after the normalization of feeding conditions. A strong
210 environmental influence seems to be common in morphological characters of fish. Amongst
211 the potential environmental components that affect morphological phenotype, the roles of
212 temperature (16) and feeding conditions (4, 16) are best documented. The composition and
213 the amount of food consumed evidently influence the conditional state, and especially the
214 extent of the fat reserve of fish, which in turn affects the body shape (4). Condition of fish
215 (fish mass relative to fish length) is however may change dynamically during the life span and
216 not only due to the variations in the food resource but also by individual feeding strategies,
217 diseases, ontogenetic stages, and even seasonally according to the reproductive and wintering

218 cycle. Several studies have investigated the effects of starvation on body shape (4, 6, 20, 21,
219 32, 34). These studies shown consistent changes in body parameters related to the condition
220 and fat metabolism of the examined individual, like body depth, and the largest fat depots in
221 the caudal and trunk region (4). Body shape parameters that are influenced by the conditional
222 state of fish might therefore limitedly be applicable for intraspecific stock discriminations.
223 Moreover, according to the above reasons, body level morphometric analyses can also
224 limitedly be used to assess the general environmental characteristic of the habitat from the
225 sample originated.

226 Compared to the shape of the whole body, scale shape is presumably less sensitive to short
227 term environmental effects and instantaneous processes, as well as it is less dependent upon
228 the conditional state of fish. In accordance with the observations of Ibáñez et al.(12), present
229 results showed that although scale shape might also recover partly during the compensatory
230 growth (i.e. with the normalization of feeding conditions), this process is much slower and
231 presumably is not as complete as it is in condition related body shape parameters. Moreover,
232 the ring structure of scales conserves individual life histories of fish, and therefore, by a
233 detailed analysis of scale shape by annuli might provide an excellent possibility of
234 investigating variability of environmental impacts and individual life histories both within and
235 among stocks of fish.

236 Experiments with the zebrafish proved that intraspecific scale shape variations are generated
237 by the interactions of genetic and environmental factors and reflect phenotypic plasticity.
238 Accordingly, information gainable from the morphological analysis of scale samples collected
239 in the field are generally inappropriate to clarify whether the deviation found between scale
240 shapes of two stock of the same species could come from genetic or environmental
241 differences (see also 18).

242 Although, in intraspecific studies, shape analysis of scales seems to have the same limits as
243 the shape analysis of the whole body, namely, based on these analyses only, no decision can
244 be made on the relative importance of genetic and environmental factors being responsible for
245 among group differences, the former method still bears several advantages. Scale sampling is
246 not as stressful for fish as whole body investigation, and therefore, the introduction of the
247 method is highly recommended when investigating protected or endangered fish species. In
248 addition, as the scale method is much easier, time and cost efficient, than the traditional whole
249 body methods, it may be favorable in other cases as well. However, the scale method is not
250 applicable for all fish species. Species that do not have scales (e.g. acipenseroids) or have
251 very small scales [e.g. European eel *Anguilla anguilla* (L. 1758)] can only be examined by the
252 traditional, full-body inspection or examination of other hard formulas (e.g. otolith 1), where
253 the individual does not survive the investigation.

254 To conclude, genetically and dietetically different experimental groups of zebrafish could
255 successfully be distinguished according to the shape of their scales, and the results showed
256 that both genetic and environmental factors may notably influence scale shape formation. It is
257 suggested that scale shape analysis might be used as an explanatory tool to detect potential
258 intraspecific variability of environmental influences impacting genetically homogeneous
259 groups of individuals. However, results also indicated that due to its sensitivity to
260 environmental factors, the applicability of a morphometric scale analysis in identifying
261 intraspecific stock membership could be limited. In order to improve the applicability of the
262 method and to assess its potentials, more laboratory inventories are needed testing the type
263 and extent of effects that the most important environmental stressors (e.g. food, temperature,
264 pH) might have on scale shape.

265

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277

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365 **Table 1.** Feeding regimens applied in the experiments. Fish were fed with SDS (Special Diets
 366 Services Limited International Dietex GB) dry food of increasing granulate size (SDS 100-
 367 400 and SDS Small Gran) supplemented with live *Artemia* nauplii (SERA GmbH). The
 368 remaining food was removed one hour after each feeding. Time is calculated from the
 369 fertilization.

Age of fish	Control regimen	Reduced regimen
1st and 2nd weeks	twice a day SDS 100 and freshly hatched <i>Artemia</i> nauplii	once in every second day SDS 100
3rd to 5th weeks	twice a day SDS 200 and freshly hatched <i>Artemia</i> nauplii	once in every second day SDS 200
6th to 7th weeks	twice a day SDS 300 and freshly hatched <i>Artemia</i> nauplii	once in every second day SDS 300
8th to 12th weeks	twice a day SDS 400 and freshly hatched <i>Artemia</i> nauplii	once in every second day SDS 400
after 12th weeks	twice a day SDS Small Gran and freshly hatched <i>Artemia</i> nauplii	once in every second day SDS Small Gran

370

371 **Table 2.** Experimental design. Description of feeding regimens are given in Table 1.

Group name	Stock	Feeding regimen	Sample size	Rearing time
AB	AB line	Control	99	12 weeks
LF BASKA	LF BASKA stock	Control	99	12 weeks
N1	AB line	Control	20	12 weeks
H1	AB line	Reduced	20	12 weeks
N2	AB line	Control	20	12 weeks
H2	AB line	Reduced	20	12 weeks
N3	AB line	Control	20	12 weeks
H3	AB line	Reduced	20	12 weeks
N4	AB line	Control	20	12 weeks
H4	AB line	Reduced	20	12 weeks
REH2 (originated from H2)	AB line	Control	20	12 weeks
REH3 (originated from H3)	Ab line	Control	20	12 weeks

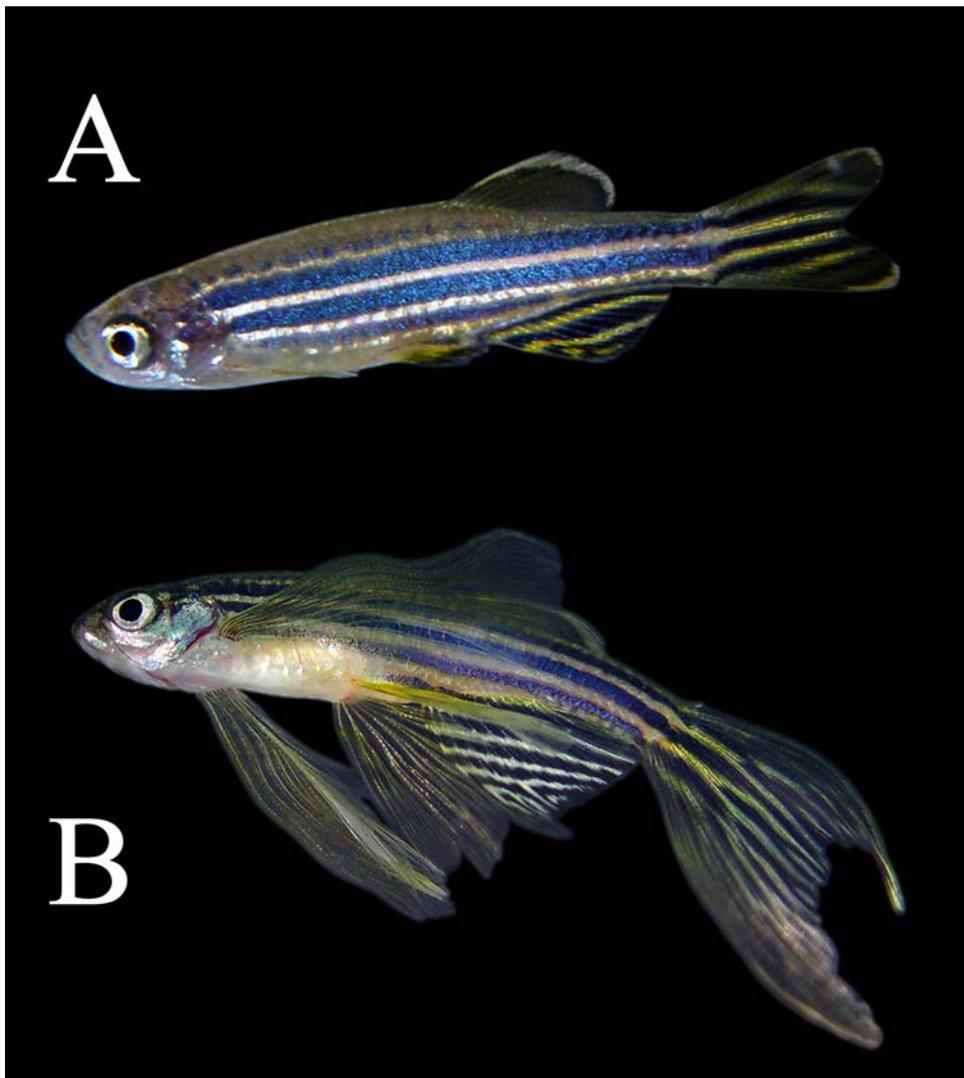
372

373 **Table 3.** Classification rates and significance of the experimental zebrafish group
 374 comparisons. Explanations for group names are given in Table 2.

Groups compared	Sample size per group	Pure classification		Cross-validated classification	
		Rate (%)	χ^2 (p)	Rate (%)	χ^2 (p)
AB vs. LF BASKA	99	81.3	77.7 (<0.001)	78.8	65.7 (<0.001)
N1 vs. H1	20	90.0	25.6 (<0.001)	72.5	8.3 (0.004)
N2 vs. H2	20	100.0	40.0 (<0.001)	95	32.7 (<0.001)
N3 vs. H3	20	100.0	40.0 (<0.001)	97.5	36.2 (<0.001)
N4 vs. H4	20	100.0	40.0 (<0.001)	100.0	40.0 (<0.001)
N2 vs. REH2	20	90.0	25.6 (<0.001)	75.0	19.6 (<0.001)
H2 vs. REH2	20	100.0	40.0 (<0.001)	95.0	32.7 (<0.001)
N3 vs. REH3	20	95.5	33.2 (<0.001)	93.0	28.9 (<0.001)
H3 vs. REH3	20	100.0	40.0 (<0.001)	95.5	33.2 (<0.001)

375

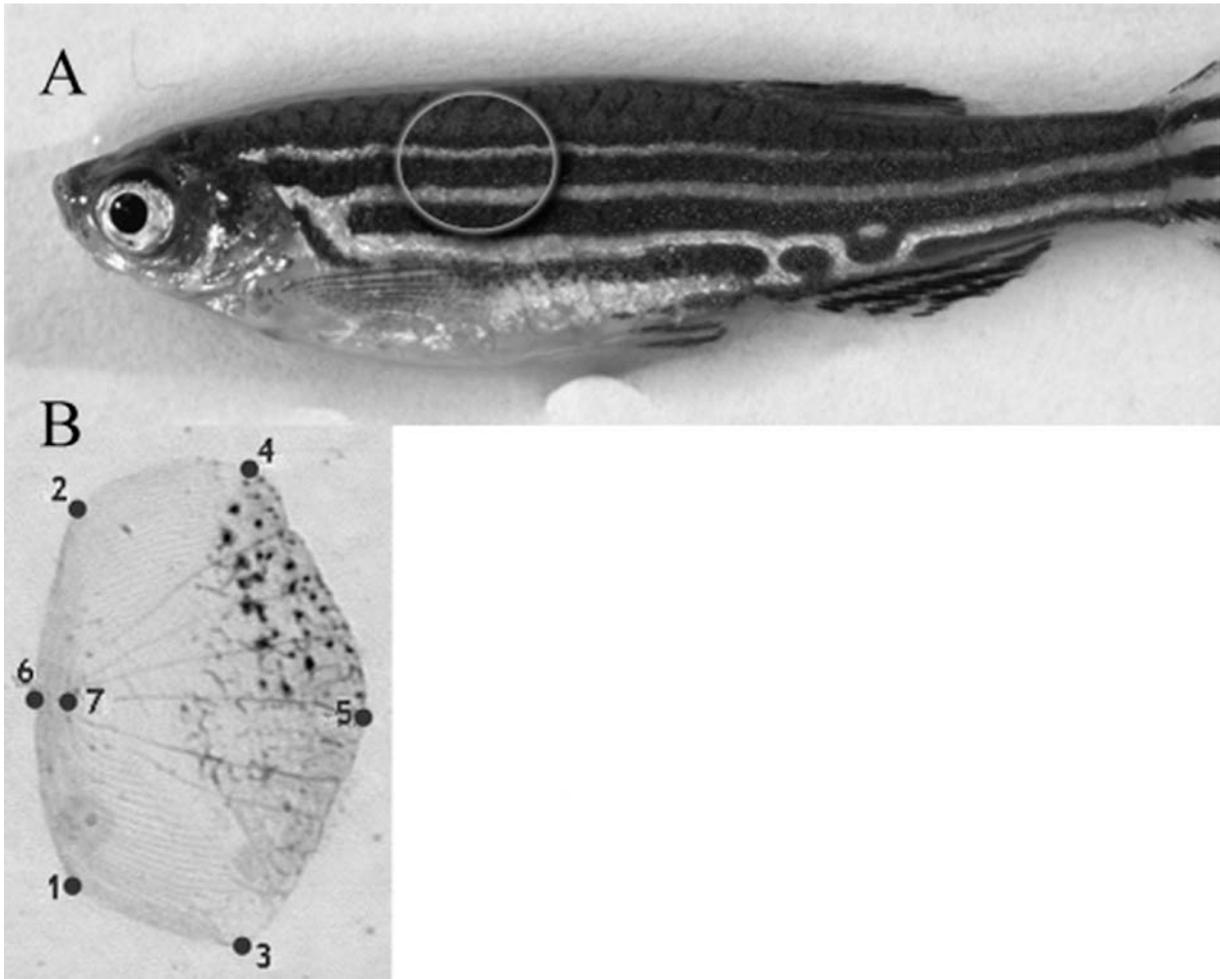
376



378

379 Figure 1. Investigated zebrafish stocks: A) AB line; B) LF BASKA.

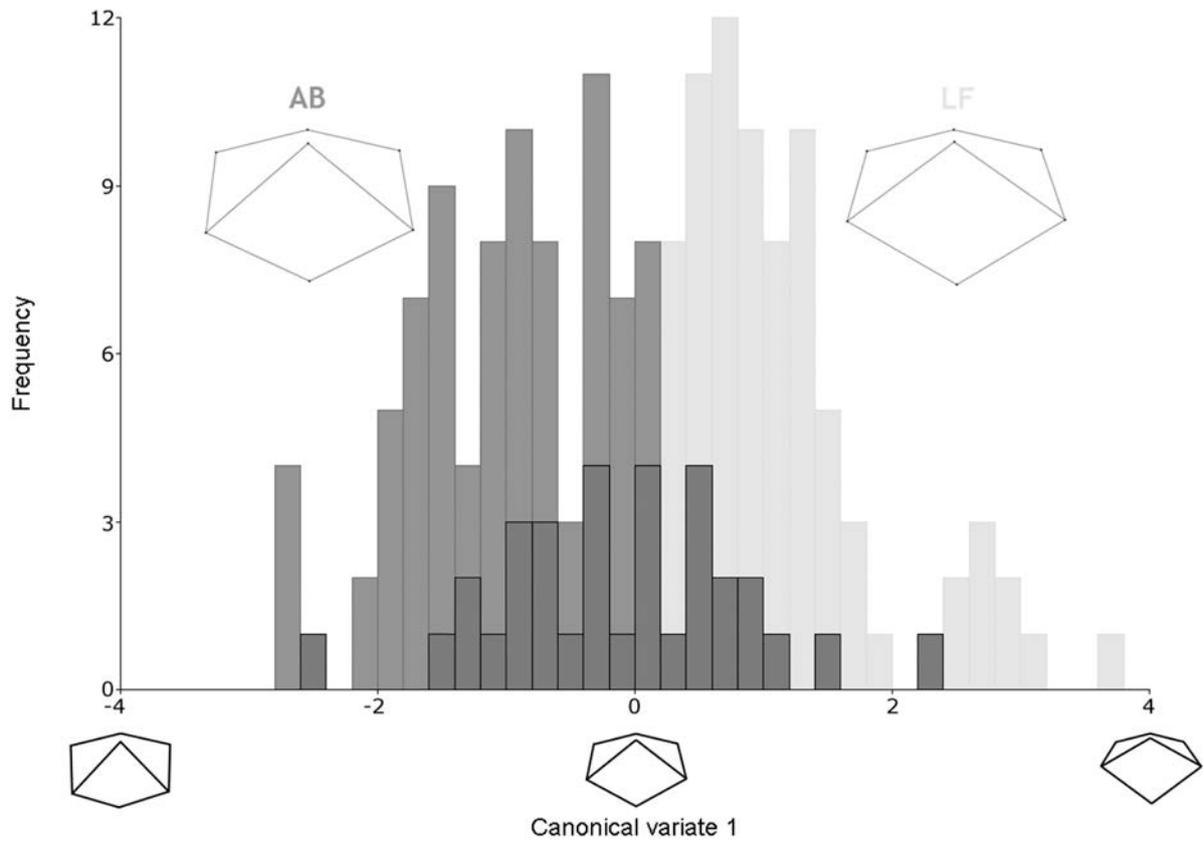
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382 Figure 2. A) Scale sampling area on zebrafish and B) the recorded scale landmarks.

383



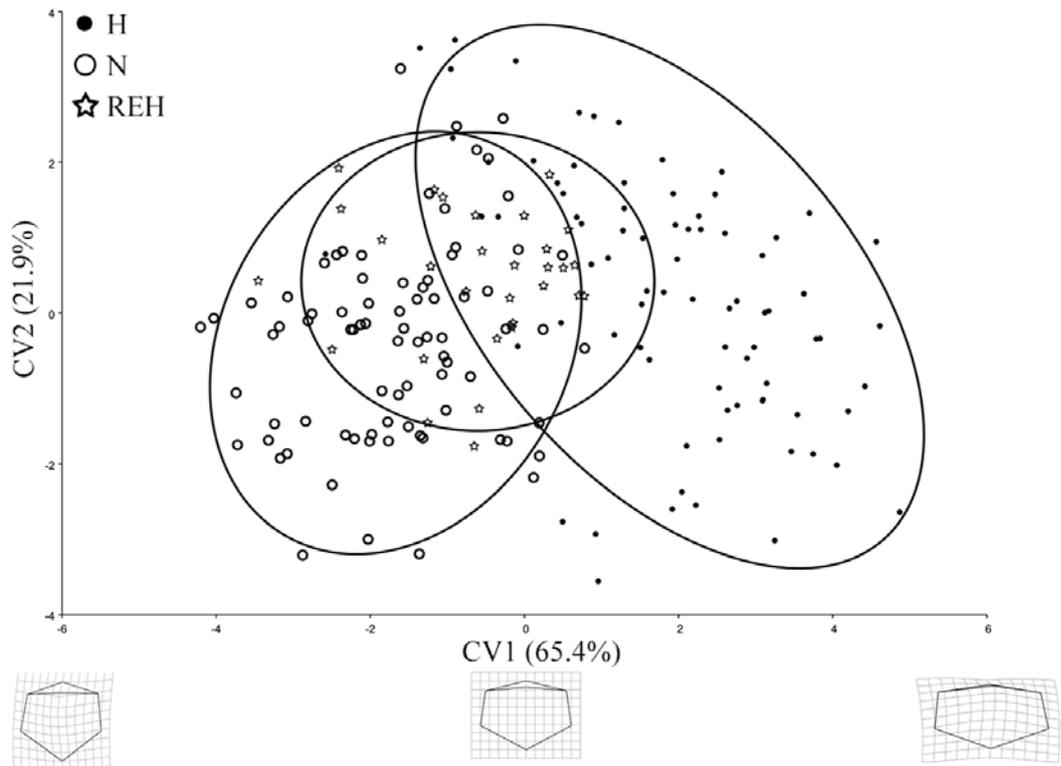
384

385 Figure. 3. Average scale shape differences between the AB (dark grey columns) and LF

386 BASKA (light grey columns) zebrafish stocks according to the Canonical Variate Analysis.

387 The darkest columns indicate overlaps between the two groups.

388

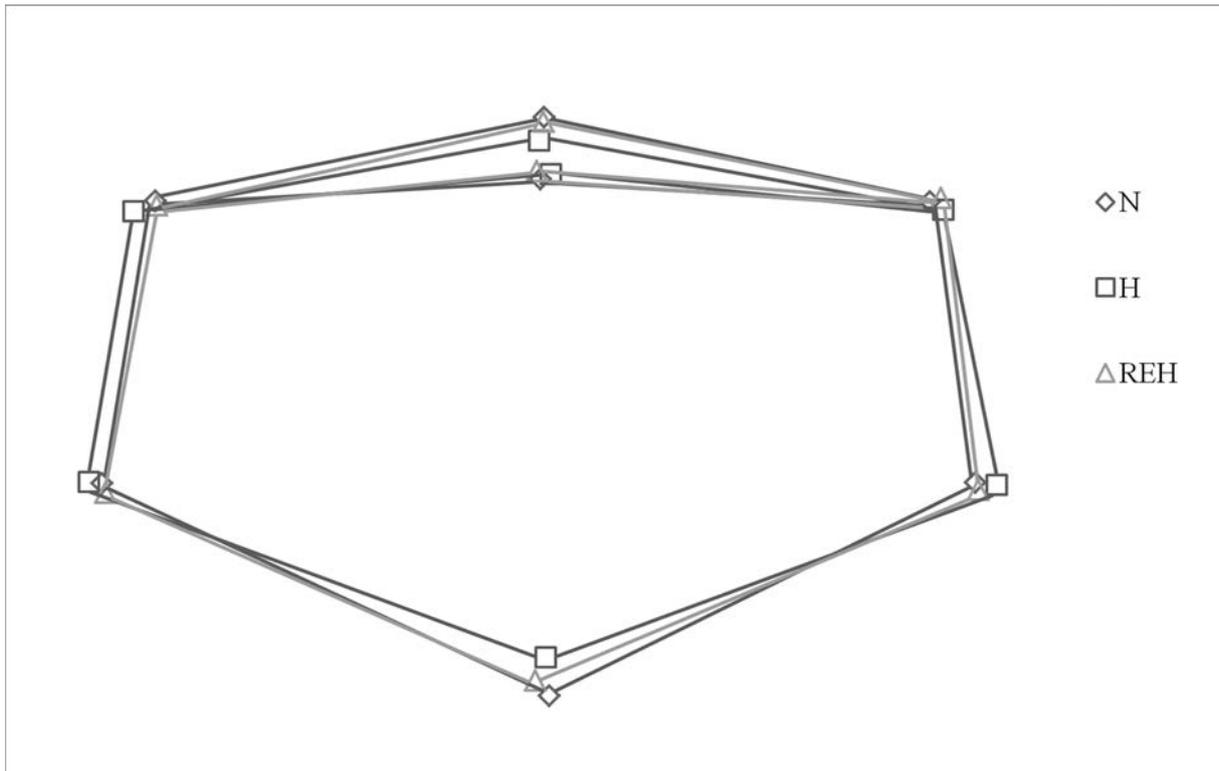


389

390 Figure 4. Canonical Variate Analysis plot comparing scale shapes of zebrafish kept on
 391 optimal (N) and reduced (H) diets and on reduced diet followed by optimal diet (REH).

392

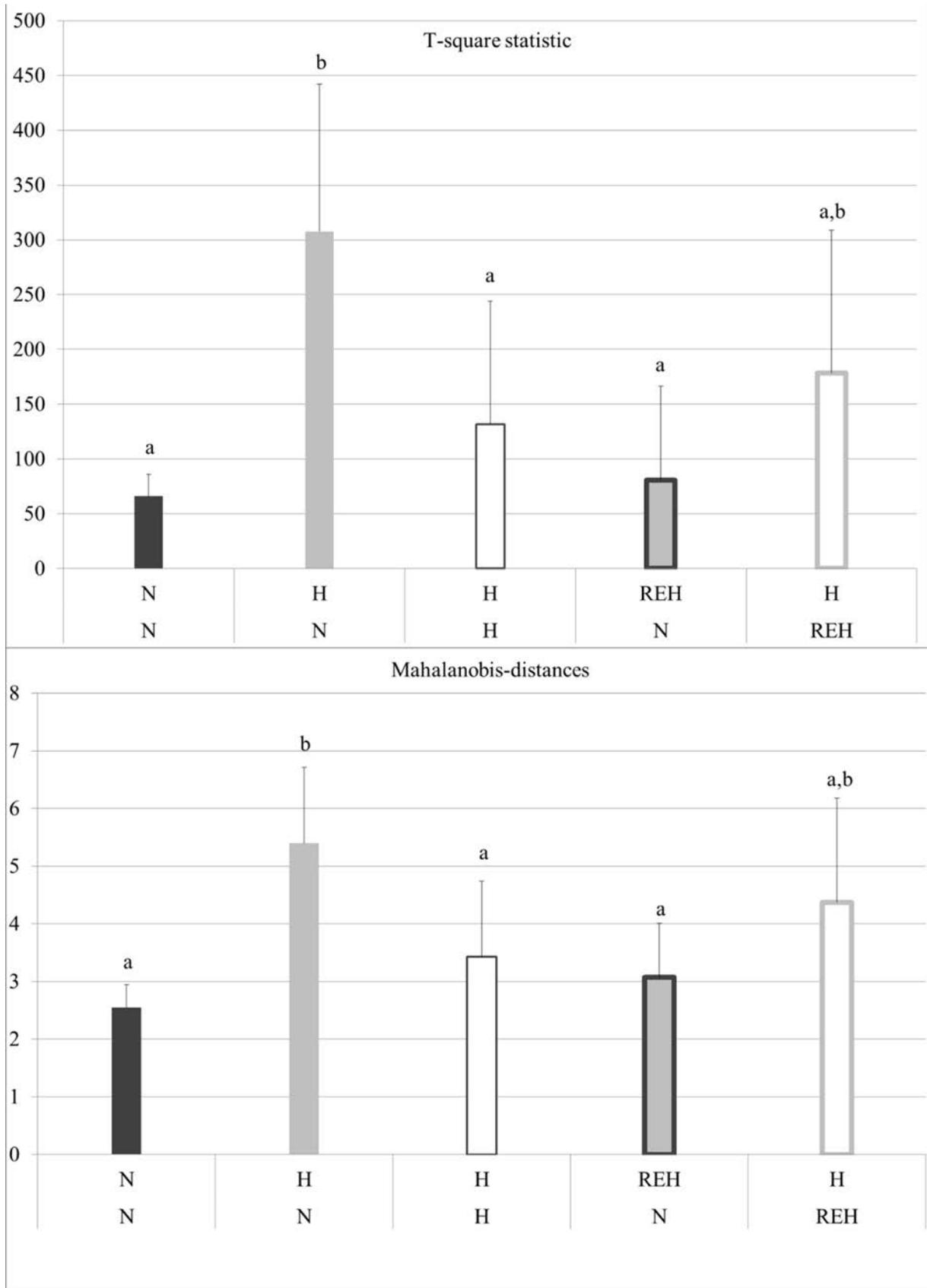
393



394

395 Figure 5. The mean scale shapes of zebrafish reared on optimal (N), reduced (H) and reduced
396 diet followed by optimal diet (REH).

397



398

399 Figure 6. Between treatment group types differences in the scale shape of zebrafish based on
 400 the T-square statistics (T) and Mahalanobis distances (D) (mean±SD). Values marked with

401 different letters are statistically different at $p < 0.05$ according to the one way ANOVA (for T-
402 square statistic: $F_{4,39} = 9.2$, $p < 0.001$; for Mahalanobis distances: $F_{4,39} = 8.4$, $p < 0.001$) followed
403 by Tukey HDS post hoc test. N – optimal diet; H – reduced diet; REH – reduced diet followed
404 by optimal diet.
405