1 **The phenotypic determinants of diet variation between divergent lineages of**

2 **threespine stickleback**

3

4 **Abstract:** Lineages with independent evolutionary histories often differ in both their morphology

5 and diet. Experimental work has improved our understanding of the biomechanics of foraging in

6 fishes and established links between traits and foraging performance (trait-utility). However,

7 because the expression of foraging-relevant traits and their utility can be highly context-specific,

8 it is often unclear how dietary divergence arises from evolved phenotypic differences. Here, we

9 explore the phenotypic causes of dietary divergence between two genetically and phenotypically

10 divergent lineages of threespine stickleback (Gasterosteus aculeatus) with independent

11 evolutionary histories. First, using individuals from a line-cross breeding design, we conducted

12 150 common-garden foraging trials with a community of multiple prey species and performed

13 morphological and behavioural analyses to test for prey-specific trait-utility. Second, we tested if

14 the traits that explain variation in foraging performance among all individuals could also explain

15 the dietary divergence between the lineages. Overall, we found evidence for the utility of several

16 foraging traits, but these traits did not explain the observed dietary divergence between the lineages

17 in common garden. This work suggests that dietary divergence observed in the wild results not

18 only from differences in morphology but also from behaviours that underlie prey choice in species-

19 rich prey communities.

20

21 **Keywords:** Trait utility, dietary divergence, foraging, trait evolution

Introduction

22

23 Lineages with independent evolutionary histories often show divergence in both morphology and

24 resource-use (Grant et al. 1976; Herrel et al. 2008; Bassar et al. 2010; Muschick et al. 2012), but

25 the functional links connecting morphology, behaviour, and diet are often unclear. The

26 ecomorphological paradigm provides a mechanistic framework for understanding the linkages

27 between morphology, performance, and fitness (Arnold 1983). In relation to foraging, both

28 morphological and behavioural traits determine an organism's performance in a particular

29 environmental context, where performance measures can include rates of prey encounter and

30 attack, the kinematics of prey capture, prey processing and overall rate of consumption. Extensive

31 experimental work, particularly involving biomechanics (Higham et al. 2021), has improved our

32 understanding about the relationships between traits and foraging performance in fishes

33 (Wainwright and Richard 1995; McGee et al. 2013; Muñoz 2019). However, compared to

34 morphology and foraging performance, diet is more proximal to the outcomes of fitness relevant

35 tasks (i.e. energy acquisition for survival and reproduction) (Storz et al. 2015), and we often have

36 a poor understanding of how functional trait variation translates into realised diet variation in

37 natural populations. This makes it challenging to understand the underlying ecological causes of

38 evolutionary divergence of consumer diets, particularly if diet variation is complex in nature, and

39 trait utility is inferred from experiments in simple environmental contexts.

40

41 Comparative studies can help us develop hypotheses about the functional links between

42 morphology and diet, but are often unable to establish how morphological variation translates into

43 feeding performance, diet variation, and ultimately fitness variation. This is largely because dietary

44 variation in natural consumer populations is determined by both contemporary resource

45   availability, and the phenotypes of individuals that are expressed in a given environmental context

46   (Araújo et al. 2011). Dietary divergence between populations can result from multiple abiotic and

47   biotic environmental factors, such as habitat structure, prey community composition, and

48   individual interactions with predators and competitors (Araújo et al. 2011; McWilliam et al. 2013;

49   Tewfik et al. 2016). Dietary divergence might correlate with functional trait divergence among

50   populations, if either trait expression depends on resource conditions (Olsson et al. 2007; Levis et

51   al. 2017) or if the traits determining diet in each population have underlying genetic causes

52   (Bolnick et al. 2003). In the latter case, consumer populations might locally adapt to prey

53   communities via, for example, evolution of functional traits in response to local resources (Grant

54   et al. 1976; Herrel et al. 2008; Sanford and Worth 2010; Holding et al. 2018). As a result, testing

55   whether dietary divergence between populations is due to evolved phenotypic differences, plastic

56   trait expression or differences in the resource base, requires rearing and testing the dietary variation

57   of individuals in foraging environments with common prey communities.

58

59   Common garden foraging experiments are frequently used to identify the underlying causes of

60   dietary variation, and are ideally performed in an environmental context that is relevant for

61   understanding the observed dietary variation in nature (Bassar et al. 2010; Arnegard et al. 2014).

62   Such experiments can provide insights into the genetic basis of dietary differences. However,

63   divergent populations typically differ in many heritable traits, which can covary within

64   populations, making it challenging to specify links between functional traits and diets. Performing

65   common garden experiments with phenotypically variable experimental populations, can increase

66   the chance of detecting trait utility, and provide a better mechanistic understanding of functional

67   trait-diet relationships (Arnegard et al. 2014). In this respect, a line-cross breeding scheme between

68   ecologically divergent populations is a particuarly useful way to generate relevant phenotypic

69   variation in a consumer population (Lynch et al. 1998). This is because hybrids can express

70   phenotypes that are either intermediate between parental lineages, or exceed the phenotypic range

71   of their parents (i.e. transgressive segregation). Hybridization can also change the covariances

72   among traits with putative utility (Holzman and Hulsey 2017). The insights gained from such

73   individual level analyses of trait utility are useful for explaining patterns of dietary divergence

74   observed between the parental populations in nature.

75

76   Here, we experimentally test for dietary divergence between two lineages of European

77   freshwater threespine stickleback *(Gasterosteus aculeatus* complex*)*, and explore the underlying

78   phenotypic drivers of dissimilar resource-use in a complex resource context (Figure 1). Marine

79   sticklebacks have repeatedly established freshwater populations that have then adapted to local

80   (resource) conditions (McKinnon and Rundle 2002). Due to the glaciation history of Europe, the

81   timing of freshwater population establishment varies widely across the continent. Stickleback

82   populations were likely established in the Western-European watersheds (e.g. Rhine, Rhône,

83   Seine) prior to the last glacial maximum, and in the North-eastern watersheds (i.e. the Baltic

84   drainage) only after the retreat of the ice sheets ~12,000 years ago (Lucek et al. 2010; Marques et

85   al. 2016, 2019). Recent range expansion and anthropogenic introductions have brought some of

86   these lineages into secondary contact in the pre-alpine lakes of the Swiss plateau, providing a

87   useful study system to explore ecological differences between lineages with independent

88   evolutionary histories of freshwater adaptation (Lucek et al. 2010; Best et al. 2017; Anaya-Rojas

89   et al. 2019; Hudson et al. 2021b).

90

Materials and Methods

Study system

In this study we focus on the stickleback populations of two large pre-alpine lakes; Lake Constance and Lake Geneva (Figure 1 A). Historical ichthyological and genomic evidence suggest that both lakes were colonised only in recent historical times (mid 19th century) by two anciently divergent stickleback lineages (Hudson et al. 2021b). Lake Constance was colonised primarily by fish that belong to a lineage of recent freshwater invaders (~17-5 Kya; (Fang et al. 2018, 2020)), originating from a river system that drains into the Baltic Sea (Lucek et al. 2010). On the opposite side of the Swiss plateau, Lake Geneva was primarily colonised by fish that originated from the upper Rhône, and belong to an older, pre-glacial Atlantic freshwater lineage (Lucek et al. 2010; Marques et al. 2016, 2019). Both lakes also received smaller contributions from the other lineages that are associated with contemporary ecotype formation, which is advanced and strongly developed in Lake Constance (Marques et al. 2016, 2019) but only subtly in Lake Geneva (Lucek et al. 2014a). Lake Constance itself is now populated by a population that lives and grows in the open water of the lake but migrates to spawn both in littoral zones and inflowing streams. This form originates from the East European lineage, whereas resident stickleback populations in the inflowing streams of Lake Constance are of hybrid origins (Marques et al. 2019). In contrast, Lake Geneva itself, as well as its streams, are populated by stickleback that mainly derive from the ancient pre-glacial freshwater lineage (Lucek et al. 2010). The habitat utilisation of the lake population is confined to the inshore littoral zones.

In accordance with their divergent evolutionary histories, Lake Constance and Lake Geneva stickleback differ in various ecological, physiological and morphological phenotypes (Lucek et al.

114   2013; Alexander et al. 2016; Best et al. 2017, Hudson et al. 2021a). Most notably, stickleback of

115   Lake Constance are hyper-abundant in the pelagic zone (Alexander et al. 2016), and express

116   phenotypes that are more typical of marine populations (e.g. extensive body armour, large body

117   size), whereas fish from Lake Geneva are restricted to nearshore habitats and express phenotypes

118   more typical of freshwater populations (e.g. reduction of body armour) (Berner et al. 2010; Lucek

119   et al. 2010, 2013, 2014b). Given these ecological and phenotypic differences between the lineages,

120   it has been previously hypothesised that the lineages express divergent resource-use in their natural

121   environment, with the pelagic foraging population of Lake Constance specialising on zooplankton,

122   and the more littoral populations of Lake Geneva specialising on benthic invertebrates (Best et al.

123   2017; Hudson et al. 2021b).

124

125   *Line-cross to generate a phenotypically variable consumer population*

126   To increase the phenotypic variance of our experimental population we created a line-cross

127   between a pelagic population from Lake Constance and a littoral resident population from Lake

128   Geneva stickleback using stock populations maintained in husbandry (Figure 1B) as described in

129   Hudson et al. (2021a). The stock populations originate from fish sampled in 2013 and 2015

130   (Ecotype / Sampling locations: lake ecotype / Marina Rheinhof, Lake Constance (47°29'51.5148N,

131   09°33'33.2064E); littoral lake ecotype / Le Grande Canal, Lake Geneva (46°23´48.372N,

132   06°53´14.2224E)) (Best et al. 2017). In 2017 we crossed fish in five different parental

133   combinations: Geneva × Geneva (G), Geneva × F_1 Hybrid (BC_G), Geneva × Constance (F_1),

134   Constance × F_1 Hybrid (BC_C) and Constance × Constance. The five resulting offspring populations

135   (from here onwards referred to as cross-types) were reared in the laboratory for two years on a diet

136   of frozen chironomid larvae before they were used in the experiment.

137

138 *Common garden foraging experiment*

139 For the foraging trials, fish (150 total, 30 individuals per cross-type) were individually placed in

140 12L tanks (20 x 30 x 20cm^3) that were supplemented with zooplankton, benthic invertebrates, and

141 plant material, and were allowed to forage for 16 minutes. The foraging trials were conducted in a

142 randomised block design, such that each temporal block contained five fish representing the full

143 line-cross (i.e. G, BC$_G$, F$_1$, BC$_C$, C), which were being tested simultaneously. The zooplankton and

144 benthic invertebrates for the trials were collected from Lake Lucerne (47°00'09.0"N, 8°20'03.2"E)

145 each day before the experiments by pelagic plankton net sampling, and by collecting plant material

146 from a littoral *Chara* bed, respectively. To homogenise the benthic prey availability within

147 experimental blocks, we rinsed off plant material through a sieve (mesh size 0.5mm) retaining

148 macroinvertebrates but washing out fine particles that would otherwise cause turbidity. The

149 separated plant material and the macroinvertebrates were then divided into eight equal parts.

150 Similarly, the zooplankton samples were split into eight equal parts using a plankton splitter. Five

151 samples of zooplankton, plants, and benthos were randomly added to the experimental tanks and

152 one sample was retained as a reference to estimate prey availability and composition. On average,

153 51g of plant material (wet weight; SE = 2g), 423 (SE = 23) benthic prey items, and 563 (SE =

154 38.1) pelagic prey items were added to each tank.

155 Shortly, after supplementing the tanks with the prey, fish were added behind a divider that

156 separated them from the prey for 10 minutes before the beginning of the feeding trial. To initiate

157 the trials, we raised the dividers simultaneously in all five experimental tanks, releasing the fish

158 into the foraging chamber. The foraging trials were video recorded for 16 minutes with two

159 cameras per tank (GoPro Hero 7) positioned from the top and the front. To conclude the foraging

160   trials, the fish were removed from the tank, euthanized with an overdose of MS-222, and frozen at

161   -80°C.

162

Analysis of foraging behaviour

164   To analyse fish foraging behaviour, we inspected the video recordings using the event-logging-

165   software BORIS (Friard and Gamba 2016). We distinguished between four different types of

166   feeding strike events: *benthic strikes* - those directed at the substrate/ground; *pelagic strikes* - those

167   occurring in the water column; *wall strikes* - those directed at the walls of the tank; and *surface*

168   *strikes* - those directed at the water surface. While observing these strikes we could neither

169   determine which prey items were being targeted, nor which strikes resulted in successful

170   ingestions. As a result, we relied on gut content analysis to determine individual diet variation (see

171   below). We additionally measured *non-foraging behaviour* as a summation of: *inactivity* - the time

172   a fish spent motionless in the plant material (e.g. hiding), and *self-reflection* - the time moving up

173   and down the glass walls of the foraging tanks, presumably interacting with their own reflection.

174

Analysis of diet

176   We removed the guts of the fish, and inspected the gut contents via dissection microscope, creating

177   a diet abundance matrix distinguishing 22 prey types (see table S1). To explore the dietary

178   variation among individuals, we Hellinger-transformed the diet-matrix, to reduce the influence of

179   prey types with low and zero values, and analysed it with a PCA (Borcard et al. 2011). Based on

180   this ordination, we grouped prey into three categories: 'plankton' ($PC1 < 0$), 'soft-bodied benthos'

181   ($PC1 > 0$, $PC2 > 0$), and 'hard-bodied benthos' ($PC1 > 0$, $PC2 < 0$) (Figure S1), which were largely

182   dominated by *Cyclops* , Chironomidae, and *Asellus* (Isopoda), respectively. We excluded five prey

183   types from the analysis (*Pleuroxus sp., Chydorus sp.* and Harpacticoida, adult insects, and digested

184   chironomidae remains) that occurred in the guts, but resulted from the feeding history in our

185   husbandry tanks despite a 24h fasting period prior to the trials. To obtain an estimate of the total

186   ingested biomass the gut contents of each fish were dried at 50° C for 12h and weighed.

187   To test for differences in diet contents between the cross-types we conducted distance-

188   based redundancy analysis (db-RDA) using the capscale() function in the R package vegan

189   (Oksanen et al., 2019). We tested for the significant differences in dietary composition of the cross-

190   types and sex on the Hellinger-transformed diet-matrix by permuting cross-type and sex within

191   blocks 999 times. We repeated this analysis with a reduced dataset containing only Lake Constance

192   and Lake Geneva fish. With the reduced dataset we also tested for lineage-effects on the absolute

193   number of ingested prey per prey categories ('plankton', 'hard-bodied benthos' and 'soft-bodied

194   benthos') using a GLMM model with block as a random effect and estimating p-values in an

195   ANOVA type II analysis with the function ANOVA() from the R package car (Fox and Weisberg,

196   2019).

197

198   *Analysis of individual morphological variation*

199   After dissection (for gut removal and sexing), we stained the fish with alizarin red and bleached

200   them with hydrogen peroxide to highlight bony features (McGee and Wainwright 2013). The fish

201   were then photographed laterally twice, once with a closed mouth, and once with their mouths

202   open and their jaws fully protruded. On these photographs we placed 11 landmarks to obtain linear

203   trait measurements using the high throughput phenotyping pipeline *phenopype* (Lürig 2021).

204   These landmarks comprise five traits and trait systems that have been previously associated with

205   foraging performance variation in stickleback (McGee et al. 2013; Schmid et al. 2018): i) gape

206 width, ii) epaxial height, iii) jaw protrusion, iv) lever ratio of lower jaw, and v) the kinematic

207 transmission of the opercular four-bar linkage. For more details see Figure S3 and Table S2.

208   To explore morphological variation among cross-types, and sexes we performed an

209 ANOVA to compare standard length between cross types, and type III ANCOVAs on the traits

210 (log transformed), including standard length as a covariate using the R package car (Fox and

211 Weisberg, 2019). We repeated this same analysis including only Constance and Geneva fish, in

212 order to specifically understand the level of phenotypic divergence between the parental lineages

213 when reared in common garden. Four fish were removed from all morphological analysis because

214 they were damaged during the staining process.

215

216 *Path analysis to identify trait utility*

217   To gain a more comprehensive understanding of how phenotypic variation contributes to

218 prey-specific variation in foraging performance among the individuals, we fitted a Bayesian path

219 analysis to explain the number of ingested 'plankton', 'soft-bodied benthos', and 'hard-bodied

220 benthos' based on sex and body size, a suite of functional traits (jaw protrusion, epaxial height,

221 gape width, lower jaw lever ratio, opercular-four bar linkage); the number of benthic and pelagic

222 strikes; the total number of available benthic and pelagic prey items; and temporal block as a

223 random effect. In this model, the total weight of the ingested biomass during the experiment was

224 included, and modelled as a function of the abundance of three main prey types. For the

225 hierarchical structure of the model see Figure S5. This full experimental path analysis allowed us

226 to include all predictor and response variables and model the dependencies between traits and

227 performance while simultaneously accounting for other sources of variation. For example, we were

228 not reliant on size correcting trait values, but could both include body size and the (biologically

229 more relevant) raw trait values into the model (Schmid et al. 2018). We did not include cross-type

230 as an explanatory variable in this analysis, because we aimed at understanding trait utility across

231 the full phenotypically diverse experimental population. This gave us more power to detect trait

232 utility relative to analyses either within or between cross-types. We form our conclusions on the

233 basis of interpreting those parameter estimates whose 95% credible intervals of effect size do not

234 overlap with zero (Schmid et al. 2018).

235

236 *Explaining between cross-types and lineage diet variation with functional traits*

237 While the full-experimental path analysis aimed at identifying traits that are associated with

238 prey-specific foraging performance at the individual level across all individuals, we also wanted

239 to understand which functional traits are driving dietary (i.e. compositional) differences between

240 cross-types and sexes. To do this, we tested for correlations between the mean trait values of cross-

241 types and sexes and their mean position on the first two axes of the constrained ordination of the

242 gut contents - 'CAP1 (full)' and 'CAP2 (full)' (Figure 3A).

243 To specifically test for the phenotypic determinants of dietary divergence between

244 Constance and Geneva fish, we conducted a second path analysis on a reduced dataset with only

245 Constance and Geneva fishes. In this path analysis 'CAP1 (Constance-Geneva)' and 'CAP2

246 (Constance-Geneva)' were the response variables, and lineage, sex, body size and the set of

247 candidate traits were explanatory variables (for path structure see Figure 5).

248

249 The experiments were conducted in accordance with animal experimentation permits, that

250 were ethically reviewed, approved, and issued by the Kanton Luzern (Permit #29801: Effects of

251 environmental variation in resource availability on ecological divergence in stickleback).

252

Results

254 *Foraging behaviour*

255 On average, fish made 28.5 feeding strikes per 16 min trial, and cross-type did not differ in the

256 number of total strikes, when non-feeding behaviours were taken into account (Cross: $F = 2.209$,

257 $p = 0.0709$) (Figure S3). Pelagic strikes were the most common (mean 13.9/trial), followed by

258 benthic strikes (11.2/trial), wall strikes (2.25/trial) and surface strikes (1.14/trial). Cross-types

259 significantly differed in the number of wall strikes (Cross: $F=3.549$, $p= 0.0086$), with Constance

260 and F_1-Hybrid fish engaging in fewer wall strikes than Geneva fish (TukeyHSD; $p = 0.031$ and p

261 $= 0.017$, respectively). Cross-types did not differ in the number of pelagic strikes (Cross: $F =1.853$,

262 $p = 0.122$), benthic strikes (Cross: $F = 1.132$, $p = 0.344$), and surface strikes $F = 1.166$ p, $p =$

263 0.3284). In subsequent analyses, in order to ensure that fish with low feeding activity were not

264 driving patterns of dietary variation, fish in the lower quartile of feeding activity (i.e. the 25% of

265 fish with the lowest feeding activity; < 8 feeding strikes; 41 fish) were excluded. Furthermore,

266 because many fish had zero occurrences of wall and surface strikes, these strike types were not

267 considered in subsequent analyses.

268

269 *Path analysis: identifying functional traits*

270 To identify traits that explain foraging performance (i.e. functional traits in the experimental

271 context) we built a full-experimental path model, that allowed us to identify correlations between

272 morphological and behavioural variation and the foraging performance on different prey categories

273 (i.e. the number of ingested 'hard-bodied benthos', 'soft-bodied benthos' and 'plankton') (Figure

274 2). We identified four morphological traits and behaviours that appeared relevant and functional

1

275   in this experimental context: The number of ingested 'soft-bodied benthos' was positively

276   associated with the number of *benthic strikes*, and the opercular-four bar linkage. The number of

277   'hard-bodied benthos' ingested was positively associated with the number of *benthic strikes* and

278   fish *gape width*, and negatively associated with the number of *pelagic strikes* and fish *epaxial*

279   *height.* Additionally, males ingested more 'hard-bodied benthos' than females. The number of

280   ingested 'plankton' was positively associated with both the number of *benthic and pelagic strikes*

281   (i.e. overall activity), and negatively associated with the *lower jaw lever ratio*. Males ingested

282   more 'plankton' than females, and there was a direct negative effect of *body size* on the number of

283   ingested 'plankton' (i.e. not mediated via another trait). The total ingested biomass was positively

284   correlated with the number of ingested 'hard-bodied benthos' but not with the other prey types. To

285   see the results of the full path model results including all significant links see Table S5 and Figure

286   S5.

287

288   *Morphological variation among cross-types and parental lineages*

289   Using ANCOVAs we explored the morphological variation between sexes and the five cross-

290   types. The three linear traits (gape width, jaw protrusion, and epaxial height) scaled positively with

291   standard length, whereas the two ratio-based functional trait systems (lower jaw lever ratio, and

292   the opercular four-bar linkage) were size independent. Sex affected all traits except epaxial height.

293   Gape width and jaw protrusion differed between cross-types, and jaw protrusion was additionally

294   affected by a significant cross-by-sex interaction (see table S3 for a complete list of the ANCOVA

295   results).

296

297   Repeating the same analysis including only Constance and Geneva fish (table S4), we found that

298   in the parental lineages only gape width differed between the sexes, with males having larger gapes

299   than females (Ancova; Sex: F = 9.2486, p= 0.004). The lineages did not significantly differ in

300   gape width and epaxial height, and only marginally in the lower jaw lever ratio (Ancova; Lineage:

301   F = 3.64, p = 0.06). Jaw protrusion was affected by a lineage-by-sex interaction (Ancova; Lineage:

302   F = 10.02, p = 0.004), such that in Geneva fish, males had larger jaw protrusion than females

303   (Ancova$_{\text{Geneva only}}$; Sex: F = 1.77, p =0.19), while the sexes did not differ in Constance fish

304   (Ancova$_{\text{Constance only}}$; Sex: F = 10.02, p = 0.004).

305

306   *Dietary divergence in common garden*

307   To identify the axes of dietary composition that were best explained by cross-types and

308   sex, we conducted a constrained ordination to the diet matrix containing all fish, as well as a

309   reduced diet matrix only containing Constance and Geneva fish only. Cross (F = 2.6951, P = 0.005)

310   but not sex (F = 2.0450, P = 0.070) had a significant effect on the diet composition in the both

311   model containing the full line-cross (Figure 3A), and in the model containing only the Constance

312   and Geneva lineages (Cross: F=3.9533 P = 0.01; Sex: F=1.2649 P=0.250) (Figure S2). For both

313   models, the first axes of variation ('CAP 1 (full)', and 'CAP1 (Constance - Geneva)') represent a

314   compositional gradient from 'soft-bodied benthos' to 'plankton' and 'hard-bodied benthos'. The

315   second axis ('CAP2 (full)', and 'CAP2 (Constance - Geneva)') represents a shift from 'hard-

316   bodied benthos' to 'plankton'.

317   Consistent with the multivariate analysis of diet composition, Geneva fish ingested more

318   'hard-bodied benthos' (Lineage: χ^2=30.39, p = 3.53e-08) than Constance fish, but fewer 'soft-

319   bodied benthos' (Lineage: χ^2=3.86, p =0.05). Males of both lineages ingested more 'soft benthos'

320   than females (Sex: χ^2=17.93, p =2.29e-05), and only males from the Constance lineages ingested

321   more 'plankton' than females (Lineage:Sex: χ^2=17.60, p =2.718e-05) (Figure 3 B-D, Table S6).

322

323   *Explaining among cross-type and linage dietary variation with functional traits*

324   To test whether the observed differences in the dietary composition among the cross-types

325   can be explained by differences in the functional traits, we tested for correlations between mean

326   functional trait values and the mean position on the axes of dietary variation of cross-types and

327   sexes (Table S7). The mean position on the dietary axis 'CAP1 (full)' was positively correlated

328   with the among cross-types and sex variation in the number of pelagic strikes (Pearson's

329   correlation coefficient; r = 0.77, p = 0.01). Thus, the shift from a diet containing more 'soft-bodied

330   benthos' to one with more 'plankton' was associated with variation in mean pelagic foraging

331   activity between the cross-types and sexes (Figure 4A). 'CAP2 (full)' was negatively correlated

332   with body size (r = -0.75, p = 0.01), gape width (r = -0.81, p = 0.00), and epaxial height (r = 0.65,

333   p = 0.04) (Figure 4B-C). Both gape size and epaxial height scale with body size, and the patterns

334   might thus be partially driven by body size variation among the cross-types (Figure 4C). However,

335   as the full experimental model path analysis suggests that body size has a direct negative effect on

336   the performance on 'plankton', the positive effect of body size on 'hard-bodied benthos' is

337   mediated via gape width (Figure 2). Because epaxial height scales with body size, the negative

338   effect of epaxial height on feeding 'hard-bodied benthos' found at the individual level, is likely

339   overshadowed by the differences in body size among populations.

340   In a second path analysis we specifically tested for determinants of divergence in the diet

341   composition between Constance and Geneva fish (Figure 5). This analysis revealed a direct

342   correlation between Lineage and the first axis of dietary divergence ('CAP1 (Constance -

343 Geneva)') but not the second axis, consistent with the results of the permutation test. However,

344 there was no indirect link between lineage and the diet axes, suggesting that the dietary differences

345 between Constance and Geneva fish are not mediated by the traits included in the analysis (Table

346 S8).

347

Discussion

349 In our common garden experiment, we observed divergent resource-use between two

350 phenotypically and genetically distinct lineages of lake dwelling stickleback when foraging in a

351 complex resource setting (Figure 3). To investigate the underlying phenotypic causes of this

352 dietary divergence, we tested for associations between traits and foraging performance (trait

353 utility) on three relevant prey categories (Figure 2), using individuals from a line-cross to increase

354 the phenotypic variance. We found that morphological and behavioural traits explained both

355 performance variation among individuals, and dietary variation among cross-types (Figure 2).

356 However, these same traits did not explain the observed dietary divergence between the parental

357 lineages (Figure 4, 5). In the following sections, we discuss these findings in the context of the

358 ecomorphological paradigm.

359

360 *Trait utility: Identifying functional traits*

361 Previous work has identified traits and trait systems that predict the biomechanical forces

362 and kinematics that fish predators apply on their prey, and that may thus play an important role in

363 explaining prey-capture success of sticklebacks (McGee et al. 2013; Thompson et al. 2017; Schmid

364 et al. 2018). However, how variation in these traits (and their predicted functions) translates into

365 variation in foraging performance depends on the habitat-context and composition of available

366   prey. We therefore tested a set of candidate traits for their utility with respect to catching plankton,

367   soft-bodied and hard-bodied benthos in a mixed resource context. We emphasise that we measured

368   foraging performance as the number of prey items ingested during the experiment, and not

369   biomechanical performance parameters related to foraging (e.g. suction forces, strike velocities,

370   etc).

371   In general, our results confirm that traits variation can explain some variation in foraging

372   performance among individuals, albeit in a prey-specific manor (Figure 2). However, some of the

373   trait-performance relationships we found were contrary to what we expected based on their

374   predicted biomechanical function. Firstly, we found that the number of ingested plankton was

375   positively correlated with overall feeding activity (benthic and pelagic strikes), which is

376   unsurprising given that the plankton, unlike benthic invertebrates, were distributed throughout the

377   tank, and so fish could ingest plankton using both benthic and pelagic strikes. However, the number

378   of ingested plankton was negatively associated with both body size, and the lower jaw lever ratio.

379   This result is surprising, since an increasing lever ratio conveys a higher degree of kinematic

380   displacement advantage of the lower jaw, and is therefore typically associated with populations

381   feeding on evasive limnetic prey, such as copepods (Alfaro et al. 2004). Secondly, the number of

382   ingested 'soft-bodied' benthic prey items was positively associated with benthic feeding activity

383   (isopoda were largely associated with the plant material) and the calculated kinematic transmission

384   of the opercular-four bar linkage, which affects the force and speed of jaw opening and protrusion

385   (Westneat 2004). Like the lower jaw lever ratio, variation in the opercular-four bar linkage has

386   been previously associated with limnetically foraging stickleback populations and success on

387   zooplankton feeding (McGee et al. 2013; Thompson et al. 2017). Finally, the number of ingested

388   hard benthos was negatively correlated with pelagic activity and epaxial height, and positively

389   correlated with benthic activity and gape width. Epaxial height and gape are counteracting traits

390   in generating the suction force applied on prey items: epaxial musculature generates the suction

391   force, but larger gape increases the volume of water this force is applied to, reducing the overall

392   suction force (Wainwright et al. 2007). Therefore, foraging on benthos (i.e. prey whose capture

393   requires high suction force) is typically associated with large epaxial height relative to gape.

394   However, the ingestion of large prey items is also limited by gape width (i.e. gape limitation),

395   which is likely causing the positive correlation between gape width and 'hard-bodied benthos'.

396   Some of these surprising relationships between morphological and performance variation

397   might be due to variation in behaviour, which is known complicate relationships between *maximal*

398   *biomechanical* performance (predicted by morphological traits) and *foraging* performance

399   (Garland and Losos 1994). In our experiment, for example, individuals that largely engaged in

400   non-feeding behaviours had a low feeding activity (number of total strikes) (Figure S4). In this

401   trivial case, behavioural variation (engaging in non-feeding behaviours) disrupted the relationship

402   between morphology and foraging performance, because individuals with low feeding activity

403   exhibit poor performance irrespective of their morphology. More importantly, variation in prey

404   choice in species-rich prey communities might alter trait-performance relationships due to

405   variation in preference and species-specific encounter rates. For example, previous work with

406   stickleback has both predicted (McGee et al. 2013) and observed (Schmid et al. 2019) that jaw

407   protrusion improves pelagic foraging efficiency on plankton. However, in our current study with

408   in similar experimental setup as Schmid et al. (2019) (e.g. same tank volume, and plankton

409   community from Lake Luzern), albeit with a benthic community added, jaw protrusion was neither

410   an important predictor of diet variation for any of the prey categories (Figure 2) nor of dietary

411   divergence between the two lineages (Figure 5).

412   In natural settings, variation in habitat choice among consumers can largely determine the

413   prey availability and composition, and, consequently, which traits are functionally important for

414   prey capture. Such variation in how individuals interact with their environment might result in

415   differential selection pressures, and the behavioural variation that is underlying these interactions

416   can be highly context dependent. Although such effects of behaviour are often studied in the

417   context of choosing abiotic environmental conditions (e.g. Bogert effect in relation to

418   thermoregulation and physiological adaptation) (Muñoz 2021), behaviour can similarly situate

419   individuals into foraging habitats with contrasting prey communities that influence the evolution

420   of morphological-foraging traits. Thus, behavioural variation might be a primary phenotypic driver

421   of how traits relate to performance and, ultimately, fitness (Garland and Losos 1994). For example,

422   in the emergence of trophic novelty in Caribbean pupfishes, genes associated with foraging

423   behaviour underwent selective sweeps prior to those associated with foraging morphology

424   (Richards et al. 2021).

425

426   *Do functional traits explain dietary differences between cross-types and lineages?*

427   For traits to explain dietary differences between groups of individuals (e.g. lineages) they

428   i) must be functional (i.e. they explain performance differences among individuals) and ii) differ

429   between the groups. We found that four of the behavioural and morphological traits that explained

430   performance variation among individuals, also explained dietary variation among the cross-types

431   (split by sex) (Figure 4). Among cross-type/sex variation in the number of pelagic strikes was

432   positively correlated with variation in the first diet axis ('CAP1 full'), which captures a shift from

433   'plankton' to 'soft benthos'. Body size, gape, and epaxial height were negatively correlated with

434   the among cross/sex variation on the second diet axis, ('CAP2 full'), which represents a decreasing

435   proportion of large benthos (predominantly isopods). Body size, gape, and epaxial height covary,

436   and this makes it challenging to interpret their independent effects. However, the path analysis has

437   revealed that - at the individual level - epaxial height was negatively associated with feeding on

438   'hard-bodied benthos' (Figure 2). Because epaxial height scales with body size, and because cross-

439   types and sexes differ in size, this could explain the positive relationship at the cross/sex level. The

440   same may be true for gape, however, the path analysis has revealed that gape mediates the positive

441   relationship between size and hard benthos at the individual level, (i.e. large individuals have high

442   performance on 'hard-bodied benthos' *because* they have a large gape width; note the path from

443   body size to 'hard-bodied' benthos via gape in Figure 2). Thus, we conclude that the dietary

444   variation among cross-types/sexes at least partially arises from variation in pelagic feeding activity

445   (predicting the ingestion of plankton), and gape width (limiting the ingestion of large benthic prey

446   items).

447   While some traits explained among cross-type variation in diet, those same traits did not

448   explain the dietary divergence between the Constance and Geneva lineages (Figure 3, 5, S2).

449   Previous work on wild-caught fish has documented a wide range of morphological differences

450   between the lineages (e.g. body depth, gill raker length, eye diameter, lateral plate number), and

451   differences in foraging efficiency on plankton (Lucek et al. 2013; Best et al. 2017;). In the current

452   study, using common-garden reared fish, we found limited morphological divergence between the

453   two lineages for several functional foraging traits (e.g. see Jaw protrusion in Table S4). On the one

454   hand, perhaps there was insufficient morphological variation to explain the observed diet

455   divergence. On the other hand, much of the divergence in functional foraging traits observed in

456   the wild may result from phenotypic plasticity, and may only be expressed if the individuals

457   develop in their natural environment (e.g. the pelagic Lake Constance stickleback). Such context-

458   specific expression of the traits that underlie foraging performance is common in many species

459   (Olsson et al. 2007; Levis et al. 2017), and can obscure our detection of foraging trait utility,

460   particularly for consumers feeding on diverse prey communities in natural populations.

461   So, what explains the dietary differences between Lake Constance and Lake Geneva fish?

462   Although we observed clear dietary divergence between Lake Constance and Lake Geneva fish

463   (Figure 3, 5, S2), it is possible this could be explained by variation in the choice of foraging habitat

464   and/or prey, rather than a strict morphology-mediated ability to catch prey (Figure 5). In our

465   experiment, Lake Constance fish predominantly ingested 'soft-bodied benthos' while Lake

466   Geneva fish ingested more 'hard-bodied benthos' (Figure 5, Table S6). The lineage-specific

467   differences in plankton consumption depended on the sex (Table S6), with the lowest consumption

468   by Constance females (Figure 3B). In the wild, Lake Constance fish feed predominantly on pelagic

469   prey outside of the breeding season (Bretzel et al. 2021, Hudson et al. 2021b). However, Lucek et

470   al. (2012) found that those Lake Constance fish that move into small streams to spawn during the

471   breeding season largely forage on benthos (and specifically Chironomidae). Some of the

472   experimental dietary differences between the lineages may reflect the ability to deal with the

473   different anti-predator strategies of benthic prey species. Many benthic chironomidae are tube-

474   dwelling, which provides protection against predation (Hershey 1987; Robinson 2000). However,

475   this mechanism was largely lost in the experiment when the invertebrates were separated from the

476   plant material, potentially turning them into 'easy-to-catch' prey, even for fish that may otherwise

477   be less proficient at capturing benthos (i.e. Lake Constance fish). However, isopods, which have

478   both cryptic coloration and chitinous exoskeletons, were mainly preyed upon by Geneva fish, but

479   we were unable to find a trait that could explain this prey preference or ability. Another possibility

480   is that the dietary differences between the lineages might reflect different degrees of neophobia

481   (Thomas et al. 2010). All the fish were reared in the laboratory on a fixed diet of chironomids and

482   so did not encounter any of the other prey items during their lifetime. More generally, it also always

483   possible that our set of candidate traits does not include the most relevant morphological or

484   behavioural traits that are responsible for the dietary divergence between the lineages.

485

486   *Conclusion*

487   Differential resource-use is a common feature of phenotypically divergent consumer populations,

488   however, we often lack a mechanistic understanding of what drives populations to interact

489   differently with their prey communities in nature. The ecomorphological paradigm suggests that

490   variation in performing ecologically relevant tasks like foraging is the product of functional

491   phenotypic differences among individuals. While our individual level analysis supports this notion

492   (i.e. Figure 2), we also found that dietary differences in common garden between parental lineages

493   could not be explained by functional traits identified at the individual level. We suspect this is due

494   to unmeasured behavioural differences between the members of each lineage affecting prey choice,

495   which can only be expressed under complex resource conditions (i.e. diverse prey assemblages).

496   The role of behavioural variation in connecting functional traits with fitness variation is still very

497   much ambiguous; behavioural variation may lead to differential selection by exposing individuals

498   to different selective agents, and simultaneously shield traits from selection by disrupting links

499   between form and function (Muñoz 2021). In nature, behavioural variation such as prey and habitat

500   choice may largely determine prey availability and composition, and, consequently, dictate which

501   traits become functionally important. Thus, to understand the functional underpinnings of

502   performance variation, and ultimately fitness, traits must be understood in the context of the

503   behavioural variation of individuals expressed in the appropriate environmental contexts. For

504   foraging traits in particular, this will require more work on the expression and functional

505   significance of behavioural traits in as natural prey-community contexts as possible.

506

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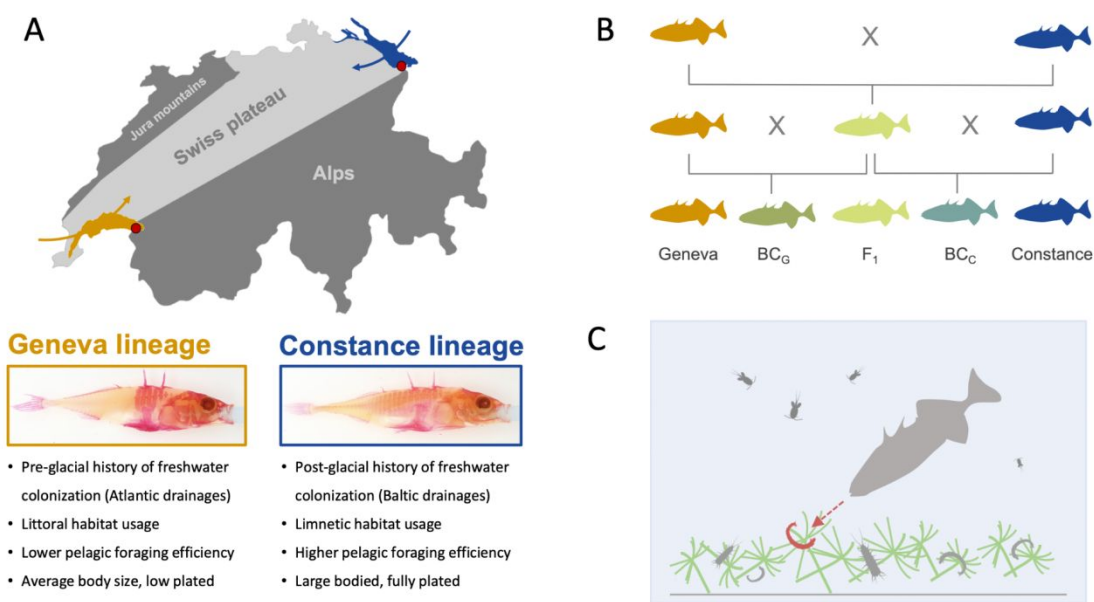
650   **Figure 1.** The Swiss plateau was colonized by two lineages of stickleback originating from

651   different European watersheds in recent historical times via Lake Geneva (orange) and Lake

652   Constance (blue) (A). These lineages differ in their history freshwater colonization, and in

653   various ecological, morphological and physiological traits. The sampling locations for the stock

654   populations used in this experiment are indicated with red dots. To obtain an experimental

655   population with an increased phenotypic variance, we bred Lake Constance and Lake Geneva

656   fish in a line-cross scheme (B). Individual variation in diet and foraging behaviour was then

657   assessed in a common garden foraging experiment with a complex prey community, and related

658   to morphological variation at an individual and cross-type level (C)
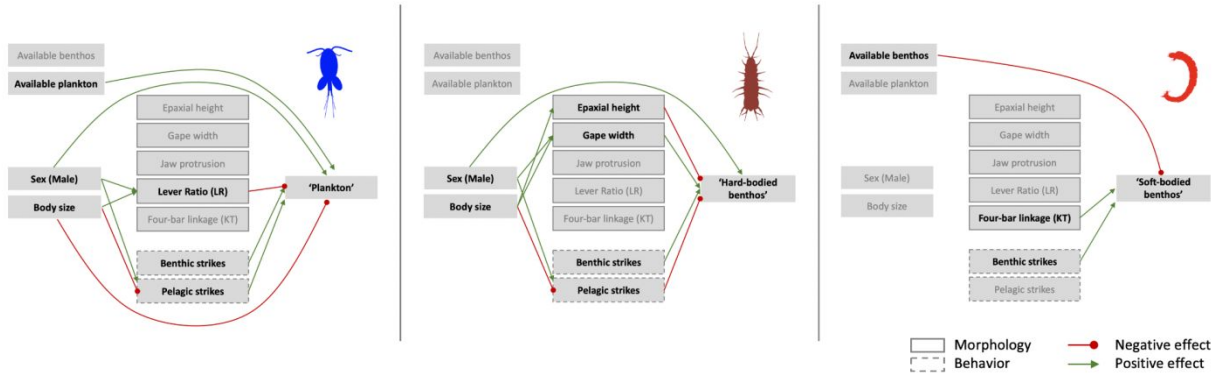
659

660   **Figure 2.** Traits that explain the number of ingested prey items per prey category. For

661   visualization purposes we only show the arrows directly or indirectly connecting explanatory

662   variables with the number of ingested prey items. The number of ingested prey items is

663   associated with morphological and behavioural variation for all prey types, and trait utility is

664   prey-specific. For the parameter estimates and the complete set of significant correlations see

665   Table S3 and Figure S4.

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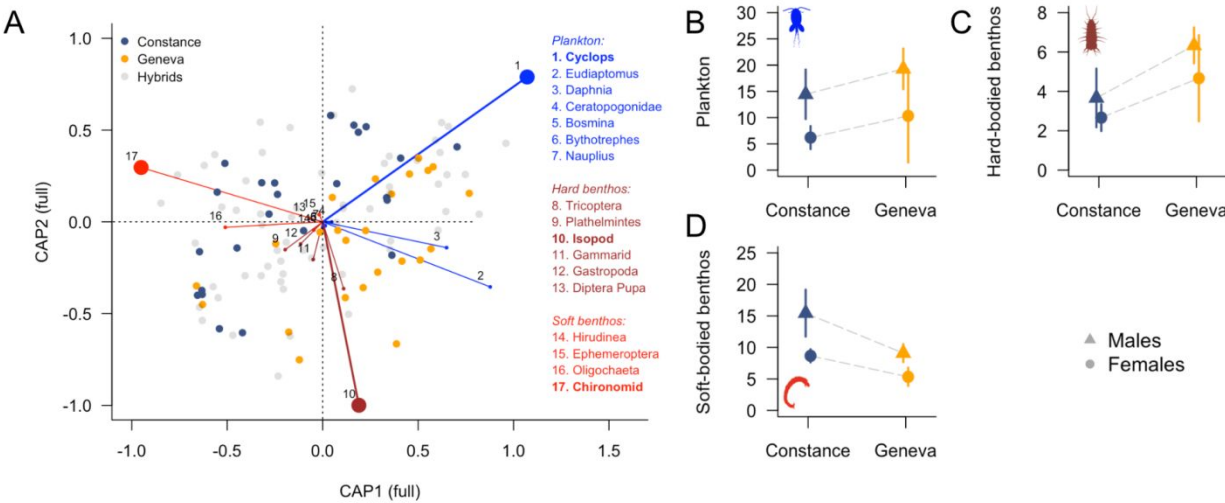
Figure 3. Dietary variation between the crosses and lineages, by analysing the axis of dietary

composition that best explain the differences between the cross-types. With respect to

composition (A), we found that Geneva fish have more hard-bodied benthos, whereas Constance

fish have more soft-bodied benthos and plankton, in their diet. With respect to numbers of prey

consumed (B-D), Geneva fish had significantly more hard-bodied benthic prey and fewer soft-

bodied benthic prey than Constance fish (C-D), whereas the differences in plankton numbers

between the lineages depended on the sex (Table S5).

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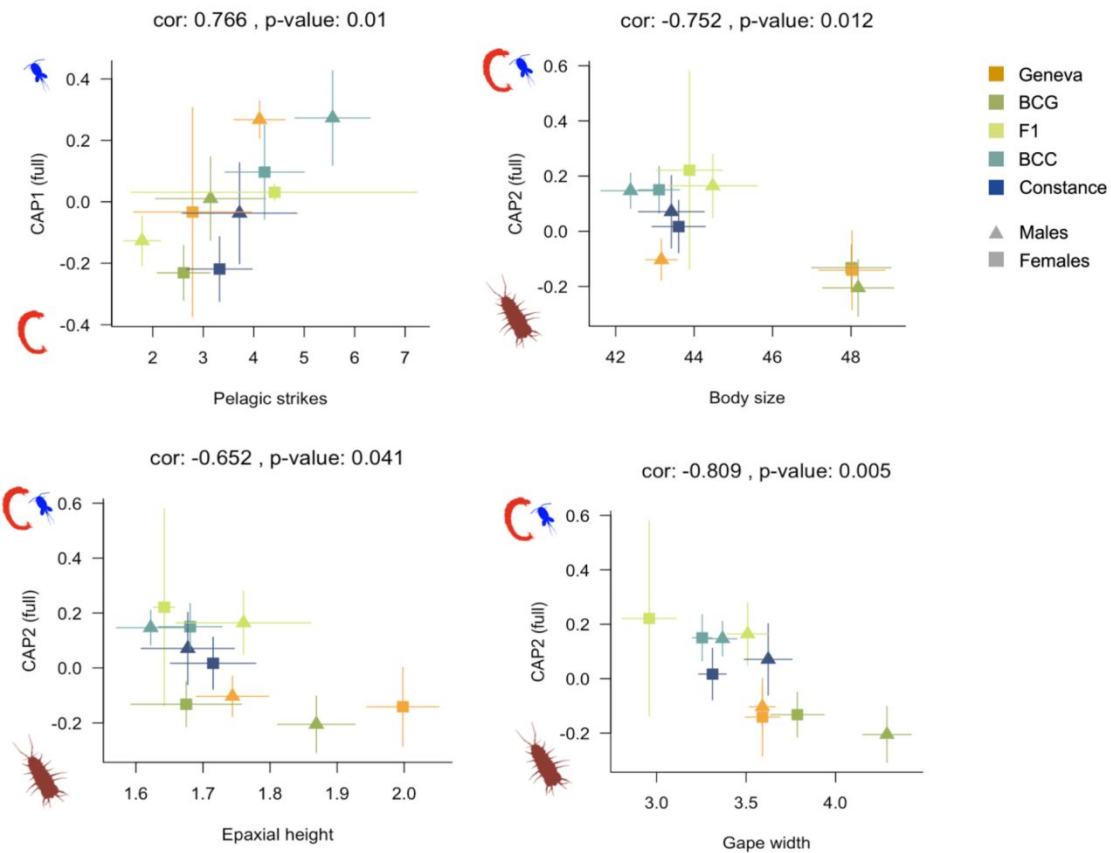
Figure 4. Correlations among cross/sex variation in dietary composition and functional traits. CAP1 reflects the proportion of plankton in the diet and is correlated with variation in the number of pelagic strikes. CAP2 reflects the proportion of hard-bodied benthos in the guts and is correlated with the among-cross variation in body size, epaxial height and gape width.

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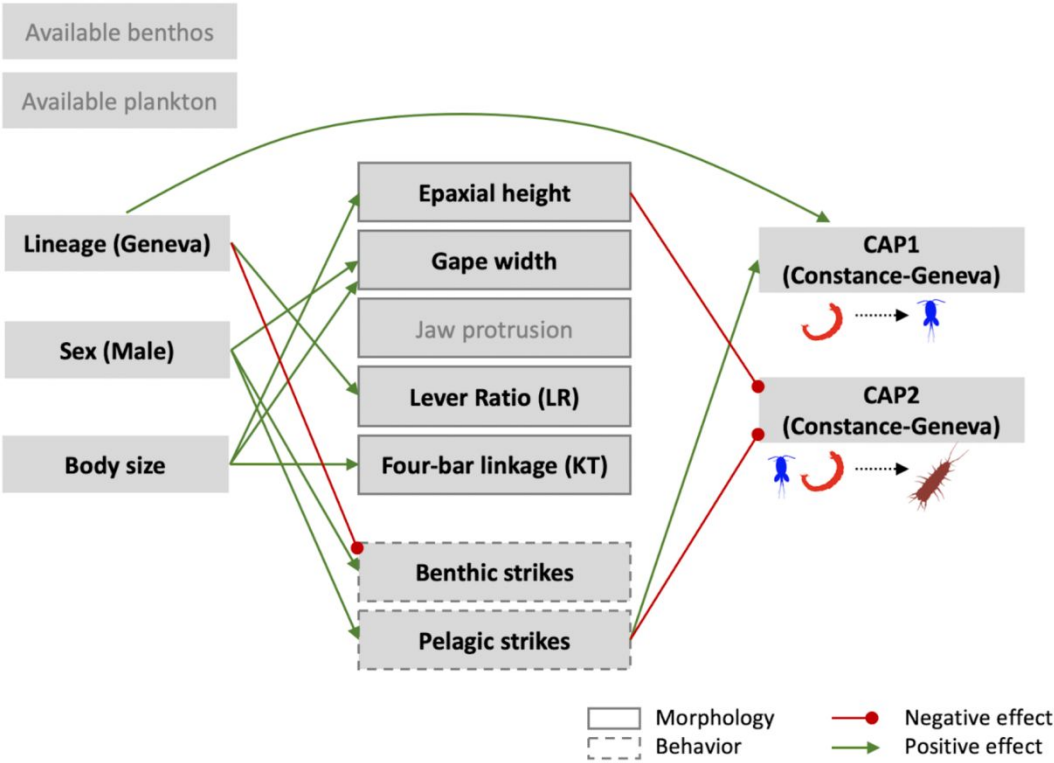
681　**Figure 5** Path analysis to uncover the phenotypic determinants of dietary variation between Lake

682　Constance and Lake Geneva fish in common garden. Lineage has a direct effect on the first axes

683　of dietary divergence between the Lake Constance and Lake Geneva lineage, however, there is

684　no indirect links connecting Lineage with dietary variation, indicating that the dietary divergence

685　is not associated with any of the traits found be functional at the individual level.

686