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1   **Contemporary ecotypic divergence during a recent range expansion was facilitated**

2   **by adaptive introgression**

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18

19   **Abstract**

20   Although rapid phenotypic evolution during range expansion associated with colonization

21   of contrasting habitats has been documented in several taxa, the evolutionary

22   mechanisms that underlie such phenotypic divergence have less often been investigated.

23   A strong candidate for rapid ecotype formation within an invaded range is the threespine

24   stickleback in the Lake Geneva region of central Europe. Since its introduction only ~ 140

25   years ago it has undergone a significant expansion of its range and its niche, now forming

26   phenotypically differentiated parapatric ecotypes that occupy either the pelagic zone of

27   the large lake or small inlet streams respectively. By comparing museum collections from

28   different times with contemporary population samples, we here reconstruct the evolution

29   of parapatric phenotypic divergence through time. Using genetic data from modern

30   samples we infer the underlying invasion history. We find that parapatric habitat-

31   dependent phenotypic divergence between the lake and stream was already present in

32   the first half of the twentieth century, but the magnitude of differentiation increased

33   through time, particularly in anti-predator defense traits. This suggests that divergent

34   selection between the habitats occurred and was stable through much of the time since

35   colonization. Recently increased phenotypic differentiation in anti-predator defense traits

36   likely results from habitat dependent selection on alleles that arrived through

37   introgression from a distantly related lineage from outside the Lake Geneva region. This

38   illustrates how hybridisation can quickly promote phenotypic divergence in a system

39   where adaptation from standing genetic variation was constrained.

40

41

42   **Introduction**

43   The colonization of new habitats combined with natural selection favouring different

44   phenotypes in new environments can quickly lead to the formation of new ecotypes that

45   are distinct from their ancestral population (Schluter, 2000; Carroll *et al.*, 2007; Nosil,

46   2012). Subsequent adaptive phenotypic evolution can be rapid (Thompson, 1998;

47   Palumbi, 2001; Hairston *et al.*, 2005; Carroll *et al.*, 2007; Dlugosch & Parker, 2008; Hendry

48   *et al.*, 2008), especially when measured over short time scales (Hairston *et al.*, 2005) and

49   if selection acts on standing genetic variation (Facon *et al.*, 2006; Arnold *et al.*, 2008;

50   Barrett & Schluter, 2008). The rate and extent of adaptive change depends moreover on

51   the genetic and environmental constraints as well as the time that is available for

52   selection to act (Johnston & Selander, 1964; Hendry *et al.*, 2000; Koskinen *et al.*, 2002;

53   Nosil *et al.*, 2009; Matesanz *et al.*, 2012). Rapid phenotypic changes that allow individuals

54   to match their environment the best may similarly occur instantaneously through

55   adaptive phenotypic plasticity, wherein identical genotypes can express different

56   phenotypes in different environments (Hairston *et al.*, 1999; West-Eberhard, 2003; Keller

57   & Taylor, 2008; Badyaev, 2010; Calsbeek *et al.*, 2011).

58

59   Although rapid ecotype formation has been frequently studied (e.g. Johnston & Selander,

60   1964; Hendry *et al.*, 2000; Koskinen *et al.*, 2002; Matesanz *et al.*, 2012), the evolutionary

61   changes through time have only rarely been investigated due to rarity of historical data

62   documenting these evolutionary shifts (but see Hairston *et al.*, 1999; Badyaev, 2010;

63   Calsbeek *et al.*, 2011 for examples). It is furthermore less clear whether ecotype formation

64   commonly occurs through a gradual process, where genetic constraints may lead towards

65   a novel adaptive optimum over time (Schluter, 1996) or rapidly through selection on

66   standing genetic variation (Barrett & Schluter, 2008) and/or phenotypic plasticity (West-

67   Eberhard, 2003). Adaptive phenotypic evolution is indeed thought to be constrained along

68   so called "lines of least resistance" or g_{max}, i.e. the leading eigenvector of the **G** matrix,

69   which summarizes the additive genetic variances and covariances (Lande, 1979; Lande &

70   Arnold, 1983; Schluter, 1996; see Steppan *et al.*, 2002 for a review). Biologically, this axis

71   accounts for the largest proportion of genetic variance and is shaped by selection and drift

72   (Lande & Arnold, 1983; Steppan *et al.*, 2002; Marroig & Cheverud, 2005; Facon *et al.*,

73   2006; Arnold *et al.*, 2008; Barrett & Schluter, 2008). In the absence of quantitative genetic

74   data, the **G** matrix and g_{max} may be surrogated by the **P** matrix an p_{max} respectively, which

75   are based on phenotypic data from wild populations (Cheverud, 1988). This is especially

76   valid when phenotypic traits are relatively heritable (Lande, 1979), as has been found for

77   many taxa (Cheverud, 1988; Leinonen *et al.*, 2011a; Kolbe *et al.*, 2011).

78

79   Here we study a case of recent ecotype formation in the threespine stickleback

80   (*Gasterosteus aculeatus* species complex) from the Lake Geneva region in Switzerland,

81   where stickleback was introduced only ~140 years ago and form now ecologically and

82   phenotypic distinct parapatric lake and stream ecotypes (Lucek *et al.*, 2010; 2013; 2014a).

83   Lake dwelling stickleback differ from their stream counterparts in both feeding and

84   defence related phenotypes. Specifically, they have longer gill rakers allowing them to

85   feed on a more planktonic diet (Gross, 1977; Berner *et al.*, 2010; Lucek *et al.*, 2013) and

86   extended defense structures, i.e. elongated spines and an increased number of lateral

87   plates, protecting them from gape-limited piscivorous predators (Lucek *et al.*, 2010;

88   2013). Historically, however, only a single lateral plate morph with few lateral plates was

89   documented in the Lake Geneva region, which is consistent with the phenotypes in the

90   native range of the introduced South European lineage (Lucek *et al.*, 2010 and references

91   therein). Phenotypic divergence between lake and stream stickleback occurs commonly

92   for feeding and defence related traits, suggesting that they reflect the major divergent

93   selective pressures between the distinct habitats that work on external phenotypes.

94   However, evidence stems mainly from evolutionarily much older stickleback populations

95   elsewhere in the world that emerged shortly after the retreat of the glaciers ~12'000

96   years ago (e.g. Reimchen *et al.*, 1985; Berner *et al.*, 2009; Kaeuffer *et al.*, 2012; Ravinet *et*

97   *al.*, 2013, but see Hendry *et al.*, 2013).

98

99   By comparing the **P** matrices of historical museum and contemporary collections from

100   several lake and stream stickleback populations, we ask if we can demonstrate gradual

101   changes through time towards the currently observed phenotypic differentiation during a

102   recent biological invasion (Lucek *et al.*, 2013). We focus on traits related to anti-predator

103   defence and feeding ecology, two independent trait categories that have previously been

104   found to experience distinct selective regimes and which are furthermore heritable

105   (Reimchen, 1994; Walker, 1997; Lucek *et al.*, 2013). We predict the angle between p_{max}

106   and the common line of least resistance to either change gradually, increasing over time

107   (Schluter, 1996) if genetic constraints restrict ecotypic divergence. Alternatively, p_{max} of

108   different populations from the same habitat type should align independently of time if

109   habitat dependent ecotypic differentiation was completed rapidly, within the first few

110   decades after colonization. In a second step, we infer the genetic history of this biological

111   invasion using contemporary populations. We test if the contemporary phenotypic

112   differentiation between ecotypes was a result of repeated colonization and secondary

113   contact, where distinct habitats may have been colonized by genetically and

114   phenotypically different colonizing lineages (Taylor & McPhail, 2000), or rather reflects *in*

115   *situ* evolution. Finally, we test if both lateral plates and the *Ectodysplasin* (*Eda*) locus,

116   which controls the lateral plate phenotype (Colosimo *et al.*, 2004; 2005) show patterns of

117   habitat dependent selection and/or reflect patterns of adaptive introgression from an East

118   European lineage that got introduced in the eastern part of Switzerland (Lucek *et al.*,

119   2010; Rieseberg, 2011; Pardo-Diaz *et al.*, 2012).

120

121   **Material and methods**

122   *Sample collection*

123   Eight historical populations from 1921-1979 with ≥ 20 individuals each were available

124   from the Natural History Museums in Geneva and Lausanne (Table 1), with a total of 438

125   individuals originating from both lake and stream habitats. Due to the type of

126   preservation used and legislative restrictions, none of the museum specimens were

127   available for DNA extraction. In addition, a total of 659 individuals from sixteen

128   contemporary populations within the Lake Geneva system were collected between 2007

129   and 2013 using hand nets and minnow traps (Figure 1). Hereafter we refer to these two

130   categories of samples as either of "historical" or of "contemporary" origin, where we use

131   the term "population" to refer to a single sampling event for a geographic site at a given

132   point in time. Some contemporary populations were collected during an earlier study of

133   the invasive range of stickleback in Switzerland (Lucek *et al.*, 2010). Further effort was

134   then made to sample the same sites as those from which historical populations were

135   available. However, only at five of these sites could stickleback be observed and collected

136   (Table 1). For the population GEL3, only juvenile individuals were caught. Consequently

137   only genetic data was generated for this population. Populations were assigned to be

138   either lake or stream dwelling based on the habitat where they were sampled.

139   Contemporary individuals were preserved in 70% ethanol after taking a fin clip, which

140   was stored in absolute ethanol for genetic analysis.

141

142   *Morphological analysis*

143   Sixteen linear morphological traits were measured to the nearest 0.01 mm using a digital

144   calliper. These traits were either related to anti-predator defense, feeding ecology or

145   general body shape and swimming performance (Mori & Takamura, 2004; Lucek *et al.*,

146   2013; see Figure 2). Because all linear traits were significantly correlated with standard

147   length (results not shown), a size correction was applied. Each trait was first scaled by the

148   average trait value separately for each population as suggested by Houle (1992).

149   Subsequently, a linear regression of each trait against standard length was performed

150   separately for each population, retaining the residuals for further analysis (Berner *et al.*,

151   2010b). In addition to the linear measurements, the number of lateral plates on the left

152   flank was counted using a dissection microscope and then separately analysed.

153

154   Pairwise phenotypic comparisons between historical and contemporary populations from

155   the same site were performed for the five cases where such data was available: GES2,

156   VDL4, VDL6, VDS6, VSS3 (Table 1). The degree of phenotypic divergence was estimated

157   using pairwise P_{ST} based on principal component scores, following Kaeuffer et al. (2012),

158   where the 95% confidence interval of each P_{ST} was calculated using a resampling

159   approach with 1000 replicates. P_{ST} is used as a unit-less and scale-free proportional

160   measure of pairwise phenotypic divergence. In addition to P_{ST}, divergence between the

161   historical and contemporary $\boldsymbol{p}_{max}$ within a sampling site was estimated as the angle

162   between them. The significance of these angles to deviate from zero was determined using

163   a bootstrap resampling procedure with 1000 replicates following Berner (2009). This

164   approach calculates the probability of obtaining an angle between p_{max} that is equal to or

165   greater than our observed angle given the resampling variance if the true angle was zero.

166   For each iteration, the observed angle was subtracted from the bootstrapped angle for

167   each iteration. *P* values were subsequently calculated as the proportion of iterations

168   where the absolute angle difference is equal or greater than the observed angle. The

169   occupied morphospace of each population was additionally estimated as the ellipse size of

170   the 95% confidence interval based on the two leading PC axes. The same PC axes were

171   then used to estimate the shape of the **P** matrix, measured by its eccentricity – the ratio of

172   its two leading eigenvectors – where for example increased directional selection can

173   result in higher eccentricity (Jones *et al.*, 2003). These calculations were performed using

174   all linear traits combined. In addition, feeding and defence related traits were separately

175   analysed because habitat dependent phenotypic divergence between parapatric lake and

176   stream stickleback populations occurs repeatedly in these trait categories whose selective

177   regime is relatively well understood (Lucek *et al.*, 2013). Both feeding and defence related

178   traits are furthermore known to be associated with high heritabilities (Leinonen *et al.*,

179   2011b; Lucek *et al.* 2014b). To account for multiple testing of the same hypothesis, the *p*

180   values for the angles between p_{max} were corrected using a Benjamini & Yekutieli

181   correction (Narum, 2006).

182

183   The overall multivariate phenotypic changes between *habitat* (lake and stream) or *time*

184   (historical and contemporary) were estimated by calculating the angles between the

185   leading eigenvectors p_{max} of each population against the overall p_{max}, where all individuals

186   were pooled. The overall $\boldsymbol{p}_{max}$ may consequently capture the major evolutionary

187   constraints in the entire data set, which may be similar for some populations but not

188   others. Following Schluter (1996), the angle between two $\boldsymbol{p}_{max}$ was calculated as the

189   inversed cosine of their dot product that is divided by the summed length of both $\boldsymbol{p}_{max}$.

190   The statistical significance of each comparison was established using 1000 bootstrap

191   replicates and then corrected for multiple comparisons using a Benjamini & Yekutieli

192   correction. Obtained angles were subsequently tested for a significant association with

193   either *habitat*, *time* or their interaction using linear models, where the best fitting model

194   was determined using a stepwise backward procedure.

195

196   Lastly, a linear model was used to test if the lateral plate counts differ significantly

197   between historical and contemporary populations (i.e. *time*) as well as between lake and

198   stream populations (i.e. *habitat*). Because lateral plate counts do not follow a normal

199   distribution (Shapiro test: W = 0.422, $p < 0.001$) and because the variance in the number

200   of plates differed between historical and contemporary populations (Fligner-Killeen test:

201    $\chi_{21} = 8.26$, $p = 0.004$) the number of plates were boxcox-transformed to meet the

202   assumptions of normality. Moreover, a variance function structure was included in the

203   model to allow for different variances between historical and contemporary populations

204   using the "varIdent" function in the R package "nlme" (Pinheiro and Bates 2000).

205   Population was included as a random effect. A stepwise backward deletion procedure was

206   then used to remove non-significant terms ($p > 0.05$). Fixed effects were subsequently

207   tested for significance using two-tailed, Type II F values (Fox, 2002). Model selection was

208   done using maximum likelihood while parameter estimates of mixed models were

209   obtained via restricted maximum likelihood (Pinheiro and Bates 2000). All statistical

210   analyses were performed in R 2.15.1 (R Core Team, 2012).

211

212   *Genetic analysis*

213   In total, 403 individuals from 16 contemporary sites within the Rhone drainage were

214   genotyped at nine microsatellites (Table 1), of which three markers (Stn26, Stn96,

215   Stn130) are putatively linked to QTLs related to spine lengths (Peichel *et al.*, 2001). In

216   addition, 147 individuals from five populations within the invasive range of stickleback in

217   Switzerland outside the Rhone drainage system were included to test for potential gene

218   flow between drainage systems. DNA for all individuals was extracted using a 10% Chelex

219   solution, following the manufacturers protocol (Biorad, California, USA). All

220   microsatellites were amplified in one multiplex kit. Detailed information on the marker

221   identity, the multiplexing setup and the PCR protocol can be found in Lucek *et al.* (2014a).

222   Alleles were visualized on an ABI 3130XL and scored with GENEMAPPER 4.0 (Applied

223   Biosystems, Switzerland).

224

225   Pairwise genetic F_{ST} were calculated with GENODIVE 2.0B22 (Meirmans & Van Tienderen,

226   2004) using 100,000 bootstrap replicates to assess significance. The pairwise F_{ST} values

227   were subsequently adjusted for multiple comparisons using a Benjamini & Yekutieli

228   correction. To test for an isolation-by-distance pattern of genetic differentiation within

229   the Lake Geneva drainage, the pairwise F_{ST} values were correlated with pairwise minimal

230   waterway distances using a Mantel test with 10,000 bootstrap replicates to assess

231   significance. The genetic structure was further assessed using an admixture model

232   implemented in STRUCTURE 2.3.4 (Falush *et al.*, 2007) with 30,000 burn in steps followed

233   by 300,000 MCMC steps. The analysis was performed assuming any number of genetic

234   clusters (K) between 1 and 15, with 10 replicates for each assumed K. The optimal

235   number of clusters was determined based on the log likelihood of each run and its

236   variation among runs for the same K, following Evanno et al. (2005). To further infer the

237   genetic relationships among populations, a neighbour joining tree, based on Cavalli-Sforza

238   distances among populations was constructed. Statistical support for each node of the

239   inferred tree was obtained using a bootstrap procedure with 1000 replicates in PHYLIP

240   3.69 (Felsenstein 2012).

241

242   In addition, 639 individuals from all contemporary populations within the Rhone drainage

243   were genotyped for *Stn382*, following the protocols of Colosimo et al. (2005). The primers

244   flank a 60 bp indel in intron 1 of the *Eda* gene, yielding either a 158 bp allele – Eda_L,

245   associated with the low plated phenotype or a 218 bp allele – Eda_C, associated with the

246   fully plated phenotype (Colosimo *et al.*, 2005). PCR products were separated on a 1.5 %

247   agarose gel, and genotypes were scored by eye. Because the *Eda* gene and its associated

248   marker *Stn382* are potentially under strong selection (Barrett *et al.*, 2008), *Stn382* was

249   excluded from the abovementioned population based analyses. To test for an association

250   between the presence of the Eda_C allele and *habitat,* a generalized linear mixed model was

251   used, treating population as a random factor and assuming a binomial error distribution.

252   The significance of the differences between groups was assessed using a likelihood ratio

253   test.

254

255   Finally, assuming that the presence of the Eda_C allele in the Lake Geneva region coincides

256   with the presence of other alleles from the Lake Constance region (Lucek *et al.*, 2010), the

257   frequency of both Eda_C and Lake Constance specific microsatellite alleles were compared

258   between habitats. The latter were defined as the private alleles of both Constance

259   populations combined in comparison to the population from the Lake Geneva system that

260   showed the lowest degree of introgression in the STRUCTURE analysis, i.e. VDS6. The

261   frequency of these Constance private alleles was subsequently estimated for all other

262   populations in the Lake Geneva region. To further test for habitat-specific and potentially

263   adaptive introgression of the Eda_C allele within the Lake Geneva system, the difference

264   between the allele frequencies of both marker types was calculated for each population.

265   Comparisons between habitats were performed using t tests.

266

267   **Results**

268   *Morphology*

269   Within a sampling site, **P** matrices commonly changed through time both in their shape

270   and their leading eigenvector p_{max} (Table 2, Figure 3). However, changes in the **P** matrix

271   and the levels of pairwise phenotypic divergence (P_{ST}) varied among sites and trait

272   combinations. Combining all traits, the 95% confidence interval of the P_{ST}s between

273   historical and contemporary samples from the same site exceeded zero in four (Table 2)

274   and p_{max} differed within sites over time, i.e. showed a significant angle between historical

275   and contemporary p_{max} in all but one case. Using either only feeding or defence related

276   traits, p_{max} did not differ between historical and contemporary populations except for

277   VSS3 for both trait categories and GES2 for feeding related traits. The size and shape of

278   the **P** matrix changed over time (Figure 3), with the phenotypic variation captured by the

279   two leading PC axes decreasing over time in many populations, especially when all traits

280   were analysed together and for defence traits only (Table 2; Figure 3). Phenotypic

281   variation increased however in three of five populations for feeding related traits (VDS6,

282   VDL4, VDL6) and eccentricity changed over time in many sites.

283

284   When comparing the p_{max} of each sampled population against the overall p_{max} combining

285   all traits, the best model explaining the angles between them retained only *time* as a

286   significant factor when all traits were used: Historical populations have a significantly

287   smaller angle against the overall p_{max} than contemporary populations ($F_{1,18}$ = 10.4, p =

288   0.005, Table 3, Figure 4a and d). When defence related traits were analysed on their own,

289   the retained best model included only *habitat*, with lake populations having significantly

290   larger angles against the overall p_{max} ($F_{1,18}$ = 25.2, p < 0.001, Figure 4b and e). Considering

291   feeding related phenotypic traits on their own, the best statistical model showed a

292   significant interaction between *habitat* and *time* ($F_{1,16}$ = 5.53, p = 0.032), where historical

293   lake populations had larger angles than contemporary ones against the overall *p_{max}*,

294   whereas this difference was inversed for the stream populations (Figure 4c and f, Table

295   3). *Habitat* was furthermore marginally significant ($F_{1,16}$ = 4.53, p = 0.049) with lake

296   populations showing relatively larger angles than stream populations.

297

298   *Genetic structure*

299   The global F_{ST} values within the Lake Geneva system, calculated separately for each

300   microsatellite marker, did not statistically differ between putatively QTL linked and

301   neutral markers (Wilcoxon W = 9, p = 0.999). Consequently all microsatellite markers

302   were pooled for the subsequent analyses. When analysing the contemporary samples

303   from Lake Geneva together with those from France and the Swiss Rhine catchment, two

304   genetic clusters (K=2) was the best supported K in STRUCTURE as inferred by the method of

13

305 Evanno *et al.* (Evanno *et al.*, 2005). The two clusters separate the Rhone drainage from

306 populations in the Aare/Rhine drainage (Bern, Constance) except for Neuchatel (VDS2;

307 Figure 5a), which was mainly assigned to the Rhone drainage cluster with clear

308 indications of genetic admixture with the Aare/Rhine drainage stickleback. Low levels of

309 introgression from the Rhone drainage into the Aare/Rhine drainage were moreover

310 observed in the Bern populations. STRUCTURE suggests that introgression also occurred

311 from the Aare/Rhine drainage into the Lake Geneva catchment, particularly into the lake

312 populations. The neighbour joining tree further suggests a single genetic clade for all

313 populations from the Rhone drainage. The Neuchatel and Bern populations are resolved

314 as intermediate between Geneva and Constance, consistent with a hybrid origin (Figure

315 5b; Lucek et al. 2010).

316

317 Populations within the Lake Geneva region were generally genetically differentiated from

318 populations in the Aare/Rhine drainage (all F_{ST} >0.200 and $p < 0.001$; Table S1). The only

319 exception was the population VDS2 from Lake Neuchatel, which was not significantly

320 differentiated from five of our populations in the Lake Geneva region, where the lowest

321 F_{ST} was observed between this population and its geographically closest population in

322 Lake Geneva (VDS2-VDL2: $F_{ST} = 0.008$, $p = 0.129$). The southern Rhone population (FrAS)

323 showed increased genetic differentiation from all populations in the Lake Geneva region

324 (all pairwise $F_{ST} \geq 0.098$ and $p = 0.001$). Within the Lake Geneva region, considerable

325 genetic structure was observed (global $F_{ST} = 0.030$, $p < 0.001$), which was not explained by

326 geographic distance (Mantel $r = 0.199$, $p = 0.069$)**.** The overall genetic differentiation

327 between lake and stream populations within the Lake Geneva region, pooling all

14

328   individuals from either habitat, was not significant (F_{ST} = 0.002, p =0.696), speaking

329   against a colonization of the two habitats by distinct stickleback lineages.

330

331   *Lateral plate phenotype/genotype-environment associations*

332   In concordance with historical reports and consistent with the genetic lineage that

333   dominates the lake, (Lucek *et al.*, 2010 and references therein), fully plated individuals

334   were absent from Lake Geneva in all historical populations (Figure 6). Nevertheless few

335   historical individuals had some additional plates close to their structural plates. Such a

336   phenotype is however not necessarily associated with the presence of the Eda_C allele,

337   which commonly underlies highly plated phenotypes (Lucek *et al.*, 2012b). In contrast,

338   fully and intermediately plated individuals were numerous among contemporary

339   populations. The best statistical model that explained the overall variation in lateral plate

340   number included significant contributions of both *time* and *habitat* with a non-significant

341   interaction ($F_{1,19}$ = 0.8, p = 0.380). Overall, stream dwelling populations had a significantly

342   lower number of plates than lake dwelling populations ($F_{1,20}$ = 22.1, p <0.001), while

343   contemporary populations had more plates than historical populations ($F_{1,20}$ = 8.1, p =

344   0.010; Figure 6). The presence of highly plated individuals among contemporary samples

345   coincides with the occurrence of the Eda_C allele, where Eda was significantly correlated

346   with the number of lateral plates (R^2 = 0.838, p < 0.001). In accordance with the observed

347   habitat dependent differences in lateral plate numbers, the presence of the Eda_C allele was

348   significantly higher in the lake than in the stream habitats for contemporary individuals

349   (likelihood ratio test = 4.24, p < 0.001), suggesting habitat dependent selection.

350

351   The frequency of Lake Constance private alleles did not statistically differ between

352   habitats within the Lake Geneva system ($t_{1,13}$ = 1.84, p = 0.089; Figure 7). In contrast, the

353   population based Eda_C allele frequency was significantly higher among lake populations

354   ($t_{1,13}$ = 2.88, p = 0.027). The frequency of Eda_C was moreover significantly higher than

355   expected from the frequency of Constance derived alleles in the lake ($t_{1,13}$ = 2.82, p =

356   0.029).

357

358   **Discussion**

359

360   Whereas empirical evidence suggests that rapid phenotypic evolution during the

361   colonization of contrasting habitats occurs frequently (e.g. Johnston & Selander, 1964;

362   Hendry *et al.*, 2000; Koskinen *et al.*, 2002; Matesanz *et al.*, 2012), the role of genetic

363   constraints (Schluter, 1996), gene flow and adaptive introgression are far less understood

364   (e.g. Lavergne & Molofsky, 2007; Rieseberg, 2011). Combining phenotypic and population

365   genetic data, we explored the evolutionary changes that underlie the rapid emergence of

366   parapatric ecotypes in stickleback in the Lake Geneva region (Lucek *et al.*, 2013). We had

367   access to preserved individuals from museum collections starting in 1921, ~50 years after

368   stickleback were introduced, and had our own collections from about 90 years later.

369   Hence, we studied phenotypic evolution over the second half of the 140 years since

370   colonization. Consistent with other studies on multivariate phenotypic evolution in

371   recently colonized habitats in different taxa (Badyaev, 2010; Eroukhmanoff & Svensson,

372   2011; Calsbeek et al., 2011), we find phenotypic shifts through time at a given site, where

373   the phenotypic covariance (**P**) matrices changed in their shape, size and in some cases

374   their orientation (Table 2, Figure 3). Overall habitat dependent phenotypic divergence in

375 ecologically relevant morphology seems to have already evolved in the first ~50 years of

376 this biological invasion as lake and stream populations had a consistently different line of

377 least resistance (p_{max}). For lateral plates, the extent of habitat dependent phenotypic

378 differentiation has increased from the historical to the contemporary samples (Figure 6).

379 This is likely a result of recent introgression of alleles from a distantly related lineage,

380 outside the Lake Geneva region, that experience divergent selection between habitats

381 (Figure 5 & 7). Thus, introgression of adaptive variation through hybridisation may have

382 contributed to the increased parapatric phenotypic divergence over the last decades

383 (Rieseberg, 2011; Pardo-Diaz et al., 2012).

384

385 *Evidence for consistent parapatric ecotypic differentiation*

386 Both the occurrence and extent of parapatric ecotype formation depends among other

387 things on the underlying environmental and selective gradients (Endler, 1977; Doebeli &

388 Dieckmann, 2003). Parallel and consistent evolutionary divergence is therefore only

389 expected when the selective regimes are very similar among populations (Langerhans &

390 DeWitt, 2004; Kaeuffer *et al.*, 2012) and adaptive genetic variation is not limiting (Barrett

391 & Schluter, 2008). Cases of parapatric lake-stream stickleback systems provide both

392 evidence for parallelism and nonparallelism in the realized trait-specific divergence that

393 occur both on smaller geographical scales and between continents (Hendry & Taylor,

394 2004; Berner *et al.*, 2009; 2010a; Kaeuffer *et al.*, 2012; Ravinet *et al.*, 2013; Lucek *et al.*,

395 2013; 2014a). Parallel habitat dependent selection in stickleback occurs especially in

396 feeding and defence-related traits (Reimchen *et al.*, 1985; Hendry *et al.*, 2002; Kaeuffer *et*

397 *al.*, 2012; Ravinet *et al.*, 2013; Lucek *et al.*, 2013), where stream populations feed

398 predominantly on benthic food and experience a macroinvertebrate dominated predation

399   regime. In contrast, lake populations feed predominantly on zooplankton and have more

400   numerous and elongated gill rakers to filter small prey items (Reimchen *et al.*, 1985;

401   Hendry *et al.*, 2002; Berner *et al.*, 2008; Lucek *et al.*, 2012a; 2014b). They moreover have

402   elongated spines and an increased number of lateral plates, likely as a consequence of a

403   predation regime dominated by piscivorous fishes and birds (Reimchen, 1980; 1994;

404   Lucek *et al.*, 2013).

405

406   Although previous studies on Lake Geneva stickleback that focused on single lake-stream

407   pairs found habitat dependent phenotypic divergence that is comparable to other systems

408   (Berner *et al.*, 2010a; Lucek *et al.*, 2013), the evolutionary history and the degree of

409   parallelism within the Lake Geneva system was not assessed. In line with our predictions

410   and the results of the abovementioned studies, we find that p_{max} for the same trait

411   categories differs significantly and consistently between lake and stream habitats when

412   comparing population based p_{max} with the overall p_{max} (*sensu* Schluter, 1996; Figure 4,

413   Table 3). Stickleback within the Lake Geneva system form a distinct genetic cluster

414   (Figure 5), where lake and stream populations do not differ genetically overall. This

415   suggests that habitat dependent phenotypic divergence likely evolved *in situ*, rather than

416   resulting from two distinct colonization events. The p_{max} for feeding and defence related

417   traits are moreover comparable within each habitat (Figure 4, Table 3), indicating parallel

418   and consistent ecotypic divergence within the Lake Geneva region. Ecotype formation

419   may consequently be similarly constrained across our studied populations where the

420   overall divergent selective regime was likely stable through time.

421

18

422   However, phenotypic changes also occur within sites, i.e. between historical and

423   contemporary populations even a hundred years after the initial colonization of the Lake

424   Geneva region (Figure 3, Table 2), where **P** matrices differ in terms of their morphospace

425   size, eccentricity and to some extent their directionality (p_{max}). Overall, we find decreased

426   phenotypic variation in defence-related traits among contemporary samples in

427   comparison to their historical counterparts (Figure 3, Table 2). Moreover, both P_{ST} and the

428   angle between p_{max} are significant between historical and contemporary populations in

429   only few cases and not for defence-related traits. Consequently, phenotypic evolution or

430   divergence that would change p_{max} seems to rarely occur at a given site for linear

431   morphology. This may suggest that adaptation to a local environment can occur rapidly

432   after its colonization especially for adaptive traits (e.g. feeding and defence-related traits

433   in stickleback), whereafter the average phenotype remains relatively stable over time and

434   may experience stabilizing selection (Jones *et al.*, 2004).

435

436   *On the tempo and mode of ecotype formation*

437   Adaptive phenotypic evolution can be rapid and emerge through phenotypic plasticity,

438   selection on standing genetic variation or a combination of both (Robinson & Wilson,

439   1996; Thompson, 1998; Hairston *et al.*, 2005; Carroll *et al.*, 2007; Dlugosch & Parker,

440   2008). Concomitantly, the **P** matrix and its axis p_{max} should rapidly and consistently

441   diverge if new adaptive phenotypes are mainly produced by phenotypic plasticity (Lande,

442   2009; Draghi & Whitlock, 2012) or adaptation from standing genetic variation (Lande &

443   Shannon, 1996; Barrett & Schluter, 2008), which can be facilitated by introgression or

444   hybridization (Guillaume & Whitlock, 2007). Conversely, a gradual increase in divergence

445   between divergently adapted populations over many generations is expected if adapted

446 phenotypes need to evolve, where the **P** matrix is realigning itself slowly towards a new

447 adaptive peak on the adaptive landscape (Schluter, 1996; Steppan *et al.*, 2002).

448

449 We find that habitat-dependent divergence may occur rapidly and may lead quickly to

450 some phenotypic differentiation between the populations inhabiting these habitats as p_{max}

451 differed already consistently between habitats among our historical samples (Figure 4).

452 However, we cannot tell from our data whether this early change was achieved by

453 phenotypic plasticity or fast but gradual evolution because our data series only starts ~50

454 years post colonization. Phenotypic plasticity may, in principle, promote the colonization

455 of distinct habitats and subsequent ecotype formation (Smith & Skúlason, 1996;

456 Ghalambor *et al.*, 2007). In such cases p_{max} may shift rapidly and p_{max} from different

457 populations may moreover align if they experience similar selective regimes (Lande,

458 2009; Draghi & Whitlock, 2012). However, the fact that we still observe changes in the

459 population specific **P** matrix even a hundred years after the colonization of the Lake

460 Geneva system may suggest a gradual differentiation over time and concordantly a

461 heritable component of ecotypic divergence (Schluter, 1996) as has been found for other

462 stickleback populations in Switzerland that evolved over a similar time scale (Lucek *et al.*

463 2014b). The relatively recent introgression from the differentiated stickleback lineage

464 dominating the East of Switzerland may have introduced additional adaptive genetic

465 variation that facilitated the observed phenotypic changes.

466

467 *Evidence for adaptive introgression*

468 Because the potential of a population to colonize and adapt to a new environment

469 depends on the availability of adaptive standing genetic variation (Sakai *et al.*, 2001;

470　　Barrett & Schluter, 2008), it can potentially be fuelled by gene flow, introducing novel

471　　beneficial alleles followed by introgression or hybridization (Lavergne & Molofsky, 2007;

472　　Guillaume & Whitlock, 2007; Rieseberg, 2011; Pardo-Diaz *et al.*, 2012). Using nuclear

473　　markers, we indeed find indications for a secondary introduction and subsequent

474　　introgression from the Aare/Rhine system into the Lake Geneva system (Figure 5), where

475　　stickleback are thought to derive from a single introduction event about 140 years ago.

476　　Based on mitochondrial haplotypes, they originate from a genetic lineage of the Southern

477　　Rhone that is fixed for both the low plated phenotype and the Eda_L allele (Lucek *et al.*,

478　　2010). In contrast, we find highly plated individuals and concomitantly the Eda_C allele to

479　　be present among our contemporary populations, where the *Eda* genotype is associated

480　　with the number of lateral plates. The Eda_C allele was likely introduced from the

481　　Aare/Rhine system given the pattern observed for the microsatellite markers (Figure 5).

482　　This is further supported by the fact that Eda_C haplotypes in the Lake Geneva region are

483　　shared with the Aare/Rhine system (Berner *et al.*, 2010a).

484

485　　Both the different lateral plate phenotypes as well as the different *Eda* alleles themselves

486　　are known to experience divergent habitat-dependent selection (Reimchen, 1994; Barrett

487　　*et al.*, 2008; Zeller *et al.*, 2012 and references therein), where even small changes in the

488　　average lateral plate number can be adaptive (Reimchen, 1994; 2000). A more fully plated

489　　body, and hence the presence of the Eda_C allele, is thought to be beneficial to protect

490　　against attacks from piscivorous predators by increasing the probability of surviving an

491　　attack (Reimchen, 1994). A lower number of plates and the associated Eda_L allele, on the

492　　other hand may increase the rate of handling failures by macroinvertebrate predators

493　　(Reimchen, 1994; Marchinko, 2009). Consistent with these predictions, we find lake

494   populations in the Lake Geneva region to have a slightly yet significantly increased

495   number of plates compared to stream populations already in the first half of the 20th

496   century (Figure 6), with phenotypes that can be expressed in the absence of the Eda_C

497   allele (Colosimo *et al.*, 2005; Cano *et al.*, 2006; Le Rouzic *et al.*, 2011; Lucek *et al.*, 2012b).

498   Coinciding with the presence of the Eda_C allele, the phenotypic differentiation is much

499   increased among contemporary populations, mainly due to the presence of many highly

500   plated individuals in lake populations. The frequency of Eda_C moreover significantly

501   exceeds that of other Lake Constance derived alleles within the lake environment (Figure

502   7). This is overall consistent with adaptive introgression (Rieseberg, 2011; Pardo-Diaz et

503   al., 2012), where positive selection leads to the disproportionate increase in the frequency

504   of an adaptive allele as for Eda_C in the lake environment.

505

506   *Conclusions*

507   Taken together, our results provide a case of rapid and repeated ecotype formation that is

508   associated with consistent evolutionary divergence among populations through time.

509   Moreover, parapatric ecotype formation has resulted in similar and hence predictable

510   phenotypic axes for ecologically relevant trait categories. Divergent habitat-dependent

511   selection appears to have led to some spatially consistent phenotypic differentiation

512   among populations inhabiting these habitats early on. More recent adaptive introgression

513   may then have facilitated further adaptive differentiation among ecotypes in anti-

514   predator related traits. Our study thus provides evidence on the role of evolutionary

515   constraints, gene flow and adaptive introgression during the colonization of novel

516   environments and subsequent ecotype formation.

517

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731

732   Figure 1: Map of Switzerland (© SwissTopo 2012) with all Swiss sampling sites indicated

733   (see table 1 for details). The colors of waterways represent the two major drainage

734   systems of western Switzerland, i.e. Rhone (blue) and the Aare/Rhine system (green).

735

736   Figure 2: Linear measurements that were taken either from the left flank a) or the ventral

737   side b) of each specimen. Traits were categorized as either anti-predator defence (red),

738   feeding ecology (green) or body shape and swimming performance (blue). Trait

739   abbreviations are as follow: FSL -length of the first dorsal spine; SSL - length of the second

740   dorsal spine; PSL - length of the pelvic spine; PGL - length of the pelvic girdle; HL - head

741   length; UJL - upper jaw length; SnL - snout length; ED - eye diameter; SL - standard length;

742   PGW - width of the pelvic girdle; BD1 - body depth measured after the first dorsal spine;

743   BD2 - body depth measured after the second dorsal spine; caudal peduncle length; BLA -

744   basal length of the anal fin; BLD - basal length of the dorsal fin; TLP - total length of the

745   pelvic fin.

746

747   Figure 3: Principal component scores based on either all linear morphological traits (left),

748   defense related traits (middle) or feeding related traits only (right) for the two leading

749   principal component axes. Only sites are shown where both historical and contemporary

750   populations were available. Ellipsoids represent the 95% CI with contemporary samples

751   in red and historical samples in blue and green. The relative changes between historical

34

752 and contemporary samples ($\triangle$) in eccentricity and phenotypic variation, i.e. the size of the

753 95% CI ellipsoid, are indicated (see Table 2 for details).

754

755 Figure 4: The angle against a common p_{max} for each population and year for either all

756 traits combined or defense or feeding related traits only (see Table S1). The major axis of

757 phenotypic variation (p_{max}) for each population is given for lake (a - c) and stream (d - f)

758 populations, separating historical (dashed) and contemporary (continuous line) sampling

759 events. *P* values are calculated from bootstrapping with 1000 replicates. Boldfaced

760 populations indicate cases where p_{max} differed significantly after applying a Benjamini &

761 Yekutieli correction for multiple comparisons (see main text for details).

762

763 Figure 5: Genetic relationship among individuals and populations within Lake Geneva and

764 Switzerland (Constance, Bern and Neuchatel) as well as a population from the Rhone river

765 downstream of Lake Geneva (France) based on nine microsatellites. a) Individual based

766 assignments using STRUCTURE for the best supported number of clusters, K = 2. The

767 respective habitat where populations were samples is indicated (L = Lake, S = Stream). b)

768 Unrooted neighbour joining tree based on Cavalli-Sforza distances calculated from

769 population allele frequencies. Bold numbers indicate the support for each node based on

770 1000 bootstrap replicates. Only values with more than 50% bootstrap support are given.

771 See table 1 for details of each population.

772

773   Figure 6: Number of lateral plates in each population for both historical and

774   contemporary stream and lake populations. *P* values for significance of differences

775   between habitats are based on *post hoc t* tests associated with fixed-effect parameters in a

776   linear model that included the non-significant interaction between *time* (historical and

777   contemporary) and *habitat* (lake and stream; see main text for details). The average

778   number of plates for each habitat is reported above the boxplots. Pie plots indicate the

779   allele frequency of the two *Stn382* alleles (white – L allele, black – C allele). No phenotypic

780   data was available for the GEL3 population (see Table 1 for details).

781

782   Figure 7: Frequency of the *Eda*$_C$ allele (circles) and other alleles that introgressed from the

783   Lake Constance region (triangles) for each contemporary population within the Lake

784   Geneva region. Sites are ordered according to the minimal waterway distance,

785   downstream of VSS3. Stream sites are depicted in black, lake sites in grey.

1   Table 1: Summary of the threespine stickleback populations studied, where population refers to a single sampling event (one point in
2   time) per site. For each site the ID, the catchment, the habitat where individuals were caught are indicated. The coordinates for each
3   location are additionally provided. For historical populations the year of collection, the number of individuals available for morphology
4   ($N_{Morphology}$) and the respective museum ID (NHMG=Natural History Museum Geneva, NHML= Natural History Museum Lausanne) are
5   given. For contemporary populations, the year of collection, the number of individuals available for morphology ($N_{Morphology}$),
6   microsatellites ($N_{\mu sat}$) and the *Eda* linked marker *Stn382* (N_{Stn382}) are indicated. Site IDs consist of two letters for Swiss sites, which are
7   consistent with a prior study (Lucek *et al.* 2010) and three letters for the French site, followed by the habitat type (L, lake; and S, stream)
8   and by a serial number.
9

Site ID	Catchment	Habitat	Coordinates		Year	$N_{Morphology}$	Museum ID	Year	$N_{Morphology}$	$N_{\mu sat}$	N_{Stn382}
					Historical			*Contemporary*			
VSS3	Geneva	Stream	46°16'45" N	7°30'55" E	1921	84	NHMG-816	2009	96	26	96
VDS6	Geneva	Stream	46°20'52" N	6°54'38" E	1958	36	NHML-163	2012	32	32	32
					1964	28	NHML-4216, -4772				
GES2	Geneva	Stream	46°11'48" N	6°11'30" E	1979	35	NHMG-2063, -2064	2009	42	32	42
VSS1	Geneva	Stream	46°12'50" N	7°18'53" E	-	-		2007	102	30	96
VSS2	Geneva	Stream	46°23'07" N	6°51'30" E	-	-		2007	59	32	59
GES1	Geneva	Stream	46°10'47" N	6°00'32" E	-	-		2008	36	32	36
GES3	Geneva	Stream	46°18'09" N	6°14'51" E	-	-		†2009	6	6	6
VSS4	Geneva	Stream	46°20'20" N	6°53'20" E	-	-		†2012	48	29	48
VDL4	Geneva	Lake	46°18'18" N	6°10'55" E	1967	20	NHML-6520	2010	30	32	23
VDL6	Geneva	Lake	46°23'45" N	6°53'18" E	1967	70	NHML-6465	2012	37	30	32
GEL1	Geneva	Lake	46°12'14" N	6°08'02" E	1978	34	NHMG-1592	-	-	-	-
GEL2	Geneva	Lake	46°12'10" N	6°07'54" E	1979	131	NHMG-2009	-	-	-	-
VDL2	Geneva	Lake	46°31'02" N	6°34'41" E	-	-		2008	40	30	40
VDL3	Geneva	Lake	46°23'38" N	6°55'18" E	-	-		2010	34	19	32
VDL5	Geneva	Lake	46°27'12" N	6°20'11" E	-	-		2010	30	16	30
VSL1	Geneva	Lake	46°23'09" N	6°51'29" E	-	-		†2012	36	28	37

GEL3	Geneva	Lake	46°17'57" N	6°14'32" E	-	-	2013	-	29	30
FrAS	Southern Rhone	Stream	45°58'04" N	5°17'40" E			2012	32	32	32
VDS2	Neuchatel	Stream	46°38'30" N	6°37'36" E			2008	-	31	-
BEL1	Bern	Lake	46°57'59" N	7°21'09" E			2007	-	30	-
BES4	Bern	Stream	46°59'31" N	7°24'42" E			2008	-	28	-
SGL1	Constance	Lake	47°29'08" N	9°32'38" E			2007	-	30	-
SGS1	Constance	Stream	47°19'33" N	9°34'41" E			2007	-	28	-

10

11   † Only the number of lateral plates was counted.

12   Table 2: Pairwise P_{ST} with its 95% confidence interval (CI) and angle between the leading eigenvectors of the P matrix for each historical
13   vs. contemporary samples within the same site are given for either all traits combined or subsets using either only defense or only feeding
14   related traits. In addition, the relative ellipse size and eccentricity for both the historical and contemporary population samples are given
15   with the respective percentage difference. See table 1 for details of each population. P_{ST} values whose 95% CI does not include zero and
16   significantly differentiated angles after a Benjamini and Yekutieli correction that was performed for site separately are highlighted in bold.

Site	Habitat	Year range	Traits	P_{ST}	95% CI	Angle	p_{Angle}	Relative ellipse size			Eccentricity		
								Historical	Contemporary	Δ%	Historical	Contemporary	Δ%
VSS3	Stream	1921-2009	All	0.000	0.000-0.227	**55.9°**	**<0.001**	0.398	0.327	-17.8	2.660	1.369	-48.5
			Defense	0.000	0.000-0.041	**16.8°**	**<0.001**	0.246	0.099	-59.8	6.432	5.305	-17.5
			Feeding	**0.218**	0.120-0.340	**11.1°**	**0.016**	0.120	0.091	-24.2	3.110	8.321	+167.6
VDS6	Stream	1958-1964	All	**0.359**	0.138-0.506	**51.8°**	**<0.001**	0.569	0.690	+21.3	1.557	1.490	-4.3
			Defense	0.041	0.000-0.175	11.5°	0.158	0.248	0.221	-10.9	4.868	11.535	+137.0
			Feeding	0.000	0.000-0.128	6.3°	0.409	0.239	0.149	-37.7	4.333	5.651	+30.4
VDS6	Stream	1958-2012	All	**0.384**	0.119-0.519	**82.9°**	**<0.001**	0.527	0.426	-19.2	1.557	2.115	+35.8
			Defense	0.000	0.000-0.059	3.8°	0.714	0.249	0.173	-30.5	4.868	2.947	-39.5
			Feeding	0.075	0.000-0.288	28.0°	0.165	0.229	0.276	+20.5	4.333	1.790	-58.7
VDS6	Stream	1964-2012	All	0.000	0.000-0.110	**81.0°**	**<0.001**	0.781	0.460	-41.1	1.490	2.115	+42.0
			Defense	0.000	0.000-0.075	7.7°	0.297	0.220	0.171	-22.3	11.535	2.948	-74.4
			Feeding	0.000	0.000-0.122	24.6°	0.222	0.137	0.279	+103.6	5.651	1.790	-68.3
GES2	Stream	1979-2009	All	**0.326**	0.041-0.709	**10.9°**	**<0.001**	0.460	0.221	-52.0	1.664	1.870	+12.4
			Defense	**0.215**	0.071-0.390	9.2°	0.062	0.185	0.095	-48.6	2.680	4.022	+50.1
			Feeding	0.000	0.000-0.163	**4.4°**	**0.001**	0.180	0.082	-54.4	5.394	6.278	+16.4
VDL4	Lake	1967-2010	All	0.000	0.000-0.139	41.7°	0.048	0.519	0.271	-47.8	3.188	1.469	-53.9
			Defense	0.000	0.000-0.108	12.4°	0.189	0.437	0.102	-76.7	5.776	3.599	-37.7
			Feeding	0.004	0.000-0.226	5.9°	0.773	0.112	0.136	+21.4	6.208	1.453	-76.6
VDL6	Lake	1967-2012	All	**0.109**	0.013-0.251	**74.4°**	**<0.001**	0.421	0.422	+0.2	1.247	1.761	+41.2
			Defense	0.010	0.000-0.088	3.8°	0.486	0.187	0.110	-41.2	5.310	6.028	+13.4
			Feeding	0.016	0.000-0.120	7.7°	0.092	0.101	0.199	+97.0	5.479	5.057	-7.7

17

18

19   Table 3: Angle between the leading eigenvector of the P matrix for each population and the leading eigenvector for all populations pooled
20   using either all linear measurements or a subset with defense or feeding related traits only. *P* values are based on bootstrapping with 1000
21   replicates. Significant values after a Benjamini and Yekutieli correction that was performed for each trait group separately are highlighted
22   in bold (see main text for details). See Table 1 for details of each population.

Site	Habitat	Year	*All traits*		*Defense traits*		*Feeding traits*	
			Angle °	***p***	**Angle °**	***p***	**Angle °**	***p***
VSS3	Stream	1921	18.4	0.057	9.8	0.023	1.2	0.139
VDS6	Stream	1958	**63.3**	**<0.001**	**6.0**	**0.003**	1.8	0.032
VDS6	Stream	1964	22.7	0.192	6.0	0.107	2.1	0.019
GES2	Stream	1979	**66.3**	**<0.001**	**13.5**	**<0.001**	2.2	0.033
VSS1	Stream	2007	**75.7**	**<0.001**	**7.3**	**0.001**	**3.0**	**<0.001**
VSS2	Stream	2007	**51.4**	**<0.001**	1.3	0.855	**4.0**	**0.002**
GES1	Stream	2008	**73.3**	**<0.001**	8.1	0.015	3.3	0.017
GES2	Stream	2009	38.4	0.027	**10.2**	**0.005**	2.9	0.013
VSS3	Stream	2009	**65.0**	**<0.001**	**12.1**	**0.002**	**2.1**	**0.002**
VDS6	Stream	2012	**64.5**	**<0.001**	3.2	0.390	12.2	0.086
FrAS	Stream	2012	**51.2**	**<0.001**	7.6	0.018	1.2	0.403
VDL4	Lake	1967	31.5	0.204	20.0	0.060	5.4	0.027
VDL6	Lake	1967	22.0	0.285	**14.2**	**0.007**	**8.9**	**0.002**
GEL1	Lake	1978	22.3	0.075	**24.4**	**0.004**	*12.5*	*0.094*
GEL2	Lake	1979	11.0	0.171	14.1	0.027	**5.8**	**<0.001**
VDL2	Lake	2008	16.6	0.136	9.7	0.030	3.2	0.042
VDL3	Lake	2010	**86.0**	**<0.001**	**17.7**	**0.001**	3.5	0.015
VDL4	Lake	2010	**63.4**	**<0.001**	**25.3**	**<0.001**	2.7	0.357
VDL5	Lake	2010	**87.6**	**<0.001**	**22.5**	**<0.001**	7.1	0.011

VDL6	Lake	2012	**86.4**	**<0.001**	**13.5**	**0.001**	**5.2**	**0.001**
Average Stream (± 1 SD)			53.9 (± 20.6)		7.8 (± 3.8)		3.5 (± 3.2)	
Average Lake (± 1 SD)			47.4 (± 32.9)		17.9 (± 5.4)		6.0 (± 3.1)	

24

25 Table S1: Pairwise F_{ST} values for all genotyped contemporary populations based on nine microsatellites (lower triangle). Significant
26 comparisons after a Benjamini and Yekutieli correction, based on 100,000 bootstrap replicates are highlighted in bold, with the actual p
27 values given in the upper triangle. See Table 1 for details of each population.

|		Lake Geneva system															Other populations					
		GES1	GES2	GES3	GEL3	VDL4	VDL5	VDL2	VDL3	VDL6	VSL1	VSS2	VDS6	VSS4	VSS1	VSS3	FrAS1	VDS2	BEL1	BES4	SGL1	SGS1
Lake Geneva system	GES1	-	0.001	0.026	0.161	0.003	0.003	0.195	0.262	0.042	0.051	0.005	0.178	0.017	0.001	0.001	0.001	0.003	0.001	0.001	0.001	0.001
	GES2	**0.079**	-	0.002	0.001	0.001	0.002	0.001	0.001	0.003	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
	GES3	0.056	**0.214**	-	0.071	0.021	0.042	0.030	0.246	0.009	0.019	0.184	0.021	0.046	0.001	0.001	0.001	0.046	0.001	0.001	0.001	0.001
	GEL3	0.006	**0.068**	0.034	-	0.762	0.141	0.450	0.294	0.208	0.165	0.636	0.403	0.158	0.001	0.001	0.001	0.004	0.001	0.001	0.001	0.001
	VDL4	**0.027**	**0.069**	0.057	-0.006	-	0.246	0.027	0.011	0.083	0.061	0.751	0.019	0.115	0.001	0.002	0.001	0.001	0.001	0.001	0.001	0.001
	VDL5	**0.043**	**0.067**	0.068	0.011	0.007	-	0.086	0.291	0.570	0.320	0.339	0.079	0.672	0.236	0.002	0.001	0.005	0.001	0.001	0.001	0.001
	VDL2	0.006	**0.049**	0.048	0.000	0.017	0.018	-	0.246	0.146	0.294	0.070	0.094	0.108	0.001	0.005	0.001	0.129	0.001	0.001	0.001	0.001
	VDL3	0.005	**0.075**	0.020	0.003	0.024	0.006	0.006	-	0.571	0.117	0.065	0.765	0.114	0.001	0.001	0.001	0.005	0.001	0.001	0.001	0.001
	VDL6	0.015	**0.041**	0.071	0.005	0.010	-0.004	0.007	-0.002	-	0.721	0.152	0.628	0.490	0.002	0.001	0.001	0.002	0.001	0.001	0.001	0.001
	VSL1	0.015	**0.050**	0.070	0.007	0.013	0.004	0.003	0.012	-0.005	-	0.231	0.175	0.693	0.014	0.006	0.001	0.011	0.001	0.001	0.001	0.001
	VSS2	**0.024**	**0.092**	0.019	-0.004	-0.006	0.003	0.014	0.015	0.007	0.005	-	0.043	0.800	0.003	0.003	0.001	0.009	0.001	0.001	0.001	0.001
	VDS6	0.006	**0.065**	0.063	0.001	0.021	0.022	0.010	-0.008	-0.003	0.007	0.017	-	0.041	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
	VSS4	0.021	**0.088**	0.044	0.007	0.009	-0.007	0.010	0.010	-0.001	-0.005	-0.007	0.015	-	0.143	0.007	0.001	0.010	0.001	0.001	0.001	0.001
	VSS1	**0.077**	**0.115**	**0.090**	**0.058**	**0.047**	0.007	**0.053**	**0.048**	**0.031**	0.021	**0.030**	**0.065**	0.008	-	0.001	0.001	0.001	0.001	0.001	0.001	0.001
	VSS3	**0.057**	**0.102**	**0.112**	**0.046**	**0.043**	**0.052**	0.030	**0.072**	**0.049**	**0.035**	**0.032**	**0.072**	0.029	**0.050**	-	0.001	0.012	0.001	0.001	0.001	0.001
Other populations	FrAS1	**0.098**	**0.222**	**0.135**	**0.121**	**0.116**	**0.153**	**0.127**	**0.141**	**0.140**	**0.112**	**0.105**	**0.154**	**0.105**	**0.127**	**0.117**	-	0.001	0.001	0.001	0.001	0.001
	VDS2	**0.024**	**0.075**	0.038	**0.025**	**0.030**	**0.039**	0.008	**0.027**	**0.028**	0.020	**0.023**	**0.045**	0.020	**0.045**	0.022	**0.083**	-	0.001	0.001	0.001	0.001
	BEL1	**0.257**	**0.335**	**0.232**	**0.255**	**0.268**	**0.229**	**0.234**	**0.230**	**0.264**	**0.259**	**0.251**	**0.296**	**0.236**	**0.238**	**0.234**	**0.266**	**0.175**	-	0.584	0.001	0.001
	BES4	**0.262**	**0.337**	**0.246**	**0.259**	**0.272**	**0.237**	**0.240**	**0.236**	**0.274**	**0.272**	**0.262**	**0.302**	**0.251**	**0.254**	**0.242**	**0.269**	**0.185**	-0.002	-	0.001	0.001
	SGL1	**0.338**	**0.394**	**0.295**	**0.344**	**0.363**	**0.331**	**0.303**	**0.299**	**0.349**	**0.352**	**0.352**	**0.379**	**0.341**	**0.343**	**0.346**	**0.396**	**0.245**	**0.144**	**0.157**	-	0.040
	SGS1	**0.357**	**0.413**	**0.312**	**0.359**	**0.378**	**0.347**	**0.327**	**0.313**	**0.364**	**0.370**	**0.368**	**0.393**	**0.358**	**0.360**	**0.370**	**0.417**	**0.265**	**0.149**	**0.163**	0.014	-

28
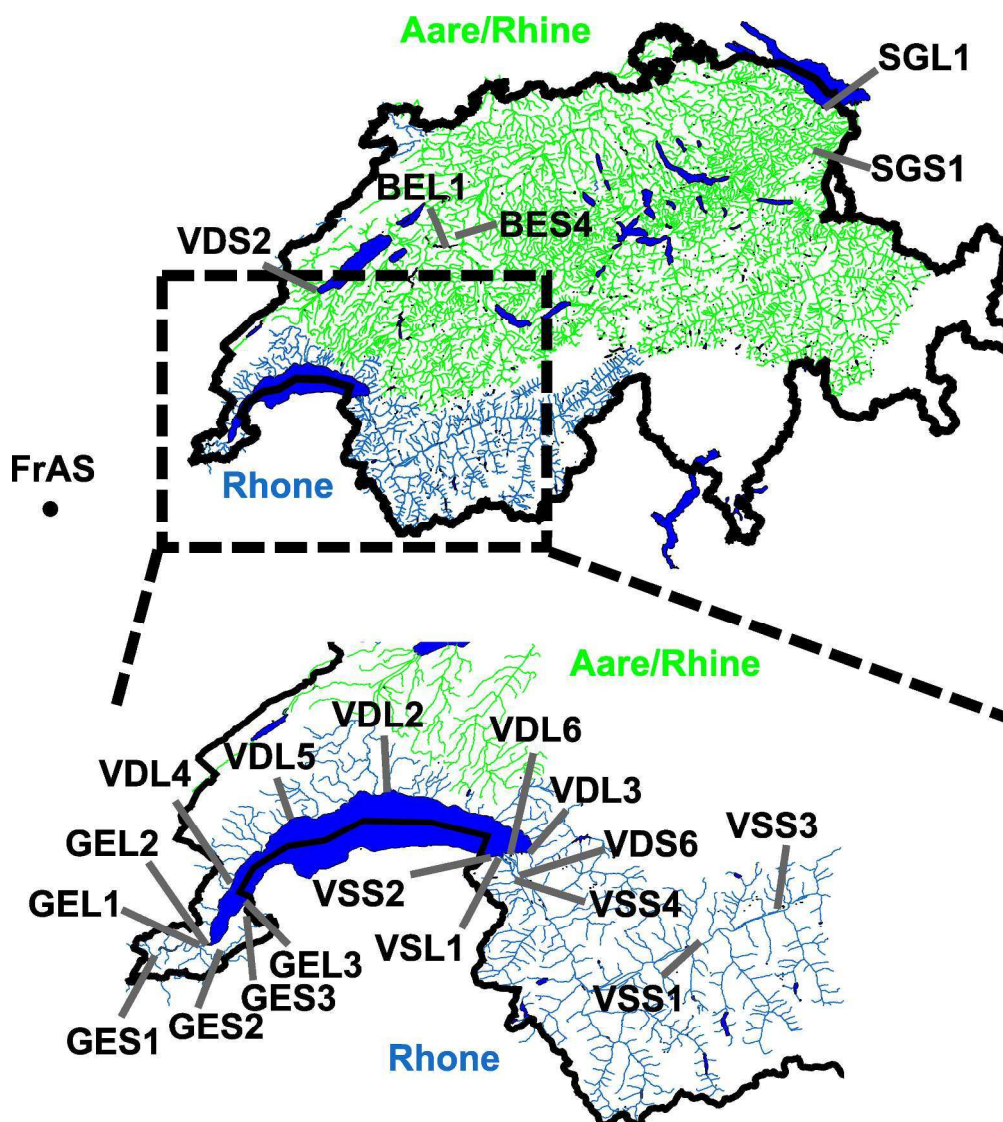
Figure 1: Map of Switzerland (© SwissTopo 2012) with all Swiss sampling sites indicated (see table 1 for details). The colors of waterways represent the two major drainage systems of western Switzerland, i.e. Rhone (blue) and the Aare/Rhine system (green).
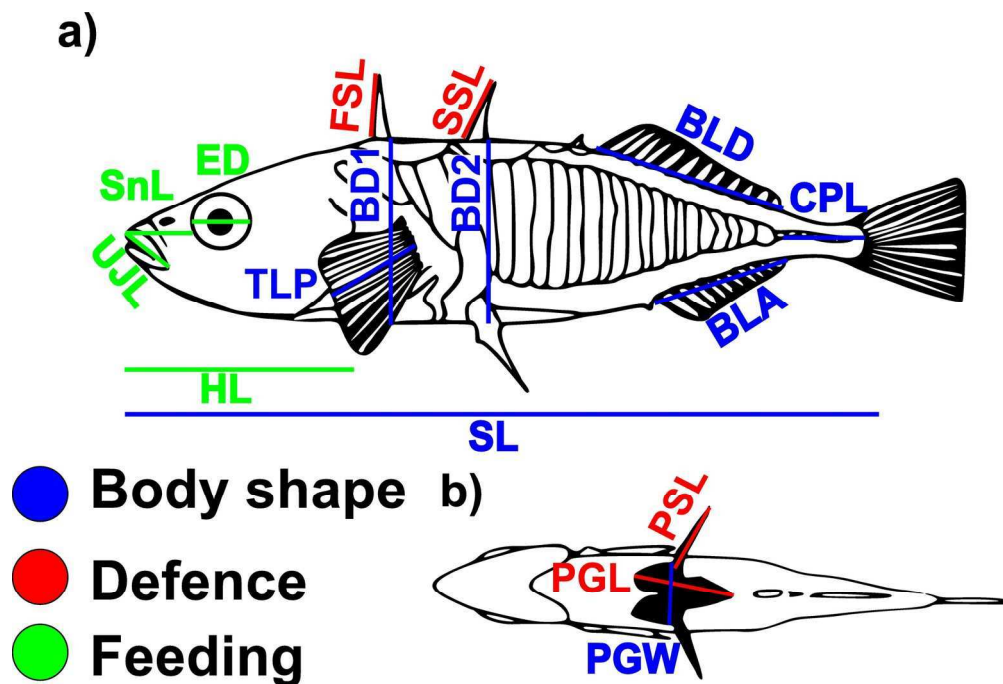
Figure 2: Linear measurements that were taken either from the left flank a) or the ventral side b) of each specimen. Traits were categorized as either anti-predator defence (red), feeding ecology (green) or body shape and swimming performance (blue). Trait abbreviations are as follow: FSL -length of the first dorsal spine; SSL - length of the second dorsal spine; PSL - length of the pelvic spine; PGL - length of the pelvic girdle; HL - head length; UJL - upper jaw length; SnL - snout length; ED - eye diameter; SL - standard length; PGW - width of the pelvic girdle; BD1 - body depth measured after the first dorsal spine; BD2 - body depth measured after the second dorsal spine; caudal peduncle length; BLA - basal length of the anal fin; BLD - basal length of the dorsal fin; TLP - total length of the pelvic fin.
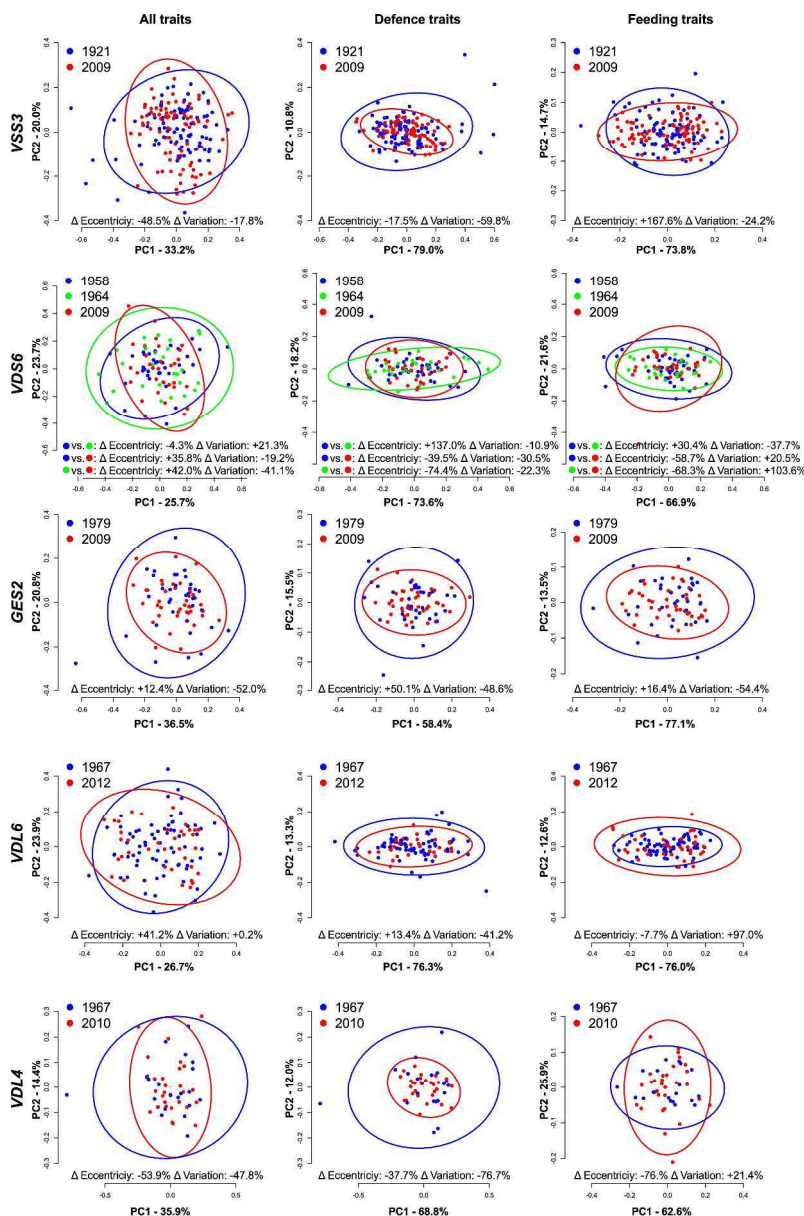163x110mm (300 x 300 DPI)

Figure 3: Principal component scores based on either all linear morphological traits (left), defense related traits (middle) or feeding related traits only (right) for the two leading principal component axes. Only sites are shown where both historical and contemporary populations were available. Ellipsoids represent the 95% CI with contemporary samples in red and historical samples in blue and green. The relative changes between historical and contemporary samples (Δ) in eccentricity and phenotypic variation, i.e. the size of the 95% CI ellipsoid, are indicated (see Table 2 for details).

513x776mm (300 x 300 DPI)
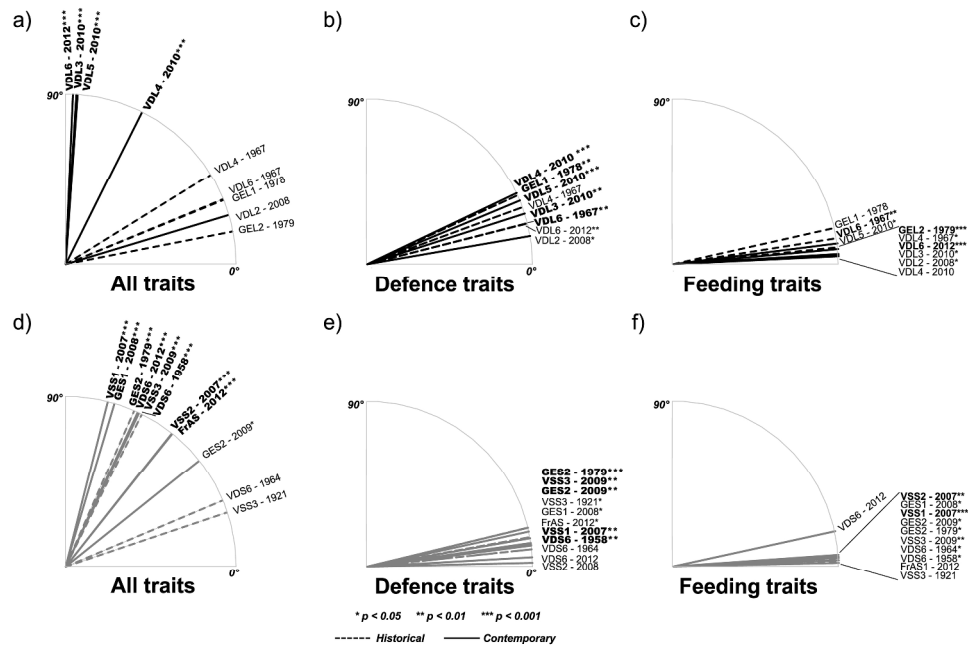
Figure 4: The angle against a common pmax for each population and year for either all traits combined or defense or feeding related traits only (see Table S1). The major axis of phenotypic variation (pmax) for each population is given for lake (a - c) and stream (d - f) populations, separating historical (dashed) and contemporary (continuous line) sampling events. P values are calculated from bootstrapping with 1000 replicates. Boldfaced populations indicate cases where pmax differed significantly after applying a Benjamini & Yekutieli correction for multiple comparisons (see main text for details).
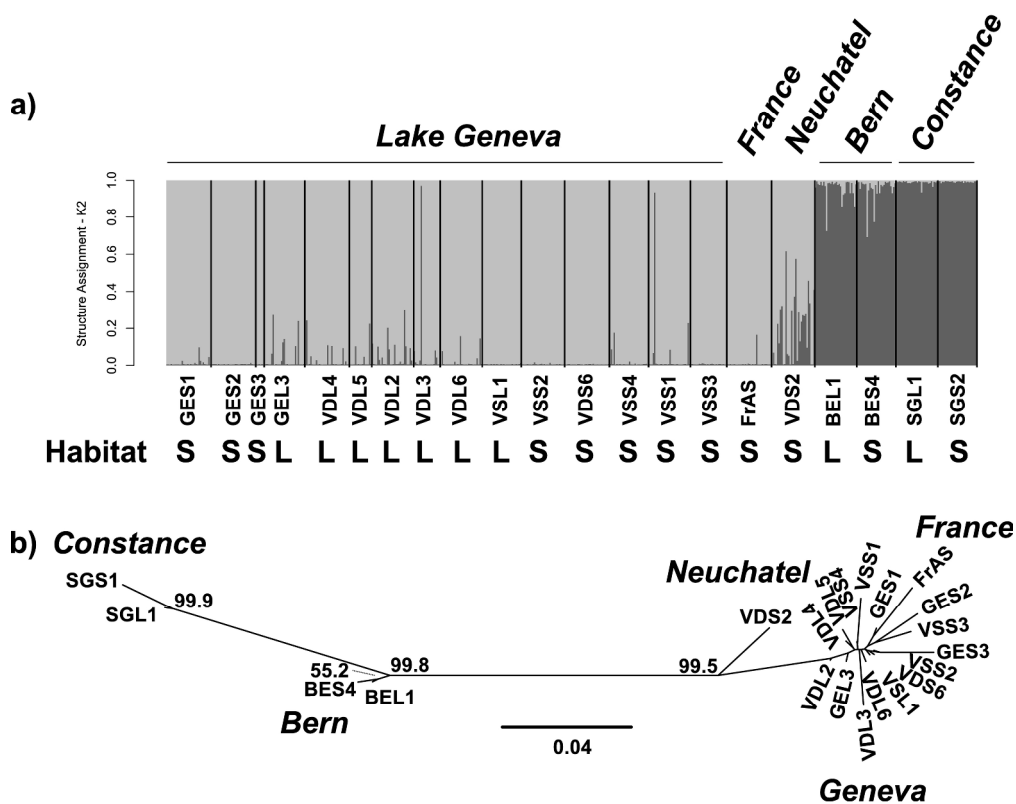
292x199mm (300 x 300 DPI)

Figure 5: Genetic relationship among individuals and populations within Lake Geneva and Switzerland (Constance, Bern and Neuchatel) as well as a population from the Rhone river downstream of Lake Geneva (France) based on nine microsatellites. a) Individual based assignments using STRUCTURE for the best supported number of clusters, K = 2. The respective habitat where populations were samples is indicated (L = Lake, S = Stream). b) Unrooted neighbour joining tree based on Cavalli-Sforza distances calculated from population allele frequencies. Bold numbers indicate the support for each node based on 1000 bootstrap replicates. Only values with more than 50% bootstrap support are given. See table 1 for details of each population.
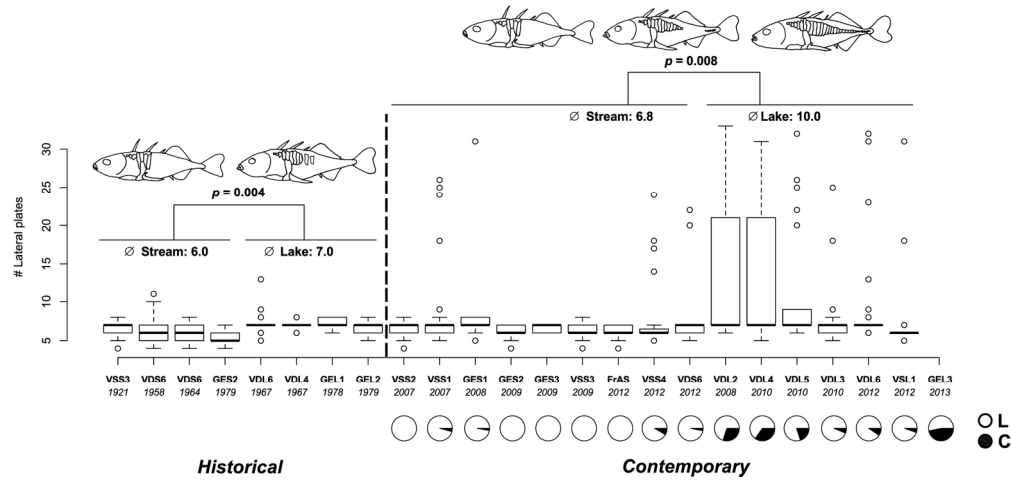265x209mm (300 x 300 DPI)

Figure 6: Number of lateral plates in each population for both historical and contemporary stream and lake populations. P values for significance of differences between habitats are based on post hoc t tests associated with fixed-effect parameters in a linear model that included the non-significant interaction between time (historical and contemporary) and habitat (lake and stream; see main text for details). The average number of plates for each habitat is reported above the boxplots. Pie plots indicate the allele frequency of the two Stn382 alleles (white – L allele, black – C allele). No phenotypic data was available for the GEL3 population (see Table 1 for details).
171x80mm (300 x 300 DPI)