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1 **Little evidence for a selective advantage of armour-reduced threespined**  
2 **stickleback individuals in an invertebrate predation experiment**

3

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35 Armour reduction provides little advantage to stickleback against an invertebrate

36 predator

37

38 **Abstract**

39 The repeated colonization of freshwater habitats by the ancestrally marine  
40 threespined stickleback *Gasterosteus aculeatus* has been associated with many  
41 instances of parallel reduction in armour traits, most notably number of lateral plates.  
42 The change in predation regime from marine systems, dominated by gape-limited  
43 predators such as piscivorous fishes, to freshwater habitats where grappling  
44 invertebrate predators such as insect larvae can dominate the predation regime, has  
45 been hypothesized as a driving force. Here we experimentally test the hypothesis  
46 that stickleback with reduced armour possess a selective advantage in the face of  
47 predation by invertebrates, using a natural population of stickleback that is highly  
48 polymorphic for armour traits and a common invertebrate predator from the same  
49 location. Our results provide no compelling evidence for selection in this particular  
50 predator-prey interaction. We suggest that the postulated selective advantage of low  
51 armour in the face of invertebrate predation may not be universal.

52

53

54 **Introduction**

55 Testing the role of natural selection in phenotypic adaptations to divergent  
56 environments has proven to be a difficult task (Endler 1986; Conner 2001).  
57 Differences in resources, habitat structure, predation and parasite infestation  
58 and competition are often thought to be responsible for divergent natural selection.  
59 The latter, a key driver of adaptive phenotypic differentiation, can lead to ecological  
60 speciation (Rundle and Nosil 2005) and eventually adaptive radiation (Schluter  
61 2000). Phenotypic differentiation driven by heterogeneity in habitat structure,  
62 available resources as well as intra- and interspecific competition has been  
63 extensively studied and documented in a wide range of organisms (Schluter 2000).  
64 However, there is limited direct evidence for a role of predation leading to speciation  
65 (Vamosi 2005; Langerhans et al. 2007; Marchinko 2009). It has been shown that  
66 predators may cause divergent selection on body shape, for instance in the Bahamas  
67 mosquito fish *Gambusia hubbsi*, where divergent predation pressures have shifted  
68 morphological traits in different directions. Fish in low predation areas tend to have a  
69 more streamlined body whereas the fish in high predation regimes have a deeper  
70 body shape. These traits were found to be the basis for assortative mating,  
71 reproductive isolation and consequently speciation (Langerhans et al. 2007).  
72 Furthermore, it has been shown that predators can shape the evolution of divergent  
73 life histories (Reznick and Endler 1982; Johnson 2001). For example, increased  
74 predation rates on old or large individuals should favor the evolution of early  
75 maturation and a high reproductive effort, while contrary trends are expected by an  
76 increased rate of predation on very young age-classes and small individuals  
77 (Ernande et al. 2004; Gårdmark and Dieckmann 2006; Walsh and Reznick 2009).

78

79 Repeated parallel cases of invasion of freshwater habitats, and the presence of  
80 replicate sets of coexisting and divergent forms have made the threespined  
81 stickleback an important model system to study adaptive diversification and perhaps  
82 the early stages of speciation (McKinnon and Rundle 2002). They exhibit a range of  
83 phenotypic traits that are related to their defense against predators. These include  
84 external bony skeletal armour traits, consisting of lateral plates (which are modified  
85 scales), pelvic and dorsal spines and a pelvic girdle. Lateral plates can be divided  
86 into structural and non-structural plates; structural plates form, together with the  
87 pelvic girdle, pelvic spines and dorsal spines, a massive defensive structure. The  
88 presence of structural plates on both sides of the fish is crucial to the effectiveness of  
89 the spines against predators. Non-structural plates reduce the probability of  
90 integumentary injuries following an attack by a predator (Reimchen 1992). In their  
91 ancestral marine habitat, stickleback are generally heavily armoured with a full set of  
92 lateral plates covering the whole flank of the body and have long dorsal and pelvic  
93 spines (Reimchen 1994; McKinnon and Rundle 2002). This extensive armour is  
94 thought to protect them in the marine habitat, which is dominated by gape limited and  
95 puncturing fish and bird predators (Gross 1978). Since the last retreat of the glaciers  
96 less than 15,000 years ago, the threespined stickleback has invaded temperate  
97 freshwater systems independently throughout the northern hemisphere (Bell and  
98 Foster 1994; McKinnon and Rundle 2002). Armour reduction has occurred  
99 repeatedly in most of these locations within this short evolutionary time. Various  
100 hypotheses have been postulated to explain the reduction in armour in freshwater  
101 relating to both biotic and abiotic factors (Barrett 2010). Biotic mechanisms include  
102 adaptations to different predator regimes (e.g. Reimchen 1980; Reimchen 1992;  
103 Vamosi 2002; Marchinko 2009), food availability (Bjærke et al. 2010) and buoyancy  
104 (Myhre and Klepaker 2009), while abiotic mechanisms include adaptations to water

105 chemistry (e.g. Heuts 1947; Giles 1983; Bell et al. 1993; Marchinko and Schluter  
106 2007). However, in most cases, more than one factor is likely involved, and the  
107 reason for the observed pattern is difficult to single out.

108

109 The selective advantage of putative defense traits has been investigated and tested  
110 in several studies. Reimchen (1992; 1994) found a positive correlation between the  
111 number of predatory fish species in a habitat and the length of pelvic and dorsal  
112 spines of sticklebacks. Most predatory fish are gape-limited, meaning that they are  
113 only able to eat prey items smaller than their own gape. Longer spines increase the  
114 effective diameter of an individual, making it more difficult for gape-limited fish and  
115 bird predators to swallow the prey (Bañbura 1994) and also increase the likelihood of  
116 injuries to the predator. Lateral plates increase the survival of sticklebacks following  
117 escape from attacks by a fish or bird predator (Hoogland et al. 1956; Reimchen 1992;  
118 Reimchen 1992; Reimchen 2000). In general, armour traits are thought to be  
119 important in marine and lacustrine habitats, where the predation regime is dominated  
120 by gape limited predators. However the mechanisms underlying the occurrence of  
121 populations fixed for the low plated phenotype in freshwater bodies, and the reason  
122 for its repeated parallel evolution remains poorly understood. Reimchen (1980; 1994)  
123 hypothesized that armour traits provide structures where grappling predators, such  
124 as dragonfly larvae, backswimmers (*Notonecta sp.*) and diving beetles, could grip  
125 their prey. Specifically, Reimchen (1980) suggested that spines might serve as  
126 objects that invertebrate predators such as *Aeshna* could use to grasp the fish;  
127 therefore a reduction in the spine phenotype (both in size and number) might confer  
128 a selective advantage in the face of strong dragonfly predation. He also suggested  
129 that external structures that enhance frictional contact with grappling predators, such  
130 as *Aeshna*, would be disadvantageous in such predation regimes. We tested the

131 hypothesis that lateral plates represent such structures, and hence expect that low  
132 plating should be advantageous in an *Aeshna* dominated predation regime. Then, in  
133 shallow and stagnant water habitats where fish predators are uncommon and aquatic  
134 insects are important predators, low plate number, smaller plate size and reduced  
135 spines should be advantageous. An experimental test of this hypothesis (Marchinko  
136 2009) showed that insect predation resulted in selection for faster growth rate,  
137 shorter spines, reduced girdle length, and selection for the allele associated with the  
138 low plate phenotype, but could not demonstrate selection on the lateral plate  
139 phenotype itself. In fact, the experiment only used juvenile fish, where the plate  
140 phenotype was not yet fully expressed. He did, though, find a selective advantage for  
141 the L-allele and argued that *Eda* might have pleiotropic effects that are under  
142 selection.

143

144 The genetic basis of the plate phenotype in stickleback is relatively well understood.  
145 A single gene, *Ectodysplasin (Eda)*, has been demonstrated to explain ~75% of the  
146 genetic variation for different bony lateral plate phenotypes between marine and  
147 freshwater threespined stickleback (Colosimo et al. 2004). Changes within and near  
148 *Eda* have been shown to be responsible for the repeated loss of lateral plates in  
149 freshwater all over the world (Cresko et al. 2004; Colosimo et al. 2005). Phylogenetic  
150 analyses of the *Eda* sequences grouped most populations all over the world  
151 according to their plate phenotype. The same analyses with 25 neutral microsatellite  
152 markers grouped the populations by geography rather than plate morph suggesting  
153 that the *Eda* locus is a likely target of selection in freshwater habitats. There are two  
154 *Eda* alleles, referred to as the “L-“ (low) and “C-“ (complete) allele. Alleles for the low  
155 plated phenotype must have been maintained at low frequencies in marine  
156 stickleback (Schluter and Conte 2009). These freshwater alleles are exported to the



157 sea through occasional hybridization events during contact between freshwater-  
158 resident and anadromous marine populations. As a consequence, freshwater alleles  
159 are now present as rare standing genetic variation in the sea, but can become  
160 positively selected for again during the colonization of new freshwater habitats  
161 (Cresko et al. 2004; Colosimo et al. 2005). However there is still only very limited  
162 empirical support for invertebrate predation induced selection on defense traits and  
163 their genetic components.

164

165

166 Despite decades of research, there remain significant gaps in our understanding of  
167 the potential of invertebrate predator induced selection on armour variants in adult  
168 threespined stickleback. The aim of the study is to test experimentally whether  
169 predation by dragonfly larvae exerts selection on armour related traits by comparing  
170 the distribution of armour variants and *Eda* alleles between different experimental  
171 predation treatments. We exposed fish from a natural population that is highly  
172 polymorphic for defense-related traits to high densities of invertebrate predators  
173 (dragonfly larvae) and compared fish that survivors and dead fish with those from a  
174 no predation control. The expectation was that low plated fish and/or those with  
175 reduced pelvic and dorsal spines have elevated survival rates under dragon fly  
176 predation.

177 We test these hypotheses in a highly polymorphic wild population. Threespined  
178 stickleback have spread rapidly across Switzerland in the last 140 years, following  
179 introductions from different European sources and hybridization, resulting in large  
180 phenotypic and genetic diversity within some populations (and also between  
181 populations; Lucek et al. 2011). This makes Swiss stickleback populations an ideal  
182 study system to address key questions in the processes involved in adaptive

183 diversification during a biological invasion. Hybridization between different lineages  
184 during secondary contact in the Bernese midlands has been documented (Lucek et  
185 al. 2010) and the great diversity in defense related phenotypes makes these hybrid  
186 populations ideal to investigate these issues.

187

## 188 **Materials and Methods**

### 189 ***Population based selection experiments***

#### 190 *Fish and dragonfly samples*

191 A total of 960 threespined stickleback were used for the experiment. All were taken  
192 from a natural population living in a stream near Bern, Switzerland. This population  
193 shows large variation in plate morphology and the length of pelvic and dorsal spines  
194 (Lucek et al. 2010). All fish were caught using either minnow traps or hand nets in  
195 spring 2010. The dragonfly larvae were collected using hand nets from the same site  
196 and other nearby ponds and streams. All dragonfly larvae used belonged to the  
197 genus *Aeshna*.

198

#### 199 *Experimental setup*

200 Eight experimental ponds of approximately 3 m x 2 m and 40 cm depth were each  
201 divided into two equal halves with nylon netting (2mm mesh size). Half-ponds were  
202 randomly assigned to the treatment (*i.e.* predation) or the control (no predation)  
203 group. Ten artificial plants, made of black plastic strips attached to a stone, and a  
204 wooden stick (about 0.5 m in length) were placed in each half-pond to provide  
205 shelter. The ponds were located within metal grill enclosures, so predation by bird or  
206 mammalian piscivores was not possible. Thirty adult stickleback were randomly  
207 assigned to each of the 16 half-ponds and allowed to acclimate to the environment  
208 for two days without predators. The fish were fed daily with a mixture of frozen

209 *Daphnia* spp. and frozen chironomid larvae. After the acclimation period, ten  
210 dragonfly larvae (*Aeshna* spp.) were introduced to the experimental treatments.  
211 Ponds were checked twice a day and the remains of all dead fish were removed and  
212 preserved in 95% ethanol. Dead or ecdysed dragonfly larvae were replaced with  
213 new ones to keep the predator density constant. The experiment was conducted in  
214 two runs: the first from 4<sup>th</sup> to 15<sup>th</sup> of May 2010 and the second from 19<sup>th</sup> to 29<sup>th</sup> of  
215 May 2010. After each run, all ponds were emptied and surviving fish were euthanized  
216 with an overdose of clove oil and stored in 95% ethanol for further analysis.

217

### 218 *Morphological analyses*

219 Standard length (SL), first dorsal spine length (FSL), second dorsal spine length  
220 (SSL), pelvic spine length (PSL) and body depth (BD, measured at the base of the  
221 second dorsal spine) were measured to the nearest 0.01 mm using digital calipers  
222 (Sylvac SA). All individuals were stained with alizarin red to ensure accurate counts  
223 of the lateral plates (Peichel et al. 2001), followed by removal of melanin pigments by  
224 bleaching in 3% H<sub>2</sub>O<sub>2</sub> for approximately 2 hours. Lateral plates were counted on the  
225 left side of each fish, and individuals were classified as low-, partially- or fully-plated  
226 phenotypes. Low plated individuals have between four and nine structural plates in  
227 the anterior region of the body, whereas fully plated individuals have continuous  
228 plating from the anterior region to the end of the caudal peduncle, forming a keel.  
229 Partial plated fish are intermediate, and either have a keel or lack one.

230

### 231 *Age determination*

232 A common way to make an accurate age determination for many fish species is with  
233 the use of scales (Helfman et al. 1997); in stickleback, however, scales have evolved  
234 into lateral plates, which cannot be used for age determination. Otoliths, which are

235 structures made of calcium carbonate in the inner ear, were used instead for age  
236 estimation. The accretion of the otoliths depends on the growth of the fish. Individuals  
237 generally show a higher growth rate in summer than in winter, resulting in seasonal  
238 rings on the otolith. By counting these rings, it is possible to determine the age of the  
239 fish in years. A procedure similar to the one described by Münzing (1959) was used  
240 to extract otoliths. Otoliths were extracted from ten randomly chosen fish from each  
241 control and each treatment, totalling 160 individuals from controls and 160 from  
242 treatments. Otoliths were mounted on slides and investigated twice under a  
243 microscope (Leitz Laborlux 11) by two different observers. Deviations between the  
244 different observers were discussed and investigated again to find an optimal  
245 determination of age.

246

#### 247 *Genetic analyses*

248 All individuals were genotyped for two markers, *Stn382* and *Idh*, using PCR protocols  
249 following Colosimo *et al.* (2005). The *Stn382* microsatellite flanks a 60 bp indel in  
250 intron 1 of the *Eda* gene, yielding either a 158 bp allele (low, or L, allele), associated  
251 with the low plated phenotype or a 218 bp allele (complete, or C, allele), associated  
252 with the complete plated phenotype. The *Idh* microsatellite marker (Peichel *et al.*  
253 2004) is diagnostic for sex, females being homozygous for a 300 bp allele and males  
254 heterozygous for the 300 bp allele and a 270 bp allele. For DNA extraction, a small  
255 piece of pectoral fin tissue from each fish was placed into a tube with 180µl of 10%  
256 Chelex 100® (Biorad, USA). The tubes were incubated twice for 15 minutes at 95°C  
257 each time, briefly vortexed between steps, spun down and 1 µl of the supernatant  
258 was transferred into new tubes. PCR amplifications were performed in 10 µl volumes.  
259 Details of PCR conditions are available upon request. The PCR products were run on  
260 a 1.5% agarose gel and genotypes scored by eye.

261

262 *Plate cover*

263 Not only does the number of lateral plates vary considerably among individuals in the  
264 studied population, so does the size of the plates. Thus, in addition to lateral plate  
265 counts, the area covered by lateral plates relative to the total surface area of the  
266 sides of the body was measured for a subset of samples. Thirty-nine randomly  
267 selected fish were scanned with a standard flatbed scanner. The surface area  
268 covered by plates as well as the total surface area of the fish was measured using  
269 Photoshop CS5 (Adobe, USA). Plate cover was then calculated as the ratio of the  
270 pixel counts of the two values.

271

272 *Statistical analyses*

273 Given that all linear measurements are correlated with body size, we first regressed  
274 measurements for each trait against SL and retained the residuals for all subsequent  
275 analyses. The distributions of all linear morphological traits (including SL) as well as  
276 plate counts were tested for normality using a one-sample Kolmogorov-Smirnov test  
277 for control and treatment independently. To account for potential differences among  
278 runs and ponds, a linear mixed model was used correcting for heterogeneity among  
279 runs. All traits were independently analyzed with treatment (predation and no  
280 predation controls) as explanatory variable and ponds nested within runs as random  
281 factors. This analysis was performed for lateral plate numbers and size corrected  
282 linear morphological traits, as well as the scores along the first two axes from  
283 principal component analysis (PCA) based on size corrected linear morphology  
284 (excluding plate count).

285

286 Standardized selection differentials ( $i$ ) for individual traits in each replicate were  
287 calculated according to the following equation (Endler 1986)

288

$$289 \quad i = \frac{\bar{X}_a - \bar{X}_b}{\sqrt{\text{var}_b}},$$

290 where  $\bar{X}_a$  represents the mean trait value for fish under selection (treatment),  $\bar{X}_b$  the  
291 mean trait value without selection (control), and  $\text{var}_b$  is the trait variance in the  
292 control. Although the original equation compares the same group before and after  
293 selection, we use the control versus treatment approach rather than pre- and post-  
294 selection, assuming that the control represents the pre-selection distribution of traits.  
295 Since all experimental fish were randomly drawn from a much larger pool of  
296 thousands of fish, it is unlikely that there were large and systematic differences  
297 between the two groups at the start of the experiment. The reason we did not  
298 measure fish pre-selection was to avoid handling, and therefore stressing and  
299 possibly injuring fish, while taking measurements. The traits that we measured in this  
300 experiment require considerable handling, and while this would have been stressful  
301 or harmful to all fish, it would have been inordinately so for smaller individuals,  
302 thereby confounding all our analyses. To accurately count plates, especially on a  
303 small fish, requires the use of a dissecting needle, and the most accurate way is to  
304 stain the fish in alizarin (which we did), which is why we only did it at the end of the  
305 experiment. A similar approach was employed by Marchinko (2009) in a comparable  
306 experiment. Significance was estimated using a re-sampling procedure with 1000  
307 replicates. These analyses were performed using R 2.12.1 (The R Project).

308

### 309 ***Individual based selection experiments***

#### 310 *Experimental setup and procedure*

311 The experiments described above were performed with adult fish and used dragonfly  
312 larvae larger than 42mm. To examine the effect of predator size, prey size and the  
313 difference between them on predation success, we performed one-on-one trials  
314 using individuals taken from a broad size distribution of both predators and prey. We  
315 conducted 50 such trials using dragonfly larvae between 36 and 52 mm in length and  
316 fish between 25 and 44 mm SL. In each trial, one randomly chosen fish was exposed  
317 to one randomly chosen dragonfly larva for a maximum of 30 minutes. Each trial was  
318 filmed and predator attack events were recorded. The dragonfly larvae were first  
319 placed in an aquarium (130 mm x 150 mm x 125 mm). After five minutes, the fish  
320 was added to the aquarium in an inverted transparent perforated plastic beaker in  
321 such a way that visual and chemical communication between predator and prey was  
322 possible, but actual predation was not. After another five minutes the beaker was  
323 removed. The trial was stopped five minutes later if the predator had successfully  
324 attacked a fish. If the dragonfly larva did not attempt to attack the fish within 30  
325 minutes, the trial was stopped. The outcome of each trial was assigned to one of  
326 three categories: 1 - the dragonfly larva did not attack the prey; 2 – the dragonfly  
327 larva actively attacked the stickleback but had no success; 3 – the dragonfly larva  
328 successfully hunted the stickleback.

329

### 330 *Samples*

331 We used 50 randomly selected threespine sticklebacks from the same site near  
332 Bern, Switzerland. These fish were caught using hand nets in early December 2010.  
333 The dragonfly larvae (*Aeshna* spp.) for these experiments were caught using hand  
334 nets in early October 2010 from the same location and a pond nearby.

335

### 336 **Results**

337 ***Population based selection experiments***

338 *Mortality*

339 The recapture rate (the ratio of the sum of the number of dead fish removed during  
340 the experiment and the number of surviving fish recovered at the end of the  
341 experiment to the total number of fish released at the start of the experiment) was  
342 98.3% for the first run and 98.1% for the second run. In both runs, there was a much  
343 higher mortality in treatments (T1 and T2, respectively in runs 1 and 2) compared to  
344 controls (C1 and C2); mortality in T1 was 35.3% compared to 3.4% in C1, and 31.5%  
345 in T2 compared to 9.8% in C2 (both differences statistically significant; T1 vs C1  $P =$   
346 0.001 and T2 vs C2  $P = 0.03$ ; Mann-Whitney U-tests). There was no significant  
347 difference in mortality between treatments from run 1 versus run 2 (T1 vs. T2;  $P =$   
348 0.460), but significantly higher mortality occurred in C2 compared to C1 ( $P = 0.009$ ).  
349 Overall the mortality rate in our controls were comparable to those in a similar  
350 experiment conducted by Marchinko (2009).

351

352 *Linear morphological measurements*

353 All linear morphological measurements (SL, FSL, SSL, PSL, BD) as well as their size  
354 corrected residuals were normally distributed for both controls and treatment  
355 combined (Kolmogorov-Smirnov tests). Within treatments, only the residuals of FSL  
356 in the control ( $D = 0.986$ ,  $p = 0.032$ ) and the residuals of SSL in the predation  
357 treatment ( $D = 0.994$ ,  $p = 0.019$ ) were not normally distributed. The two first PC axes  
358 explain 52.4% and 24.0% of the total variation respectively. Loadings between these  
359 axes differed, as all spine lengths contributed most on the first and BD on the second  
360 axis.

361



362 None of the size corrected linear measurements except BD were significantly  
363 different between controls and treatments (Table 1). BD showed a significant effect  
364 ( $F_{1,737} = 5.569$ ,  $P = 0.019$ ) with surviving individuals in the treatment being deeper  
365 bodied than fish from the control. Concomitantly, the second PC axis, to which BD  
366 makes a very high contribution, showed a significant difference among treatments  
367 ( $F_{1,737} = 4.358$ ,  $P = 0.037$ ). The first PC axis did not differ between control and  
368 treatment, nor did lateral plate counts or spine lengths. The distribution of  
369 morphological trait values for control and treatment fish for traits studied here, as well  
370 as the PC scores for linear traits along the first two PC axes are shown in figures 1  
371 and S1.

372

#### 373 *Lateral plate counts and coverage*

374 Lateral plate counts were strongly bimodal, and not normally distributed in either the  
375 treatments or the controls ( $P < 0.001$  in both cases; Kolmogorov-Smirnov tests).

376 There was no significant overall difference in the number of lateral plates between  
377 controls and treatments ( $P = 0.798$ ; Mann-Whitney-U-test) even when corrected for  
378 run and pond (table 1). Plate cover, also not normally distributed, again showed no  
379 significant difference between treatments and controls ( $P = 0.810$ ; Mann Whitney U-  
380 test). Plate cover was highly correlated with plate count ( $R^2 = 0.845$ ,  $P < 0.001$ ) in  
381 this population, which is not necessarily the case in other Swiss stickleback  
382 populations (O. Seehausen and K. Lucek, unpublished data).

383

#### 384 *Stn382 allele frequencies*

385 Allele frequencies at the *Stn382* locus were normally distributed among replicates ( $P$   
386 = 0.699, Kolmogorov-Smirnov test). The frequency of the C-allele in the control was  
387 not significantly higher than in the treatments ( $P = 0.729$ , one-tailed t-test; figure 2).

388 Further *Stn382* C-alleles were not significantly higher in frequency in the surviving  
389 fish from treatments compared to the dead fish from the treatments, which were  
390 removed during the course of the experiment ( $P = 0.400$ , paired t-test; figure 2).  
391 Analysing the differences in *Stn382* C-allele frequencies for each replicate  
392 separately, we found that the differences between replicates appeared to be  
393 stochastic. In only four out of 16 replicates did we find a significant difference in the  
394 allele frequency when comparing control and treatment for each replicate separately,  
395 using a Fisher exact test (table 2). In three cases (replicates 2, 3 and 6) this  
396 difference was in the predicted direction, and in the fourth case (replicate 16) it was  
397 in the opposite direction. However, this test might be confounded by variation in  
398 starting frequencies so we conducted the same type of tests comparing the surviving  
399 fish from treatments with those which died during the experiment and found a  
400 significant difference in *Stn382* allele frequencies in 7 of 16 replicates. In four cases  
401 (replicates 5, 13, 14, and 16) the difference in allele frequencies was in the expected  
402 direction while in three cases (replicates 3, 7, 15) it was in the opposite direction.  
403 Furthermore the difference in the *Stn382* C-allele frequency of all replicates between  
404 “dead” and “alive” did not differ from zero ( $P = 0.400$ , one sample t-test of difference  
405 against zero).

406

#### 407 *Survival of Stn382 alleles and genotypes*

408 The survival for the two *Stn382* alleles, as well as the three genotypes were  
409 measured in all the treatment replicates. The L-allele did not have a higher survival  
410 rate compared to the C-allele ( $P = 0.302$ ; one tailed t-test; figure S2). There was also  
411 no significant difference in the survival of the three genotypes (CC vs LC:  $P = 0.728$ ;  
412 LC vs LL:  $P = 0.275$ ; CC vs LL:  $P = 0.737$ ; two-tailed t-tests; figure S2 ).

413

414 *Lateral plate count and Stn382 genotype*

415 There was no apparent difference in the frequency distribution of lateral plates, when  
416 broken down by *Stn382* genotype, between the control and treatment fish (figure 3).

417

418 *Sex and age*

419 To test the possibility that the two sexes differ in the selective pressure exerted by  
420 predatory dragonfly larvae, we compared the sex ratios of fish in the treatments and  
421 controls, but found no significant difference ( $P = 0.960$ ). We then asked whether  
422 there was a difference in age structure among fish in the controls and treatments,  
423 possibly reflecting learnt behavioural avoidance of predation. Again, we found no  
424 difference between controls and treatments (figure S3;  $P = 0.104$ ).

425

426 *Selection differentials*

427 Estimated selection differentials were significantly different from zero only in a few  
428 cases among the six traits tested in sixteen replicates (FSL in three replicates, SSL in  
429 two replicates, PSL in two replicates and BD in four replicates; table 3). This  
430 suggests that in this experiment there is no indication that dragonfly larvae caused  
431 directional selection pressure on any of these morphological traits.

432

433 ***Individual based selection experiments***

434 Standard lengths (SL) of stickleback and length of dragonfly larvae as well as the  
435 difference between these lengths were normally distributed for all categories  
436 (Kolmogorov-Smirnov tests). In nearly half (23 out of 50) of the trials, there was no  
437 attempt by the predator to capture the fish (category 1). Of the remaining trials,  
438 where the predator attempted to capture the prey, in eight cases the attempt was  
439 unsuccessful (category 2), while in nineteen cases it was successful (category 3).

440 Fish did not differ significantly in size between successful and unsuccessful predation  
441 attempts (two-tailed t-tests;  $P = 0.394$ ), nor did it differ between cases where  
442 attempts were made or not (category 1 vs 2,  $P = 0.912$ ; 1 vs 3,  $P = 0.333$ ). The same  
443 was true for dragonfly larvae: successful predators were not significantly different in  
444 size from unsuccessful ones ( $P = 0.504$ ) nor did size of the predator differ between  
445 cases where no attempt was made and ones where they were (category 1 vs 2,  $P =$   
446  $0.502$ ; 1 vs 3,  $P = 0.959$ ). Finally, the size difference between the predator and prey  
447 (length of dragonfly larva minus SL of fish) also did not differ between categories 2  
448 and 3 ( $P = 0.249$ ), nor did it between category 1 and cases of attempted predation (1  
449 vs 2,  $P = 0.697$ ; 1 vs 3,  $P = 0.412$ ; figure 4). Logistic regression using the outcome as  
450 a categorical response and difference in size between predator and prey as the  
451 predictor showed no significant relationship whether or not category 1 was included  
452 in the analysis ( $P = 0.637$  and  $0.244$  respectively).

453

## 454 **Discussion**

455 The aim of this study was to experimentally test predictions of the hypothesis that  
456 predation by dragonfly larvae exerts selection on stickleback armour traits by  
457 comparing the distribution of armour trait variants between different experimental  
458 conditions. We did not find any significant selective advantage for fish with reduced  
459 armour traits.

460

461 Reimchen (1980, 1994, 2000) hypothesized a reduction in armour from marine to  
462 freshwater habitats comprising three distinct components: a reduction in the posterior  
463 plates as an adaptation for increased burst velocity, a reduction in the anterior plates  
464 associated with a shift in predator behaviour from puncturing to compression and a  
465 reduction in spine lengths as an adaptation for increased post-capture escape

466 against grappling predators. The hypothesis we addressed differed from the original  
467 hypothesis in that we tested whether dragonfly larvae exerted selection on the  
468 number of lateral plates and on the length of spines. We find no compelling evidence  
469 in this study that there is a selective advantage in the reduction of defense traits  
470 (spine lengths and lateral plate number).

471

472 We made 96 estimates of selection differentials for various armour traits: six traits,  
473 each measured in 16 replicates. In eleven of these cases, selection differentials differ  
474 from the null expectation. All of these cases point towards greater relative spine  
475 length and body depth in treatment survivors compared to control fish. Given that of  
476 96 comparisons, about five may be expected to be significant by chance, but not in a  
477 predictable direction, the fact that eleven comparisons are significant in the same  
478 direction may be interpreted as evidence for selection for longer relative spine length  
479 and increased body depth. This trend for spine length is, however, contrary to the  
480 predictions of a selective advantage of reduced body armour under dragonfly  
481 predation. It is possible that fish with longer spines are attacked less often, and  
482 hence have enhanced survival, but our experiment does not allow us to  
483 unequivocally infer this. It might, however, be argued that a  $P$  value of 0.05 is both  
484 arbitrary and too stringent to be applied to selection differentials, since only strong  
485 selection could be detected given our experimental sample sizes, whereas weak and  
486 moderate natural selection is most common in nature. Indeed the four highest  
487 selection differentials for plate number indicate a reduction in plate number in the  
488 treatment survivors relative to controls, consistent with predictions. This might be  
489 interpreted as weak evidence for a selective advantage of reduced plate cover.  
490 However, given our experimental design, sample sizes and use of standard statistical  
491 criteria, we cannot claim strong or unequivocal support for the hypothesis.

492

493 Marchinko (2009) showed that insect predation resulted in selection for faster growth  
494 rate, smaller spine size and selection for the allele associated with the low plate  
495 phenotype (L-allele), but could not demonstrate selection on the lateral plate  
496 phenotype itself. There are several differences between the two studies. Marchinko  
497 (2009) used a mix of dragonfly larvae (38% ) and *Notonecta* spp. (62%) as predators  
498 for his experiments, while we used 100% dragonfly larvae. Marchinko (personal  
499 communication) also used smaller dragonfly larvae (30 – 40 mm) than the ones that  
500 we used (36 – 51 mm), which belong to the most common species in our study site.  
501 This difference in predator composition and size should be accounted for when  
502 comparing the two studies. Marchinko (2009) also found a higher survival probability  
503 for larger fish. In contrast, we found no selection on standard length either in the  
504 population based experiments or in the individual based experiments, and did not  
505 observe a significant difference in the SL for surviving fish between control and  
506 treatment, although our results do suggest that larger body depth might provide a  
507 selective advantage to fish in the face of predation by dragonfly larvae. Our study  
508 does not rule out that predation by invertebrate predators other than large *Aeshna*  
509 spp. larvae could be a selective agent for faster growth or increased armour. That in  
510 our experiments the dragonfly larvae hunted randomly among all size classes of  
511 stickleback is in contrast with previous findings, where *Aeshna* larvae captured and  
512 consumed mostly juvenile stickleback up to a length of 25mm (Foster et al. 1988).  
513 Taken together, our results give some indications that the reduction in armour traits  
514 as a defense against dragonfly larvae predation may not be a universal pattern in  
515 stickleback.

516

517 There is some evidence for a trade-off between growth rate and armour traits  
518 (Marchinko and Schluter 2007) as well as with the *Eda* gene, with the low allele  
519 conferring a growth advantage (Barrett et al. 2008). Since overwinter survival - a key  
520 component of fitness - is correlated with a high growth rate during summer (Curry et  
521 al. 2005), lateral plates are likely to lose their adaptive value in freshwater systems  
522 where gape-limited predators are less dominant. Therefore selection on growth could  
523 outweigh the role of being protected against gape-limited predators in freshwater.  
524 Consequently, armour might experience indirect selection resulting from direct  
525 selection on growth rate (Lahti et al. 2009). Further, Bergstrom (2002) found that an  
526 increased number of lateral plates is associated with reduced velocity and  
527 displacement during the fast-start escape response. This suggests that reduced plate  
528 cover could be advantageous in some predation regimes, such as those dominated  
529 by avian predators and ambush hunting fish. The match between phenotype and  
530 swimming performance also differs between lake and stream habitats, suggesting  
531 that this coupling has a genetic basis and might be involved in adaptive divergence  
532 (Hendry et al. 2010).

533

534 Our experiments were conducted with fish from a population that is naturally highly  
535 polymorphic for armour related phenotypes. The fact that we used predators and  
536 prey from wild populations from the same location provides biological realism to this  
537 study. The fish from this population might have acquired behavioral or physiological  
538 traits, relevant to predator avoidance or escape as well as potentially coevolved  
539 predators, with adapted hunting strategies. Moreover, the polymorphic wild  
540 population allows us to rule out effects of negative epistatic effects on fitness, which  
541 may be common in crosses of divergent populations with different genetic  
542 background (Ungerer et al. 2003). The parallel instances of reduction in armour traits

543 in the threespine stickleback during the colonization of freshwater systems are likely  
544 a result of the interaction of multiple selective forces, and not just of predation. We  
545 must note that the predator-prey relationship that we examined represents only a  
546 single link in a complex network of ecological interactions. Still, our data suggest that  
547 in the face of predation from large predatory insect larvae, a reduction in body  
548 armour may offer little advantage.

549

## 550 **Acknowledgments**

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552 Canton of Bern and complied with all legal requirements in Switzerland (permit no.  
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555 manuscript for their comments.

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680

681

682

683

684 **Tables**685 **Table 1**

686 Statistical summary of the linear mixed models. For each trait the control was tested  
 687 against the treatment with ponds nested in runs as random factor to account for  
 688 differences. Results for FSL, SSL, PSL and BD are based on size-corrected values.  
 689 Statistically significant *P* values are given in bold.

Trait	$F_{1,737}$	<i>P</i>
SL	0.149	0.699
FSL	0.648	0.421
SSL	0.037	0.848
PSL	1.086	0.298
BD	5.370	<b>0.021</b>
Plates	0.031	0.860
PC1 (explains 52.4% of total variance; trait loadings: FSL, -0.495; SSL, -0.540; PSL, -0.629; BD, -0.259)	1.706	0.192
PC2 (explains 24.0% of total variance; trait loadings: FSL, -0.184; SSL, -0.214; PSL, -0.066; BD, 0.957)	4.088	<b>0.044</b>

690

691

692 **Table 2**

693 Sample sizes and *P* values from comparisons (Fisher’s exact test) of the *Stn382* C-  
 694 allele frequencies for each replicate ( $N_{Control}$ : number of fish in the control group;  
 695  $N_{Treatment}$ : number of fish in the treatment group that were alive at the end of the  
 696 experiment;  $N_{Dead}$ : number of fish in the treatment group that died during the  
 697 experiment). For each replicate, two comparisons were made: between survivors of  
 698 both control and treatment, and between the survivors from the treatment and the  
 699 dead fish from the treatment. Values in bold indicate significantly different C-allele  
 700 frequencies in the predicted direction (i.e. control > treatment and dead > survivors)  
 701 while italics indicate comparisons that were significant in the opposite direction. *P*  
 702 values larger than 0.05 are denoted “ns”.

Replicate	$N_{Control}$	$N_{Treatment}$	$N_{Dead}$	Control vs treatment	Treatment survivors vs dead
1	27	19	10	0.956	0.505
2	29	25	5	<b>0.039</b>	0.561
3	30	17	12	<b>0.023</b>	<i>≤0.001</i>
4	30	19	11	0.500	0.191
5	27	22	8	0.500	<b>0.050</b>
6	28	19	11	<b>0.016</b>	0.135
7	29	17	13	0.832	<i>0.002</i>
8	27	16	14	0.980	0.101
9	27	23	5	0.884	0.128
10	26	26	4	0.101	0.160
11	28	14	15	0.443	0.669
12	28	20	10	0.965	0.195
13	26	22	6	0.972	<b>0.005</b>
14	24	13	17	0.943	<b>0.029</b>
15	27	24	6	0.722	<i>0.004</i>

16	26	19	11	0.043	<b>0.050</b>
All	439	315	158	0.500	0.331

703

704



705 **Table 3**

706 Selection differentials (*i*) for standard length (SL), first dorsal spine length (FSL),  
 707 second dorsal spine length (SSL), pelvic spine length (PSL), body depth (BD), and  
 708 lateral plate counts (plates) for each of the 16 replicates. Statistically significant  
 709 values of *i* ( $P < 0.05$ ; *P* values based on 1000 resampling steps) are in bold. Results  
 710 for FSL, SSL, PSL and BD are based on size corrected values.

Replicate	SL	FSL	SSL	PSL	BD	Plates
1	0.11	-0.11	-1.01	-0.37	0.55	0.22
2	0.13	<b>0.54</b>	<b>0.97</b>	0.10	0.41	-0.47
3	0.28	0.19	-0.04	0.34	<b>0.68</b>	-0.39
4	0.29	-0.41	-0.25	-0.55	-0.51	0.04
5	0.42	-0.32	-0.34	-0.15	-0.01	0.06
6	0.03	-0.06	-0.34	0.37	<b>0.43</b>	-0.43
7	0.02	<b>0.92</b>	<b>0.65</b>	<b>0.62</b>	0.11	0.05
8	-0.36	0.02	-0.13	0.03	0.09	0.13
9	0.14	-0.21	0.28	-0.21	-0.24	0.10
10	-0.34	-0.66	-0.45	-0.11	-0.29	-0.40
11	0.29	0.50	0.47	0.42	<b>0.57</b>	0.12
12	0.30	-0.15	-0.42	-0.56	-0.33	0.26
13	-0.29	-0.05	-0.03	-0.02	0.46	0.01
14	-0.27	-0.21	0.02	0.33	<b>1.12</b>	0.31
15	-0.02	0.29	0.28	0.22	0.22	0.06
16	-0.18	<b>0.60</b>	0.10	<b>0.79</b>	-0.06	0.19

711

712

713 **Figure legends**

714 **Figure 1**

715 *Average trait values per replicate for both control and treatment for (a) the number of*  
716 *lateral plates, (b) standard length (in mm); residuals of (c) first dorsal spine length, (d)*  
717 *second dorsal spine length, (e) pelvic spine length, (f) body depth on standard length.*  
718 *In addition the average residuals per replicate of a principal component (PC) analysis*  
719 *using all size corrected linear morphological traits for the first (g) and the second (h)*  
720 *axis.*

721

722 **Figure 2**

723 *Allele frequencies per replicate for the Stn382 C-allele in control and treatment fish*  
724 *that were alive at the end of the experiment (“survivors”) and treatment fish that died*  
725 *during the experiment (“dead”).*

726

727 **Figure 3**

728 *Frequency distributions of lateral plate counts plotted for all three Stn382 genotypes*  
729 *in (a) controls and (b) treatments. Open bars: homozygote for the low plated (L-)*  
730 *allele, black bars: homozygote for the complete (C-) allele, grey bars: heterozygote.*

731

732 **Figure 4**

733 *Sizes of (a) fish and (b) dragonfly larvae, and (c) difference in size between predator*  
734 *and prey grouped by the outcome of individual-based experiments: 1, no attempted*

735 *predation by the predator; 2, unsuccessful attempt by the predator and 3, successful*  
736 *attempt by the predator.*

737 **Supplementary figure S1**

738 *Frequency distributions for (a) lateral plate count, (b) SL and residuals of (c) FSL, (d)*  
739 *SSL, (e) PSL and (f) BD in control and treatment for each replicate. Blue = control,*  
740 *red = treatment.*

741 **Supplementary figure S2**

742 *Survival of Stn382 (a) alleles and (b) genotypes in treatments among all 16*  
743 *replicates.*

744

745 **Supplementary figure S3**

746 *Age distributions for control and treatment, based on 180 fish from controls and 178*  
747 *fish from treatments. White, one-year old; grey, two-year old; black, three-year old.*

748

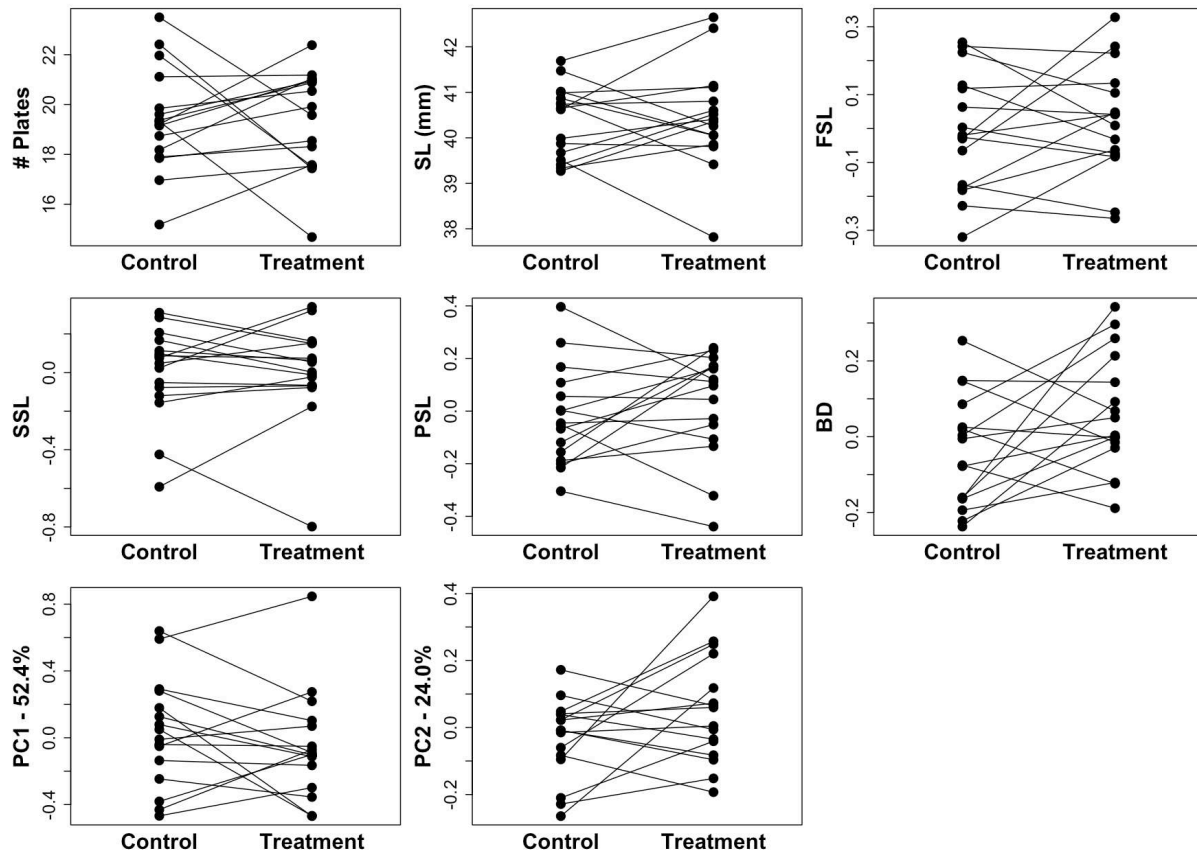
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752 **Figures**

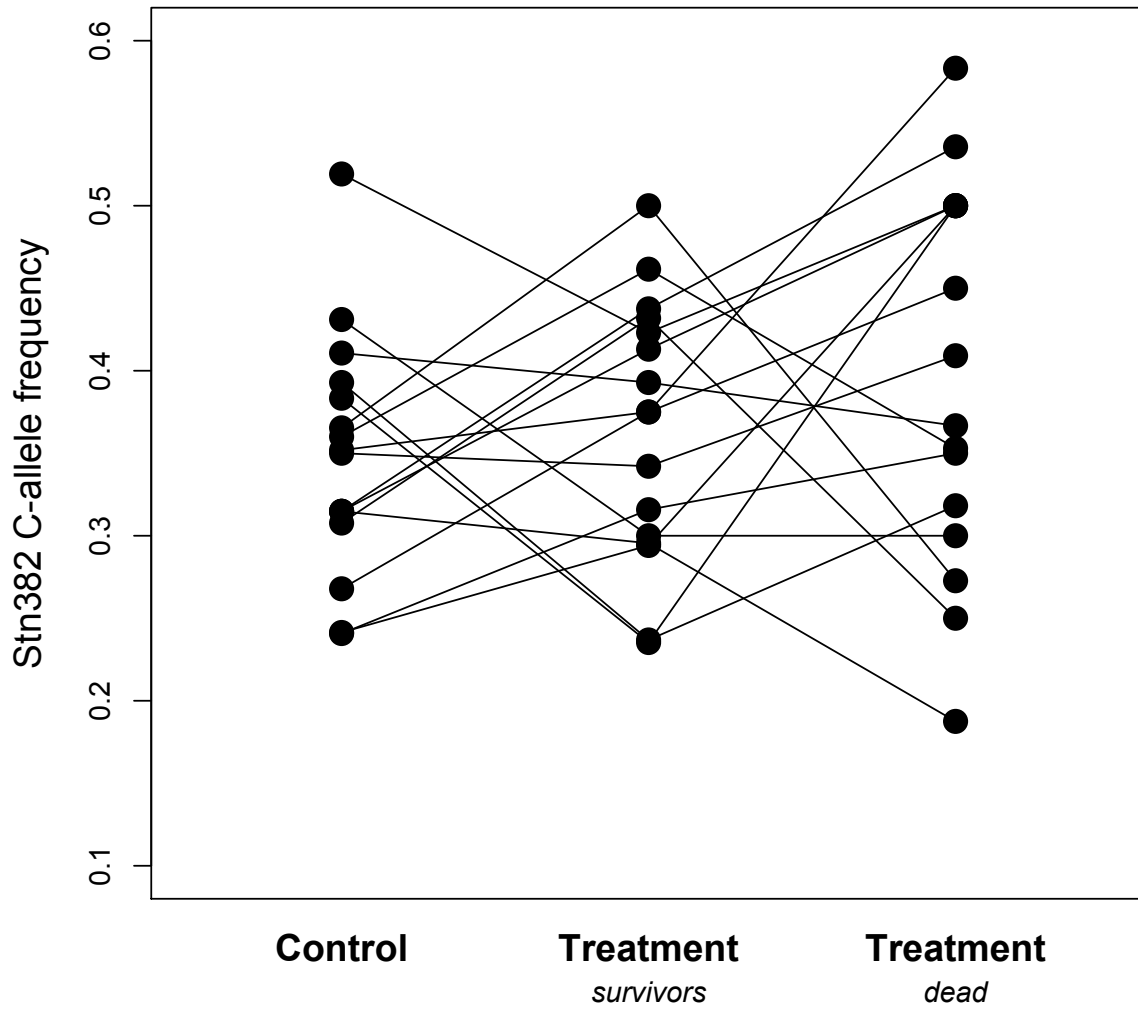
753 **Figure 1**



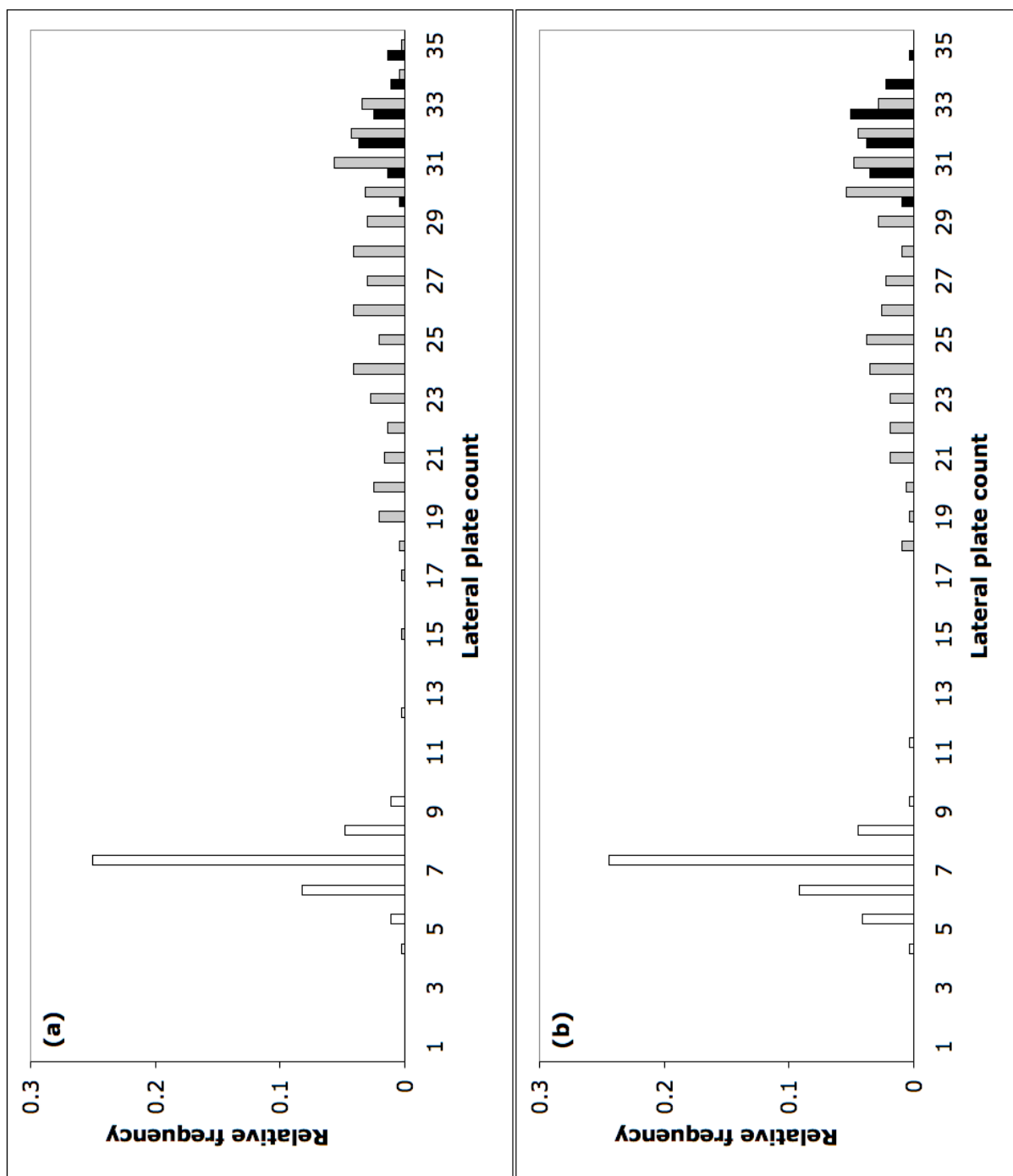
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755 **Figure 2**

756



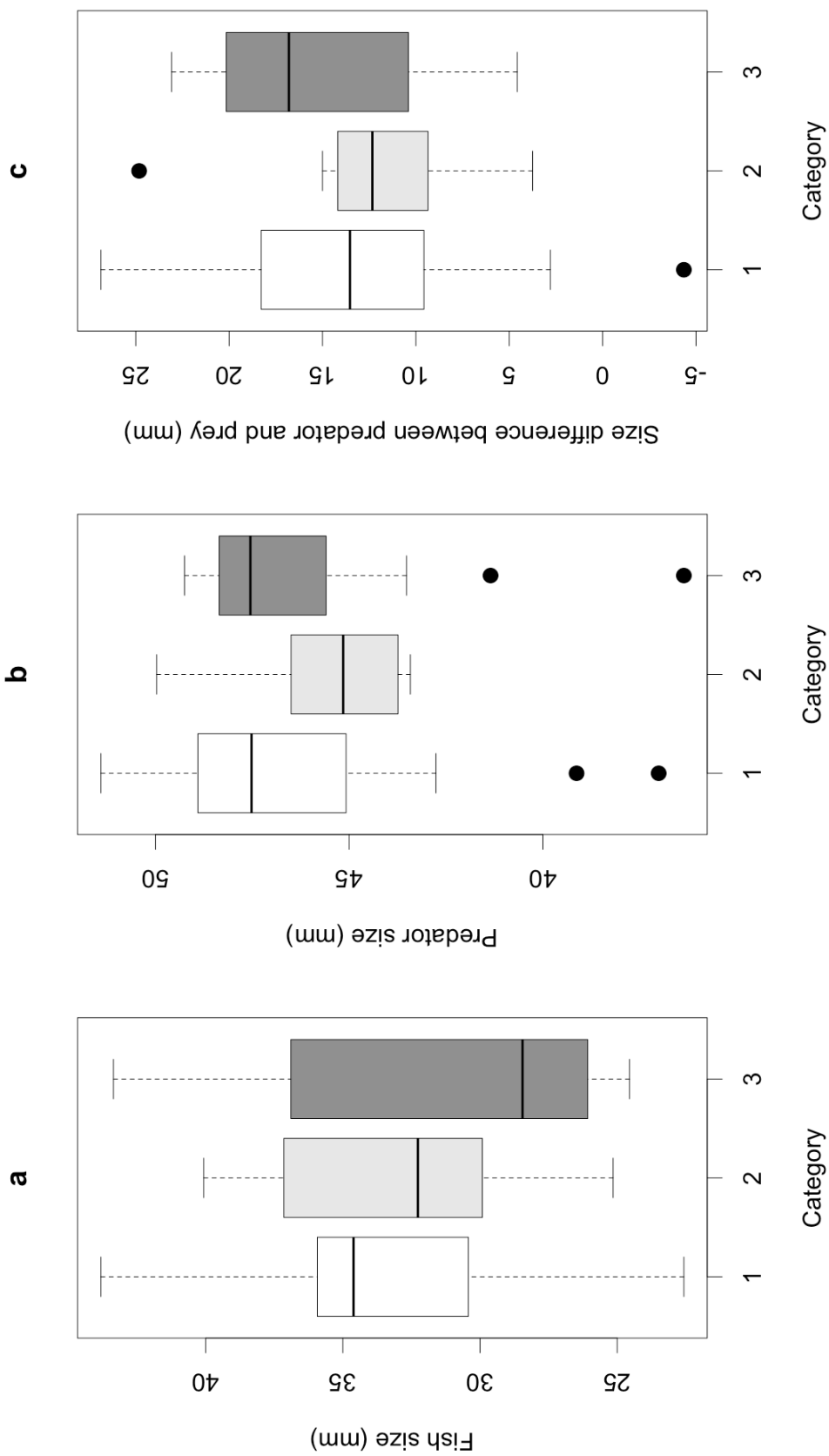
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761 **Figure 4**



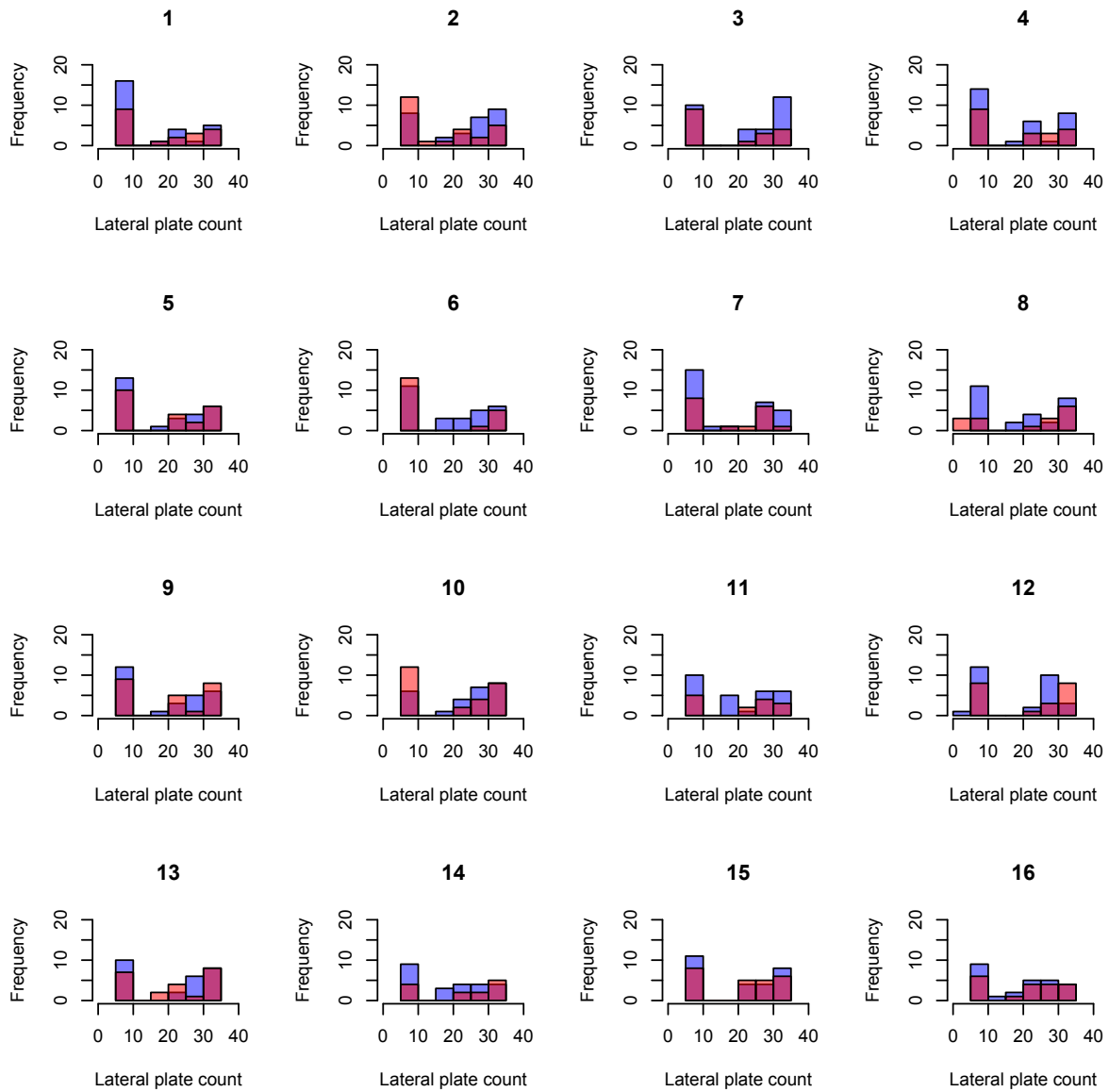
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763

764 **Supplementary figures**

765 **Figure S1**

766 **(a)**

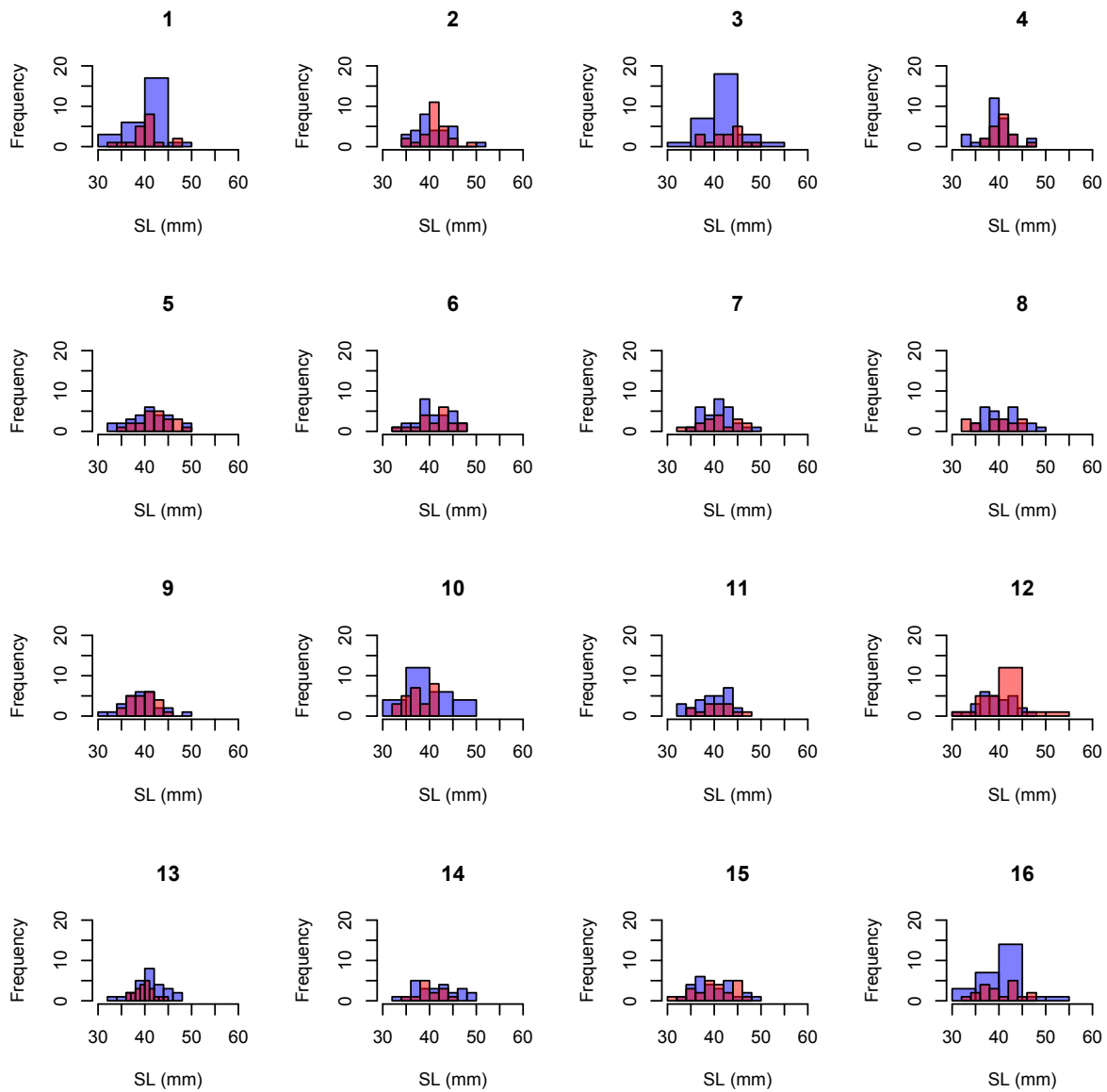


767

768



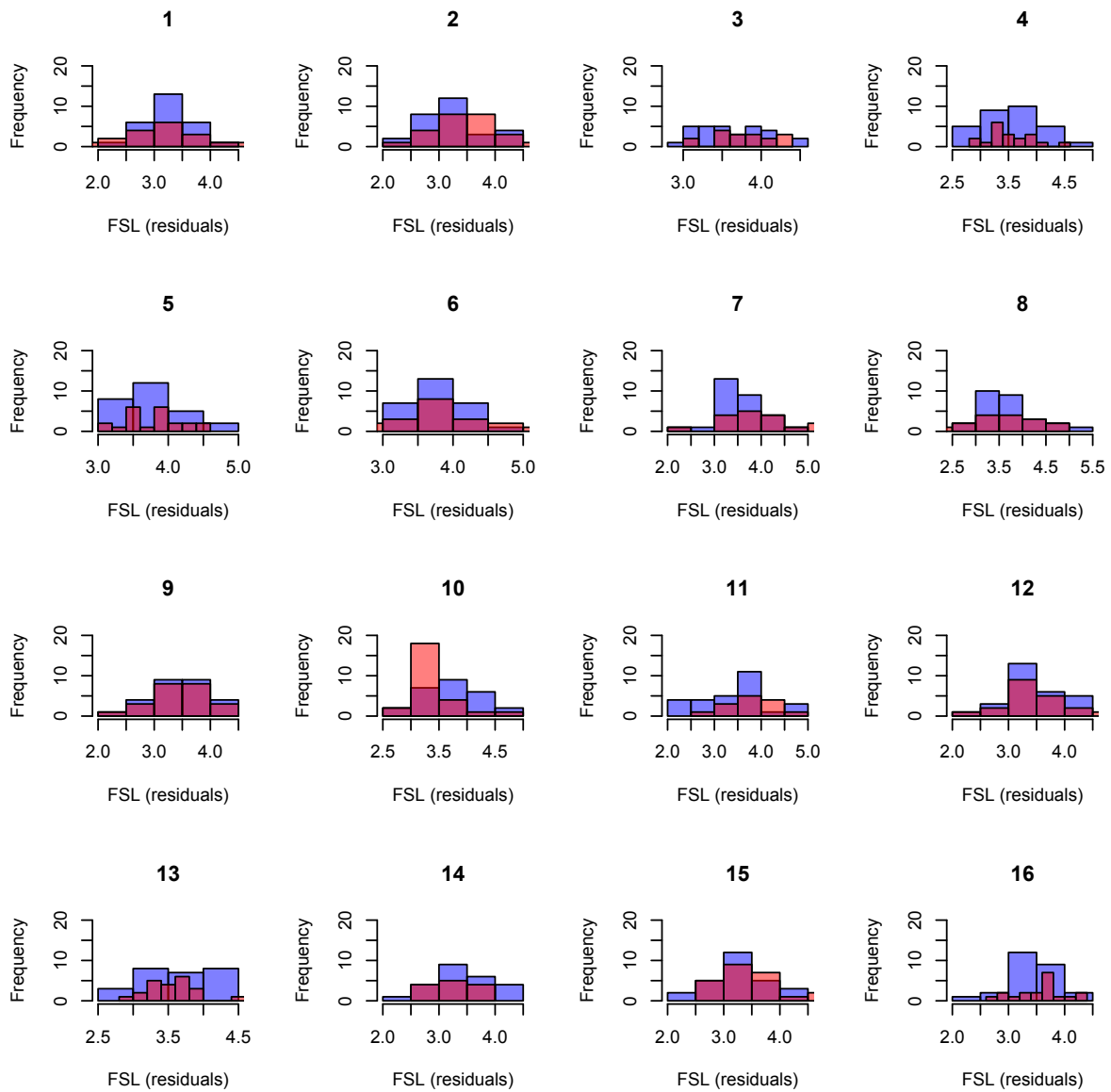
769 (b)



770

771

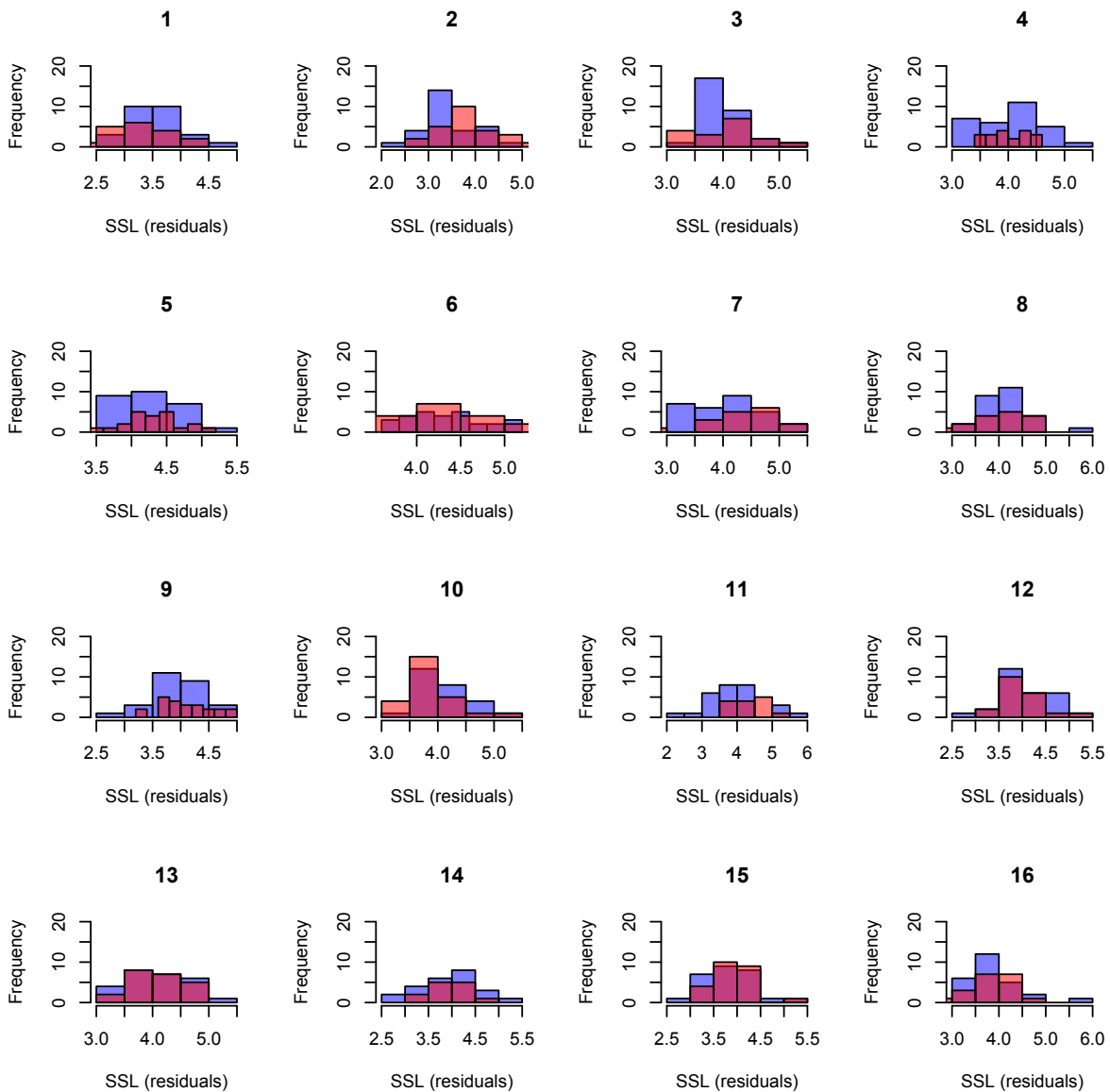
772 (c)



773

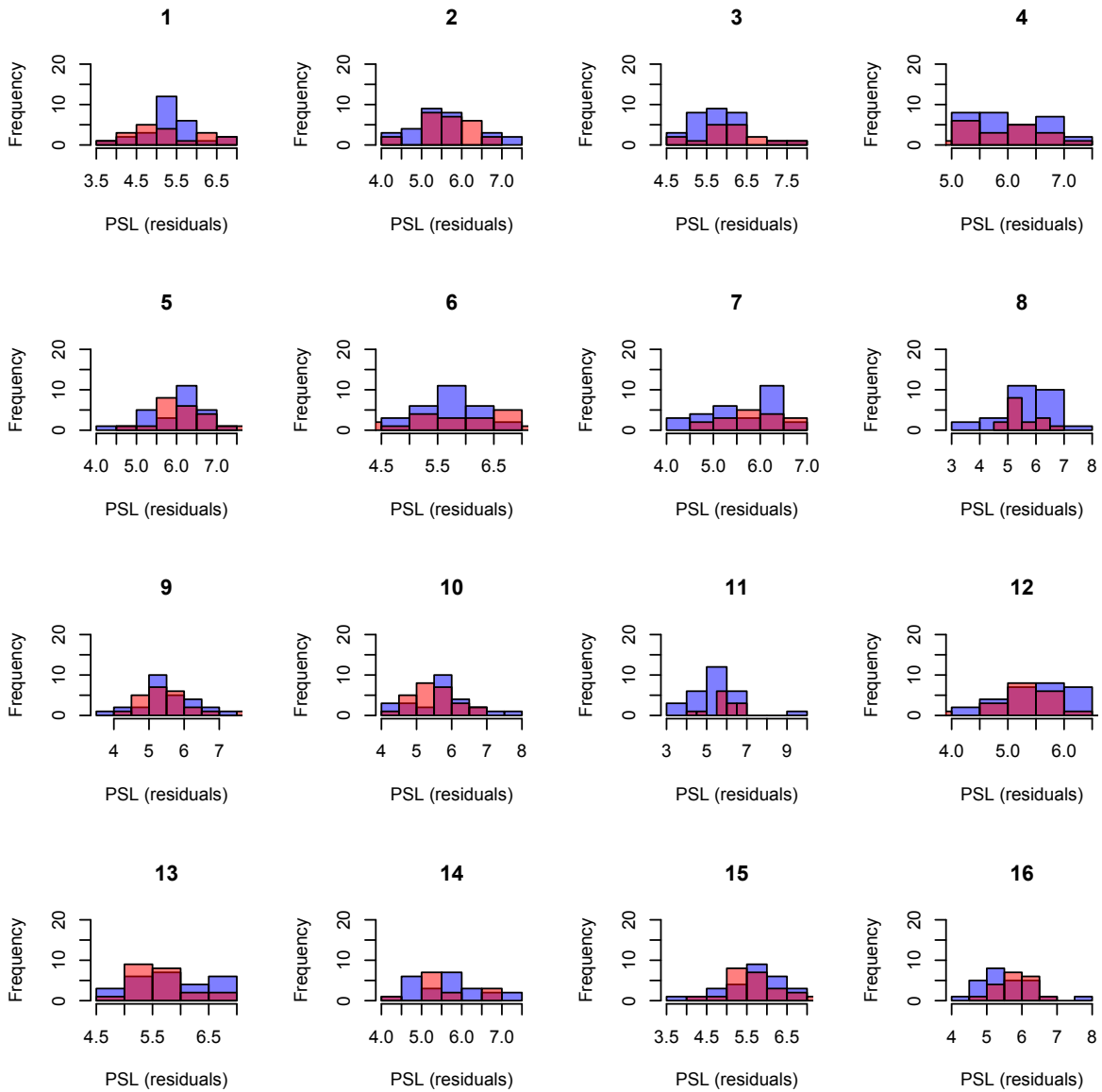
774

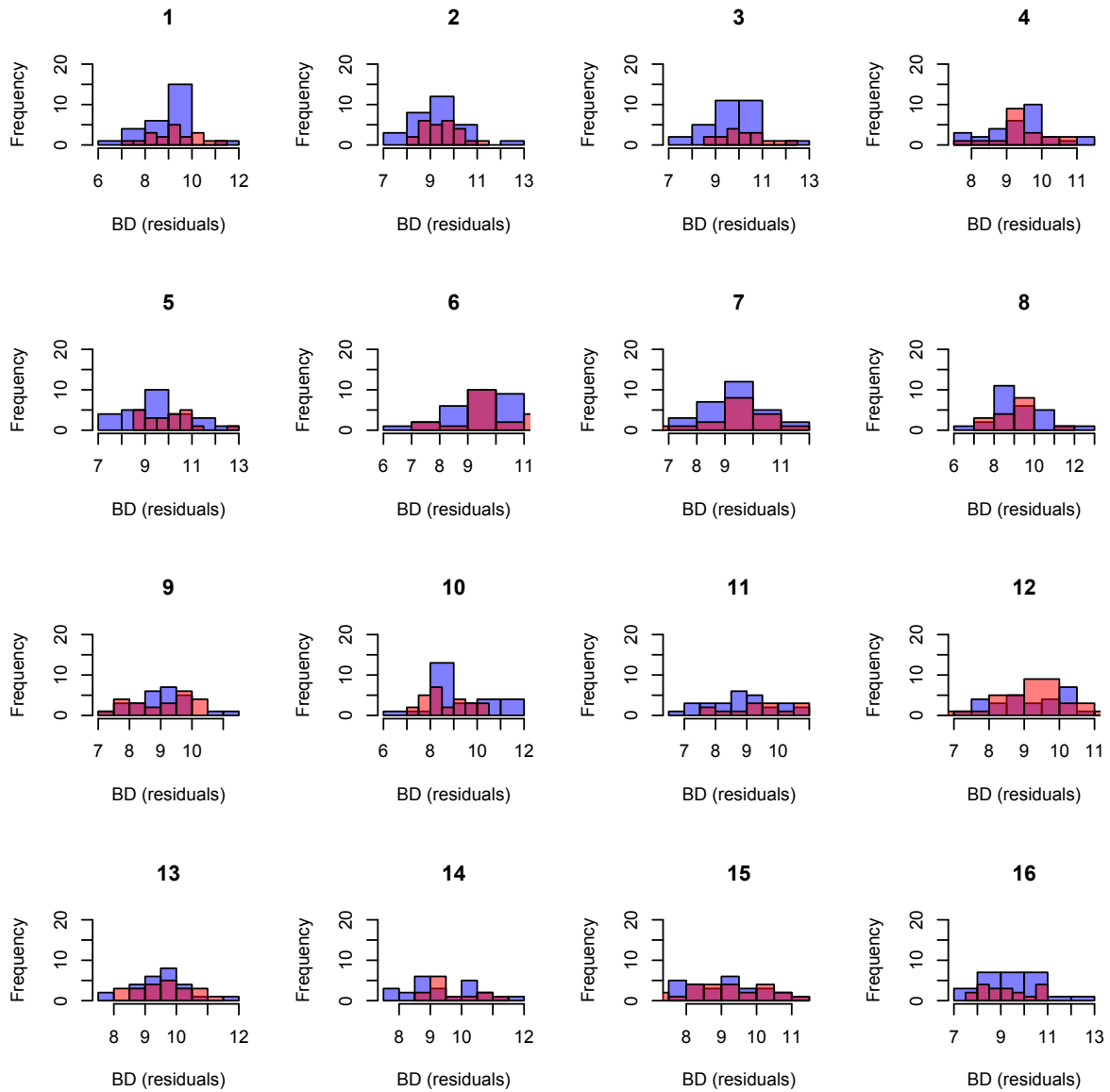
775 (d)



776

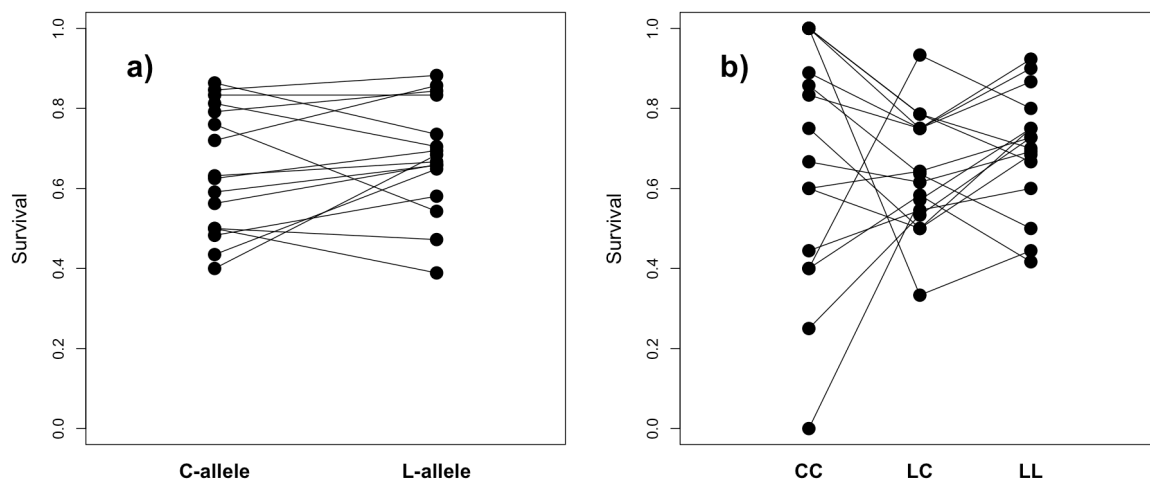
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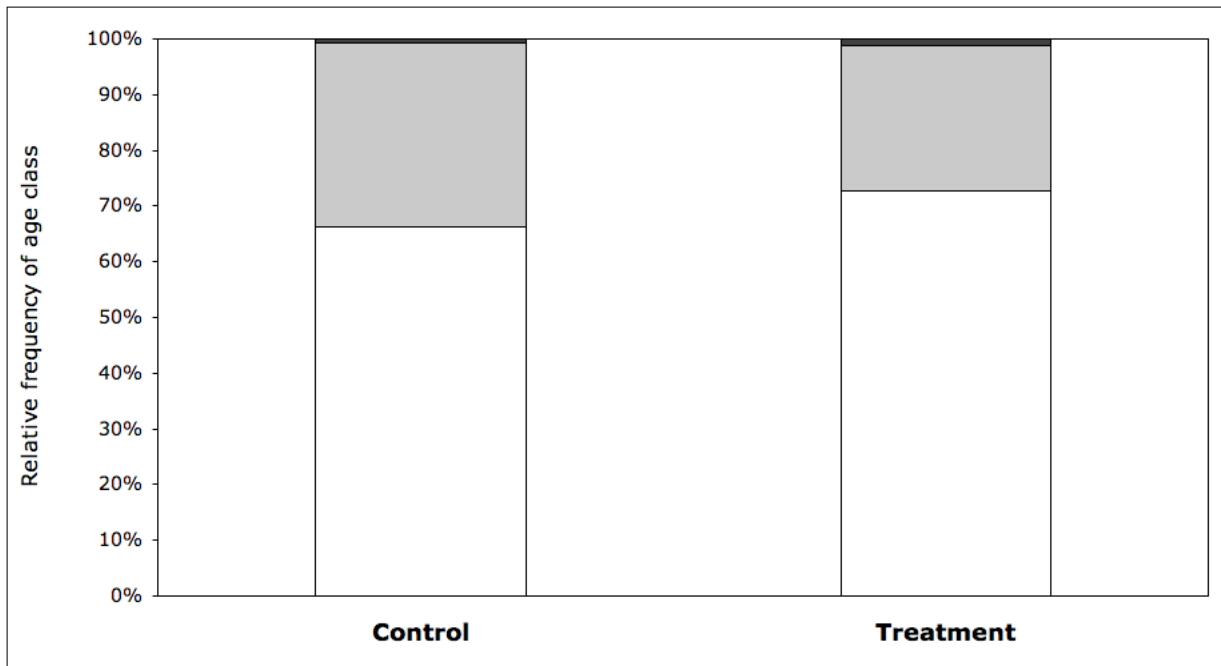
783 **Figure S2**

784



785

786 **Figure S2**



787