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1 **SIGNIFICANCE STATEMENT**

2 Pigmentation variation in the polytypic brown trout (*Salmo trutta*) has an unknown
3 genomic basis. Using redundancy analyses and genome-wide association studies, authors
4 showed that pigmentation has a large polygenic basis in this species, based on hundreds of
5 loci known to be associated to colour. Redundancy analysis also showed that backcrossed
6 wild trout converged to a locally adaptive Mediterranean pigmentation pattern despite the
7 release of Atlantic hatchery fish.

8

9 **Redundancy analysis, genome-wide association studies,**
10 **and the pigmentation of brown trout (*Salmo trutta* L.)**

11
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29
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31

32 ABSTRACT

33 The association of molecular variants to phenotypic variation is a main issue in biology,
34 often tackled with genome-wide association studies (GWAS). GWAS are challenging, with
35 increasing, but still limited use in evolutionary biology. We used redundancy analysis (RDA)
36 as a complimentary ordination approach to single- and multi-trait GWAS to explore the
37 molecular basis of pigmentation variation in brown trout (*Salmo trutta*) belonging to wild
38 populations impacted by hatchery fish. Based on 75,684 single nucleotide polymorphic
39 (SNP) markers, RDA, single- and multi-trait GWAS allowed to extract 337 independent
40 “colour patterning loci” (CPLs) associated with trout pigmentation traits, such as the
41 number of red and black spots on flanks. Collectively, these CPLs (i) mapped onto 35 out of
42 40 brown trout linkage groups indicating a polygenic genomic architecture of pigmentation,
43 (ii) were found associated to 218 candidate genes, including 197 genes formerly mentioned
44 in the literature associated to skin pigmentation, skin patterning, differentiation or
45 structure notably in a close relative, the rainbow trout (*Onchorhynchus mykiss*), and (iii)
46 related to functions relevant to pigmentation variation (e.g., calcium- and ion-binding, cell
47 adhesion). Annotated CPLs include genes with well-known pigmentation effects (e.g.,
48 *PMEL*, *SLC45A2*, *SOX10*), but also markers associated to genes formerly found expressed in
49 rainbow or brown trout skins. RDA was also shown to be useful to investigate management
50 issues, especially the dynamics of trout pigmentation submitted to several generations of
51 hatchery introgression.

52

53 *Keywords:* ancestry – admixture – genetic drift – linkage disequilibrium – melanocytes – multi-trait
54 phenotype – salmonid

55 1— INTRODUCTION

56 Testing for genotype-phenotype association is common in many biological disciplines,
57 including the study of genotype-phenotype relationships in biomedical, agronomical, or
58 evolutionary studies. Using data generated from genome-wide single nucleotide
59 polymorphism (SNP) surveys, genotype-phenotype associations are often viewed through
60 the lens of genome-wide association studies (GWAS). Despite their success (e.g., Visscher
61 *et al.*, 2017; Tam *et al.*, 2019), the use of GWAS remain challenging. The poor ability of
62 GWAS to detect variants except those with large effects, to tackle complex trait
63 architectures, and their propensity to be affected by spurious associations have been
64 commonly reported (e.g., Rockman, 2012; Wellenreuther and Hansson, 2016; Boyle *et al.*,
65 2017; Tam *et al.*, 2019). These issues, together with robustness over sample sizes, and poor
66 consideration of population stratification still limit the application of GWAS in wild
67 populations ('wild GWAS'; Santure and Garant, 2018). GWAS remain underrepresented in
68 evolutionary studies so far (Ahrens *et al.*, 2018). However, increased access to genome-
69 wide distributed markers through next-generation sequencing technologies allowed for an
70 extended application of 'wild GWAS' to detect SNPs involved in adaptive trait variation
71 (e.g., Johnston *et al.*, 2011; Hansson *et al.*, 2018; Duntsch *et al.*, 2020; Lundregan *et al.*,
72 2020; Blanco-Pastor *et al.*, 2021). This includes the Atlantic salmon (*Salmo salar*) with focus
73 on age at maturity as the trait of interest (e.g., Barson *et al.*, 2015; Sinclair-Waters *et al.*,
74 2020; but see Hecht *et al.*, 2013). Without searching to minimize findings reported in these
75 studies, the use of GWAS yielded mixed results as no or very few candidate SNPs or genes
76 linked to the trait(s) of interest were detected. For example, because of a low sample size
77 which is the rule in most studies of wild organisms, Hansson *et al.* (2018) reported no

78 association using GWAS for wing length variation in warblers, while adaptive markers were
79 previously identified.

80 It seems legitimate to question if other association methods could perform
81 reasonably well to (i) detect relevant SNPs or genomic regions associated to phenotypic (or
82 life-history) traits, (ii) identify candidate genes with functional significance for the trait(s)
83 of interest (i.e., known to be expressed in a tissue relevant for that trait), and (iii) inform
84 onto eco-evolutionary processes that operate in wild populations. In a former review on
85 multivariate data analysis using molecular markers, Jombart *et al.* (2009) reported that
86 RDA— a constrained ordination method that has been used for decades in numerical
87 ecology (Legendre and Legendre, 2012; Capblancq and Forester, 2021)— was neglected in
88 population genetics studies so far. The gap was filled over years, and RDA became a
89 standard to identify adaptive markers in genotype-*environment* association (GEA) studies
90 (Capblancq and Forester, 2021). This includes fish GEA (e.g., Hecht *et al.*, 2015; Brauer *et*
91 *al.*, 2016; Babin *et al.*, 2017; Dalongeville *et al.*, 2018; Bekkevold *et al.*, 2019). However,
92 RDA appears largely – if not totally— restricted to GEAs, while GWAS are devoted to
93 genotype-*phenotype* associations. This observation has no methodological support as
94 environmental and phenotypic data are interchangeable in RDA. Moreover, RDA could
95 potentially better manage some of the drawbacks associated to GWAS. Indeed,
96 associations detected by RDA were shown poorly dependent on false positive SNPs
97 (Capblancq *et al.*, 2018; Forester *et al.*, 2018), and robust over sample sizes tested (Forester
98 *et al.*, 2018). Performances of RDA to investigate an adaptive genotype-phenotype
99 association have yet to be tested, at least empirically.

100 In this study, we performed RDA and GWAS on a genomic dataset (>75K SNPs)
101 produced by Leitwein *et al.* (2018), coupled with a high quality phenotypic characterisation
102 of body pigmentation in 112 brown trout (*Salmo trutta*). As variation in body colour and
103 pigmentation patterns are of wide interest in biology and are often adaptive (Hubbard *et*
104 *al.*, 2010; Cuthill *et al.*, 2017; San José and Roulin, 2017; Orteu and Jiggins, 2020), the choice
105 of the brown trout appears an interesting case study to evaluate the performance and
106 complementarity of GWAS and RDA to detect relevant variants involved in adaptive
107 genotype-phenotype association. Indeed, *S. trutta* is a highly polytypic species for many
108 traits, including body colour and pigmentation, going from continuous colour (silver,
109 brownish, greenish) to complex spotty, marbled, blotchy and striped patterns (Colihueque,
110 2010). These patterns are traditionally included for subspecies and lineage description, but
111 also population characterisation and the detection of hybrids (e.g., Mezzera *et al.*, 1997;
112 Delling *et al.*, 2000; Aparicio *et al.*, 2005; Kocabaş *et al.*, 2018; Duchi, 2018; Lorenzoni *et al.*,
113 2019). Indeed, specific pigmentation patterns (e.g., spots) in trout have been shown to be
114 under simple genetic control in controlled-crosses experiments (Blanc, Poisson and Vibert,
115 1982; Blanc, Chevassus and Krieg, 1994; Skaala and Jørstad, 1988; Colihueque, 2010).
116 However, the genomic architecture of pigmentation variation (i.e., the number and
117 distribution of loci over the genome), and the genes responsible for adaptive pigmentation
118 variation are largely unknown in trout with still limited insights coming from gene
119 expression studies (Sivka *et al.*, 2013; Djurdjevič *et al.*, 2019). We thus expect that RDA
120 and/or GWAS could inform on the molecular basis of pigmentation pattern variation
121 observed among Atlantic and Mediterranean hatchery trout, but also wild-caught
122 Mediterranean trout that present different levels of admixture with hatchery individuals
123 (Leitwein *et al.*, 2018).

124 The main aim of this study is thus to document the ability of RDA and GWAS to
125 identify relevant candidate loci involved in pigmentation variation in the brown trout,
126 including wild trout from Mediterranean populations and hatchery individuals issued from
127 the Mediterranean and Atlantic lineages. We also use the information extracted by RDA to
128 illustrate shift in pigmentation patterns that occurred over multiple generations of
129 hatchery introgression in these local populations.

130

131 **2 - MATERIALS AND METHODS**

132 **2.1 - BROWN TROUT'S GENOMICS DATA**

133 Double digested restriction site-associated DNA sequencing (ddRADseq) data used in this
134 study are from Leitwein *et al.* (2018) and available at NCBI Short Read Archive under the
135 study accession SRP136716. The data set consists in 75,684 genome-wide SNPs distributed
136 over 40,519 RAD-loci (i.e., a RAD-locus may contain several linked SNPs) for 112 trout of
137 hatchery and wild caught origins. As in Leitwein *et al.* (2018), loci were retained according
138 to the following filters (a) a minimum depth of 5 reads; (b) a genotype call rate of at least
139 80%, (c) a minimum allele frequency of 2%, and (d) a maximum observed heterozygosity of
140 80%. Over the 112 trout, eighty-two wild caught individuals were fished in the headwaters
141 of three rivers within the Mediterranean Orb River catchment (France) (Gravezon, Mare,
142 and Orb rivers; Leitwein *et al.*, 2016, 2018 for details). Rivers are located less than 30km
143 apart from each other (air-line distance). This small Mediterranean catchment has been
144 seeded by alien hatchery fish of Atlantic origin for decades, and seeded by a locally derived
145 strain of Mediterranean origin over the last 15 years (Leitwein *et al.*, 2016, 2018). Atlantic
146 and Mediterranean trout refer to two distinct evolutionary lineages within the brown trout

147 species complex (e.g., Sanz, 2018). They differ from each other for body colour and
148 patterning (e.g., Lascaux, 1996; Aparicio *et al.*, 2005). Leitwein *et al.* (2018) showed that
149 these wild caught individuals consisted in released hatchery individuals of both
150 Mediterranean and Atlantic origins, F1's, F2's, 'early' and 'late' backcrossed individuals, and
151 'pure' natural Mediterranean fish (Leitwein *et al.*, 2018). 'Early' and 'late' backcrossed
152 individuals correspond to Mediterranean wild-caught fish with distinct distributions of
153 Atlantic ancestry tracts in their genome. Thirty hatchery individuals have been also
154 considered. Hatchery fish included in this study consisted in $N = 15$ hatchery trout of
155 Atlantic origin, and $N = 15$ hatchery trout of Mediterranean origin. Atlantic hatchery fish
156 originated from the Cauterets hatchery that maintain a brown trout strain distributed
157 worldwide (Bohling *et al.*, 2016). Mediterranean hatchery fish have been randomly
158 sampled in a local strain formerly established using mature trout from the Gravezon River
159 by a local fishery authority (Fédération Départementale de Pêche de l'Hérault) in 2004, and
160 now used to seed Mediterranean local rivers within the Orb catchment to minimize the
161 release of foreign Atlantic trout. Private hatcheries are also located in the Orb catchment,
162 notably on the Gravezon River, and escapees may still impact natural populations (Leitwein
163 *et al.*, 2018). Fishing was carried out by this authority under the agreement DDTM34-2021-
164 03-11786.

165

166 **2.2 - PHENOTYPIC DATA**

167 Acquisition of pigmentation data followed Lascaux (1996) and is used by numerous
168 management authorities in France. Similar analysis and quantification of pigmentation data
169 are performed in other fish species (e.g., cichlids; Albertson *et al.*, 2014). The set of

170 variables considered in this protocol is listed and illustrated on pictures in Suppl. Mat. Fig.
171 S1. Data were recorded from individual photographs of the 112 fish considered for genomic
172 analyses. Photographs were taken at fishing on slightly anesthetized trout with eugenol. A
173 camera Canon® EOS 1000D was used. After recovery, fish were released in the wild or in
174 hatchery tanks, respectively. Scales were not sampled and the age of wild-caught fish was
175 not estimated. Sizes of wild caught fish ranged from 10.1 cm to 33.7cm, indicating juveniles,
176 sub-adults and adult fish. All hatchery fish have size > 24.0 cm. Quantitative ($N = 19$) and
177 semi-quantitative variables ($N = 11$) were recorded by visual examination of photographs
178 of the left flank of each individual fish as in Westley, Conway and Fleming (2012).
179 Quantitative variables refer to punctuation patterns of the brown trout (e.g., number and
180 diameter of spots) and are recognized important for this species (Blanc *et al.*, 1982, 1994).
181 Semi-quantitative variables refer to 'ornamental' appearance patterns (e.g., parr marks,
182 fringes on fins, white ring around spots). Each quantitative punctuation variable was
183 measured independently. Ornamental variables have been coded by modalities, either
184 present/absent [1,2] or, e.g., absent/partial/complete [1,2,3] (Table 1 and Suppl. Mat.
185 Table S1). This set of variables avoided focusing only on peculiar attributes of the
186 pigmentation patterns. Other traits participating to body colour that can be also important
187 for trout (e.g., background colour, brightness) were not considered in this study because of
188 non-standard light conditions during field data acquisition.

189 In addition to pigmentation data, size (fork length) of individuals was acquired in
190 the field (age is unknown and not considered in this study). Furthermore, as sex was shown
191 to influence (Yaripour *et al.*, 2020) or not (Kocabaş *et al.*, 2011) body pigmentation in trout,
192 individuals have been sexed using a microsatellite marker targeting a salmonid sex-
193 determining locus (Yano *et al.*, 2013). Correlations among the full set of phenotypic

194 variables—pigmentation, sex, and size— were checked for minimizing collinearity in this data
195 set. A conservative $r > 0.7$ rule-of-thumb was considered to discard explanatory variables
196 (Dormann *et al.*, 2013). Pictures and phenotypic data have been deposited in Dryad (doi:
197 [completed under acceptance](#)).

198

199 **2.3 - GENOTYPE-PHENOTYPE ASSOCIATION**

200 The evaluation of the genotype-phenotype association between SNPs and pigmentation
201 patterns was performed using three distinct approaches: RDA, single- and multi-trait
202 GWAS. Hereafter, a ‘colour patterning locus’ (CPL) was coined as any candidate RAD-locus
203 found significantly associated with at least one of the uncorrelated variables considered in
204 this study.

205 *2.3.1 – Redundancy analysis* – We used RDA to investigate any association between
206 genomic and pigmentation trait data. RDA was performed with the uncorrelated
207 pigmentation variables as the explanatory and the SNPs as the response variables. Missing
208 genomic data (2.63%) were imputed by the most commonly observed genotype. Missing
209 phenotypic data (3.54%) were imputed by the mean of observed trait for quantitative data,
210 and the most commonly observed phenotype for semi-quantitative data. We classified
211 SNPs as showing statistically significant association with individual
212 pigmentation/ornamental traits when they loaded with more than 2.5 standard deviations
213 (S.D.) from the mean.

214 A forward model selection was used to select for the relevant uncorrelated
215 phenotypic variables in the RDA (Blanchet *et al.*, 2008). Indeed, increasing the number of
216 explanatory variables makes RDA similar to unconstrained ordination method (i.e.,

217 principal component analysis [PCA]) as the percentage of variation explained increases
218 when considering more explanatory variables. Models were defined, first including the
219 eleven uncorrelated phenotypic response variables, then reducing this number. The Aikake
220 Information Criterion was computed in each case to select the model minimizing deviance.
221 Permutation tests ($N = 999$) were performed by permuting individuals in each model. This
222 procedure was first established for the RDA, then for each successive RDA axis to
223 investigate if observed patterns carried significant association between relevant traits and
224 SNPs. Analyses were performed with the *vegan* package ([https://cran.r-](https://cran.r-project.org/web/packages/vegan/index.html)
225 [project.org/web/packages/vegan/index.html](https://cran.r-project.org/web/packages/vegan/index.html)), following rules provided by B. Forester in a
226 vignette on population genetics with R (<https://cran.r-project.org/>) and available at:
227 https://popgen.nescent.org/2018-03-27_RDA_GEA.html. Once relevant variables were
228 identified in models, each significant SNP was associated to the specific trait it was the most
229 significantly correlated with. As the ± 2.5 S.D. threshold is arbitrary (e.g., Babin *et al.*, 2017),
230 we looked at seventeen specific genes known to be involved in pigmentation in vertebrates
231 in order to evaluate if important candidate genes might have been dismissed by RDA. They
232 include classical players of pigmentation regularly studied in fish, mice and/or human
233 (***Mc1r***, ***Tyr***, ***Dct***, ***Oca2***, ***Mitf1***, ***Kit***, ***Tyrp1***, ***Pomca1***, ***Pomca2***, ***Pomcb***, ***Asip***, ***Slc24a5***, ***Oa1***,
234 ***Ednrb***, ***Hdac1***, ***Vps18*** and ***Scg2a***; see, e.g., Raposo and Marks 2007; Protas and Patel 2008;
235 Kelsh *et al.*, 2009; Baxter *et al.*, 2019, Wang T. *et al.*, 2020). Genes given in bold were more
236 specifically studied for gene expression differences in brown trout skin (Sivka *et al.*, 2013;
237 Djurdjević *et al.*, 2019).

238 **2.3.2 - Genome-wide association studies** – GWAS are typically based on linear mixed models
239 to fit one SNP at a time to a single trait (Hackinger and Zeggini, 2017). These are simple
240 statistical models, but one SNP may correlate with several traits. These correlations can be

241 used in multi-trait linear mixed models for GWAS to reduce false positives and increase the
 242 statistical power for association mapping (O'Reilly *et al.*, 2012; Korte *et al.*, 2012). Both a
 243 single- and a multi-trait analysis were performed in this study.

244 A single-trait association (single-trait GWAS, hereafter) was first considered by
 245 fitting each phenotypic pigmentation variable with SNPs by linear regression. We used a
 246 penalized maximum-likelihood least absolute shrinkage and selection operator (LASSO)
 247 model to select for sets of SNPs implied in each trait association, then solving:

$$248 \quad \min_{(\beta_0, \beta) \in \mathbb{R}^{p+1}} \left[\frac{1}{2N} \sum_{i=1}^N (y_i - \beta_0 - x_i \beta)^2 + \lambda \sum_{j=1}^p |\beta_j| \right] \quad (1)$$

249 in which $Y \in \mathbb{R}$ represents the response phenotypic variable, $X \in \mathbb{R}^p$ a vector of predictor
 250 variables (i.e., SNPs), λ the penalty parameter, β_0 the y-intercept of multiple linear
 251 regression, and $\beta \in \mathbb{R}^p$ a vector of β_j coefficients (Friedman *et al.*, 2010). This vector of β_j
 252 coefficients represents the effect size β_j of the j^{th} SNP conditional on the effects of all other
 253 SNPs. The penalized term λ shrinks the regression coefficient towards zero, keeping only a
 254 small number of SNPs with large effects in the model. A cyclical coordinate descent
 255 procedure was retained for model selection (Friedman *et al.*, 2010). The retained model
 256 was determined by cross-validation. $\log(\lambda)$ was estimated by minimizing the mean
 257 quadratic error. The number of positive β_j coefficients was estimated from $\log(\lambda)$, with each
 258 β_j coefficient associated to a suite of SNPs considered as involved in the association.
 259 Analyses were performed with the *glmnet* package ([https://cran.r-](https://cran.r-project.org/package=glmnet)
 260 [project.org/package=glmnet](https://cran.r-project.org/package=glmnet)).

261 A multi-trait association (Multi-trait GWAS, hereafter) was also implemented. We
262 used the MultiPhen package (O'Reilly *et al.*, 2012) to test for the linear combination of
263 phenotypes associated with the genotypes at each SNP. Such one approach may capture
264 effects hidden to single-trait GWAS. It performs a 'reversed regression', with multiple
265 phenotype predictors and genetic variant as outcome (i.e., G SNPs $X_{(ij)} = \{X_{(i1)}, \dots, X_{(iG)}\}$ are
266 explained by K phenotypic pigmentation variables $Y_{(ij)} = \{Y_{(i1)}, \dots, Y_{(iK)}\}$). SNPs are encoded by
267 allele count ($X_{(ij)} \in \{0, 1, 2\}$). An ordinal logistic regression was considered to derive the
268 probability that one SNP is associated to a multi-trait phenotype (Porter and O'Reilly,
269 2017). Permutation tests were performed to determine one genome-wide false discovery
270 rate (FDR) adjusted significance threshold to detect false positives (Dudbridge and
271 Gusnanto, 2008). A FDR-adjusted probability $P < 5 \times 10^{-8}$ was retained to consider one SNP
272 as significantly implied in a multi-trait pigmentation association.

273 In single- and multi-trait GWAS, we controlled for population stratification by using
274 nine distinct trout samples depending on the length and number of ancestry tracts resulting
275 from admixture between foreign Atlantic and local Mediterranean populations (Leitwein
276 *et al.*, 2018). The nine groups include the two original hatchery samples (Atlantic [$n = 15$],
277 Mediterranean [$n = 15$]), then seven distinct clusters of wild-caught trout. Following
278 Leitwein *et al.* (2018), wild-caught trout were grouped as follows: F1's ($n = 7$), F2's ($n = 4$),
279 'early' ($n = 20$) and 'late' ($n = 15$) backcrossed individuals resulting from crossings between
280 wild and released hatchery fish, then 'pure' wild individuals assigned to each of the three
281 local populations (Mare: $n = 4$; Orb: $n = 10$; Gravezon, $n = 15$). As some wild caught trout
282 were identified as released hatchery individuals (Leitwein *et al.*, 2018), they have been
283 grouped with individuals sampled in the hatchery type they have been assigned (Atlantic:

284 $n = 6$ caught in the Gravezon River, probably escapees from private hatchery;
285 Mediterranean: $n = 1$ caught in the Mare River).

286

287 **2.4 - MAPPING AND ANNOTATION**

288 CPLs detected by RDA and GWAS have been mapped on the Atlantic salmon genome (Lien
289 *et al.*, 2016; Genbank assembly: GCA_000233375.4) and on the high density linkage map
290 of *S. trutta* (Leitwein *et al.*, 2017). The final VCF and the PLINK files for *S. trutta* LGs are
291 available at NCBI under the study accessions SRZ187687 and SRZ187688
292 (<https://trace.ncbi.nlm.nih.gov/Traces/sra/sra.cgi?study=SRP136716>). We searched for
293 genes located in a 25kb window upstream or downstream of each CPL (arbitrary range).
294 When one association with a gene was detected, markers were assigned to the gene body
295 (exons and introns), to the upstream/downstream sequences of the genes, or other specific
296 entities (transposable elements, pseudogenes). Using *S. salar* annotations as inputs, gene
297 ontologies (GO) for biological processes and molecular functions were derived for genes
298 associated to CPLs using QuickGO (<https://www.ebi.ac.uk/QuickGO/>). When annotations
299 were unavailable on the salmon genome, a search was launched on UniProtKB
300 (<https://www.uniprot.org/uniprot/>) for the protein sequence encoded by the gene. Only
301 annotations with similarity >90% were retained.

302 The functional relevance of gene sets issues from RDA and GWAS has been
303 evaluated by screening (i) curated lists of pigmentation gene provided by Baxter *et al.*
304 (2019) for zebrafish, but also mice and human, (ii) genes found differentially expressed in
305 skin of brown trout belonging to the Atlantic and marbled (*S. t. marmoratus*) lineages, as
306 well as their hybrids (Sitka *et al.*, 2013; Djurdjevič *et al.*, 2019), (iii) genes identified as

307 differentially expressed among early life stages (i.e., when developmental signals for the
308 establishment of pigmentation patterns take place) of the closely-related yellow mutant
309 rainbow trout (*Onchorhynchus mykiss*) (Wu *et al.*, 2022), and (iv) the available literature,
310 including the associated supplementary information that might report lists of differentially
311 expressed genes. This literature review focused on vertebrates. Because many genes
312 associated to melanocyte development (i.e., melanin-producing neural-crest derived cells
313 primarily responsible for skin colour and pigmentation) are also implied in melanoma
314 development and skin colour variation when mutated (e.g., Uong and Zon, 2010; D’Mello
315 *et al.*, 2016), this literature search used specific queries. Queries were made using the gene
316 ID (or aliases taken from GeneCards, <https://www.genecards.org/>) and a specific keyword.
317 The retained keywords first aimed to cover the diversity of skin pigment cells in fish
318 (chromatophores, including melano-, irido-, leuco-, and xanthophores) (e.g., Nüsslein-
319 Vollard and Singh, 2017; Parichy, 2021), all present in salmonid skin and trout in which
320 erythrophores have also been found (Djurđjevič *et al.*, 2015). The other keywords used
321 were: skin, melanocyte, melanophore, melanosome (melanin-containing organelle),
322 melanoma (squamous, cutaneous and uveal), pigment/-ation, keratinocyte (associated to
323 melanocyte), chromatophore, iridiophore, xanthophore, follicle (hair or skin), nevus/-i
324 (melanocyte proliferation), (epi)dermis, vitiligo, piebald/-ism, erythema/-tous, and
325 sebocyte. This search was updated until August, 2021, each time using the Web of Science,
326 PubMed and Google Scholar as databases, the latter providing access to a ‘grey’ literature
327 (e.g., Ph.D. or M.Sc. thesis, patents).

328

329 **2.5 - GENOMIC DIFFERENTIATION**

330 Distributions of F_{ST} values (Weir and Cockerham, 1984) were established using all SNPs,
331 then for the subset of CPLs found significant in the RDA and GWAS. Single-locus F_{ST} values
332 were estimated considering five distinct trout samples: the two samples of hatchery fish,
333 then trout caught in each of the three rivers within the Orb catchment. The R package
334 *assigner* (https://rdr.io/github/thierrygosselin/assigner/man/fst_WC84.html) was used
335 for these computations.

336

337 **3 - RESULTS**

338

339 **3.1 - PIGMENTATION VARIABLES**

340 Eleven colour patterning variables over thirty were found not significantly correlated ($r <$
341 0.7) (Suppl. Mat. Table S2), and retained for subsequent analysis (Table 1). Sex and size
342 were also found uncorrelated to pigmentation variables (Suppl. Mat. Table S2) and also
343 retained for next step analysis.

344

345 **3.2 - REDUNDANCY ANALYSIS**

346 Forward model selection showed that a model based on eight over the eleven
347 pigmentation variables retained minimized the deviance in RDA (*Macrost*, *N.PR.Tot*, *Zeb*,
348 *Fr.An*, *L.Lat*, *Fr.P*, *Diam.PN* and *N.PN.Tot*). Sex and size were not retained by the forward
349 selection process, indicating they had no significant role in trout pigmentation variation *in*
350 *this study* (Suppl. Mat. Table S3). Constrained inertia (i.e., the percentage of variation

351 explained by the eight uncorrelated pigmentation variables) was found to represent
352 35.50% of the total inertia.

353 Patterns of variation explained by the RDA were found to significantly structure the
354 association between SNPs and pigmentation variables ($P < 0.001$). The first axis of RDA was
355 also found significant ($P < 0.001$), and to represent 29.81% of the total inertia (Fig. 1). RDA
356 axis 2 was found to explain only a tiny fraction of observed phenotypic variation (2.10%;
357 Fig. 1), and was marginally significant ($P = 0.051$). Axis 1 represented approx. 84% (ratio:
358 29.81/35.50) of constrained inertia, while axis 2 explained only approx. 6% (2.10/35.50).

359 RDA results have been represented as a triplot in which individuals are positioned
360 according to the relationship established between response and explanatory variables (Fig.
361 1). The ornamental trait *Macrost* (a large pre-opercular black stain/spot) and the
362 pigmentation trait *N.PR.Tot* (total number of red spots) explained 23.6% and 22.7%,
363 respectively, of the total loading scores of phenotypic variables onto RDA axis 1.

364 Results showed a clear distinction between colour patterns of Mediterranean and
365 Atlantic hatchery fish, but also of the three wild trout populations that did not cluster
366 together indicating that they do not present identical pigmentation patterns (Fig. 1A). This
367 observation illustrates the polytypic nature of pigmentation in trout, even at a local scale.
368 Atlantic hatchery fish were characterised by the total number of red spots, while the
369 macrostigma spots, and the total number of black spots (*N.PN.Tot*) (i.e., melanic traits)
370 characterised Mediterranean hatchery fish along RDA axis 1. This illustrates a potential
371 trade-off between red vs black ornaments, thus in the presence of two different pigment
372 cells, probably erythrophores and melanophores (Djurdjevič *et al.*, 2015). According to
373 their position along RDA axis 1, results confirmed that few wild caught individuals from the

374 Gravezon River were released Atlantic hatchery fish and that one individual from the Mare
375 River is a released Mediterranean hatchery fish (Leitwein *et al.*, 2018). Other individuals
376 from the Gravezon River and 'late backcrossed' individuals have patterns close to hatchery
377 Mediterranean fish (Fig. 1A; the hatchery was seeded by individuals caught in this river [see
378 Materials and Methods section]). Individuals identified as F1's (near equal hatchery Atlantic
379 and wild Mediterranean ancestry) and wild individuals of the Mare and Upper Orb rivers
380 presented intermediate positions between Mediterranean and Atlantic fish along RDA axis
381 1 (Fig. 1A), while the few segregating F2 and 'early' backcrossed individuals were dispersed
382 onto the RDA map.

383 The position of the wild caught Orb River individuals referred to the marginally
384 significant second axis of the RDA and to specific variables (*L. Lat*, *Fr.P*) that were mostly
385 unobserved in other fish (Fig. 1A).

386 The ordination of SNPs by RDA is further detailed in Fig.1B. A total of 1,130 distinct
387 loci (1.49% of 75,684 SNPs) were found significantly associated to the first two RDA axes
388 (i.e., > 2.5 S.D. from the mean). Only 22 loci (0.03%) were significantly associated to each
389 of the two axes for distinct phenotypic variables, indicating independence (orthogonality)
390 of RDA axes. Different numbers of SNPs were found associated with pigmentation
391 variables: 299 with *Fr.P*, 269 with *N.PR.Tot*, 225 with *N.PN.Tot*, and 213 with *Macrost*. Each
392 SNP was only associated with the variable it was the most significantly associated (i.e.,
393 pleiotropic effects not considered). As axis 2 was however found only marginally significant
394 and substantially affected by individuals from one single population (Orb River, above), only
395 SNPs associated to the first RDA axis will be considered further. This discarded loci
396 associated to *Fr.P* that explained most of the variation for the second RDA axis. This results

397 in three hundred and twenty SNPs associated with the main RDA axis (0.42% of 75,684
398 SNPs). Results are summarized in Table 2.

399 We further searched for the loading scores of SNPs associated to 17 pigmentation
400 genes regularly studied in fish within the distribution of loci over the RDA axis 1. We
401 detected only eight of these genes representing 30 SNPs for which sequencing reads were
402 available in our data (*Dct*, *Mitf1*, *Kit*, *Tyrp1*, *Pomca1*, *Pomca2*, *Pomcb* and *Scg2a*; Suppl.
403 Mat. Fig. S2). Only one SNP located downstream the *Mitf1* (microphthalmia-associated
404 transcription factor 1) gene was close to the ± 2.5 S.D. threshold considered in this study.
405 The *Mitf1* gene is a pleiotropic gene involved in the differentiation, proliferation, migration
406 and survival of melanocytes (Levy *et al.*, 2006; Cheli *et al.*, 2010). With a negative loading
407 score on the first axis of the RDA, this SNP is effectively associated to the expression of
408 black colour (Fig. 1B). The *Scg2a* (Secretogranin 2a) gene found differentially expressed in
409 trout skin (Sivka *et al.*, 2013) was relatively close to significance (Suppl. Mat. Fig. S2). This
410 may indicate RDA false negatives. However, SNPs associated to *Mitf1* and *Scg2a* were not
411 identified as putative CPLs by GWAS either (Suppl. Mat. Table S4).

412

413 **3.3 - SINGLE- AND MULTI-TRAIT GWAS**

414 After selection of the penalized term using the cyclical descent procedure, only two
415 pigmentation variables allowed for relevant LASSO model construction in single trait
416 GWAS: *N.PN.Tot* (total number of black spots: 17 candidate SNPs), and *N.PR.Tot* (total
417 number of red spots: 9 candidate SNPs) (Table 2; Suppl. Mat. Table S4). These two variables
418 were found significant in RDA, and negatively correlated (Fig. 1). The location of candidate
419 SNPs detected using single GWAS models are reported in Fig. 2A.

420 Two multi-trait GWAS models were retained (Fig. 2B). The first one jointly
421 retained *N.PN.Tot* and *N.PR.Tot* as variables for which some SNPs were found associated.
422 The second selected model considered seven of the eight variables formerly retained in the
423 RDA (except *Zeb*). Seven and thirty-one SNPs were considered as significant in these multi-
424 trait GWAS, respectively (Table 2; Suppl. Mat. Table S4). Lower number of SNPs detected
425 in both the single- and multi-trait GWAS might be indicative of associations involving large
426 effect loci.

427 Despite similarities for the phenotype variables put forward by the different
428 genotype-phenotype association methods (Table 2), only one single SNP (RAD-locus) was
429 found in common between RDA and the single-trait GWAS for number of black spots (Fig.
430 2A). No other SNP was commonly detected by distinct genotype-phenotype association
431 methods, and especially no SNP was shared among single- and multi-GWAS models.
432 However, both a multi-trait GWAS model and the RDA indicated a genotype-phenotype
433 association at the *ITGAV* (Integrin Alpha 5) gene located on LG24 of the brown trout, but
434 methods did not identify the same locus (Suppl. Mat. Table S4). Overall, the combination
435 of RDA, single- and multi-trait GWAS resulted in a total of 384 SNPs considered as CPLs
436 putatively involved in pigmentation variation in the brown trout (0.51% of the total number
437 of SNPs). These CPLs corresponds to 337 RAD-loci (0.83% of the total number of RAD-loci).
438 The 337 CPLs are listed in Suppl. Mat. Table S4. The main information regarding variables
439 and loci found associated in each method are summarized in Table 2.

440

441 **3.4 - GENOMIC DIFFERENTIATION**

442 The distributions of F_{ST} values for the 40,519 RAD-loci and the 320 CPLs associated to axis
443 1 of the RDA are reported in Fig. 3. The mean F_{ST} was estimated to $F_{ST} = 0.286$ [95% CI:
444 0.284, 0.287] for the full set of SNPs, while this estimate was $F_{ST} = 0.575$ [95% CI: 0.569-
445 0.583] for the 320 CPLs detected with the RDA. Observed mean F_{ST} values were $F_{ST} = 0.176$
446 (min. 0.02 – max: 0.38) and $F_{ST} = 0.025$ (min.: 0.00; max. 0.20) for the single- and the multi-
447 trait GWAS, respectively. Min/max values are reported rather than 95% CI because the low
448 number of SNPs associated to phenotypic variables in each GWAS. The F_{ST} value for the
449 *Mitf1*-associated locus found close to the significance for RDA axis 1 was $F_{ST} = 0.478$.

450

451 **3.5 - MAPPING ON *S. TRUTTA* LINKAGE GROUPS AND ANNOTATION**

452 Only the set of 337 RAD-loci CPLs was considered. Three hundred CPLs were mapped onto
453 35 of the forty brown trout LGs defined in Leitwein *et al.* (2017) (Fig. 4), while 37 of them
454 could not be adequately positioned on this high density linkage map because they mapped
455 to several LGs probably due to the ancestral duplication of salmonid genomes, or because
456 portions of this map remain unsufficiently characterized. The distribution of these 300 CPLs
457 on each LG varied from zero (LG13, LG14, LG15, LG20, LG39) to 23 (LG22; 20 from RDA, 3
458 from a GWAS), then 86 (LG31; 85 from RDA, 1 from a GWAS). LG22 and LG31 contained
459 36.33% of mapped CPLs, while others were found evenly distributed across remaining LGs.
460 The presence of false positives will be briefly discussed below, but, whatever the method
461 CPLs were detected, they are distributed over several LGs, suggestive of a polygenic
462 architecture of pigmentation in trout.

463 The annotations of the 337 CPLs are reported in Suppl. Mat. Table S4.
464 Supplementary Material Table S4 also provides details on gene names, their position and

465 the location of each CPL. Among them, 71 (21.07%) did not match with any gene in the
466 25kb window retained in this study, and 254 were found located in the gene body ($n = 141$;
467 41.84%) or in the upstream-downstream of genes ($n = 113$; 33.53%). The remaining portion
468 (3.56%) was found associated to pseudogenes ($n = 3$) or long non-coding (lnc) RNA ($n = 9$)
469 (Suppl. Mat. Fig. S3). Overall, this translated into CPLs associated to 218 distinct candidate
470 genes in a 25kb window (Table 2; Suppl. Mat. Table S4). More than ninety percent (90.36%;
471 197 out of 218) of these annotated genes have literature match dealing with their
472 involvement in colour/pigmentation, skin patterning, differentiation or structure. This
473 specifically includes 144 out of 218 annotated genes (66.05%; 22 detected by GWAS, 120
474 by RDA, and two by RDA and GWAS) found expressed in the skin transcriptomes of rainbow
475 trout produced by Wu *et al.* (2022).

476 An excerpt of twenty-five of these 218 genes (11.93%) is reported in Table 3. Thirty-
477 two CPLs were found close to these 25 genes that have been reported in former
478 pigmentation studies, in curated lists of pigmentation genes provided by Baxter *et al.*
479 (2019) or Wu *et al.*'s (2022) rainbow trout transcriptomes. Table 3 includes seven CPLs
480 associated to genes formerly found differentially expressed in the brown trout (Djurdjevič
481 *et al.*, 2019).

482

483 **3.6 - GO TERMS**

484 GO terms for molecular functions and biological processes of the 337 CPLs are reported in
485 Suppl. Mat. Fig. S4. Calcium and metal ion binding were found to be the most
486 representative molecular functions. GO-terms for biological functions highlighted cellular

487 adhesion processes (Suppl. Mat. Fig. S4A), signal transduction and translation, and
488 transmembrane transport (Suppl. Mat. Fig. S4B).

489

490 **4 - DISCUSSION**

491 While recognized as a polytypic species with very distinct colour morphs, few studies were
492 interested in pigmentation variation in the brown trout. Previous studies were either based
493 on experimental crosses to study patterns of inheritance of few pigmentation traits (Blanc
494 *et al.*, 1982, 1994; Skaala and Jørstad, 1988; Skaala, Jørstad and Borgstrøm, 1991), or on
495 the analysis of gene expression variation to investigate the molecular basis of pigmentation
496 variation (Sivka *et al.*, 2013; Djurdjevič *et al.*, 2019). The genomic architecture of
497 pigmentation variation was not investigated yet. The present study partly fills this gap by
498 analyzing variation at >75,000 SNPs and several pigmentation variables in 112 brown trout
499 using diverse association methods, namely RDA, single- and multi-trait GWAS whose
500 findings were never compared yet. RDA is traditionally not considered to illustrate
501 genotype-phenotype associations, but single- and multi-trait GWAS remain rarely
502 compared, especially in fish (Yoshida and Yáñez, 2021). A single empirical study on the
503 brown trout does not aim to discard the effectiveness of GWAS in fish (e.g., Barson *et al.*,
504 2015; Lemopoulos *et al.*, 2019) or pigmentation studies (e.g., Kim *et al.*, 2019; Wang S. *et*
505 *al.*, 2020; Wang *et al.*, 2021). However, RDA showed interesting properties in GEAs such as,
506 for example, robustness over sample sizes that may also benefit genotype-phenotype
507 association studies in wild organisms. In the case of the brown trout, however, the effects
508 of ontogenetic and environmental variations, but also sexual maturation on pigmentation

509 patterns were not or poorly investigated in this study (e.g. parr marks, present on too few
510 individuals to be relevant in the model), and certainly deserve more attention.

511

512 **4.1 - RDA: A POWERFUL GENOTYPE-PHENOTYPE ASSOCIATION METHOD**

513 Capblancq and Forester (2021) coined redundancy analysis as the Swiss Army Knife for GEA,
514 and the present study demonstrates it is a quite useful association analysis to investigate
515 the molecular basis of a genotype-phenotype association in wild populations. In the brown
516 trout, RDA highlighted the same main phenotypic variables than GWAS (total number of
517 black or red spots), completed by other traits that were also selected the multi-trait GWAS.
518 The presence of red and black spots reported by each approach is commonly reported to
519 distinguish Atlantic and Mediterranean trout, respectively (Lascaux, 1996; Aparicio *et al.*,
520 2005).

521 Unlike PCA (e.g., Duforet-Frebourg *et al.*, 2016), or F_{ST} outlier methods (e.g., Hassl
522 and Payseur 2016; Ahrens *et al.*, 2018), RDA promoted only candidate SNPs with large F_{ST}
523 values involved in observed phenotypic variation. For example, a classical application of a
524 F_{ST} outlier method generally selects for the top 1% or 5% loci with the largest F_{ST} values
525 irrespective of a relationship with a trait of interest (see Neethiraj *et al.*, 2017 for a
526 pigmentation study). Applied to brown trout data, most RDA candidates will have been not
527 considered with an outlier approach while many other would have been despite lack of any
528 functional relationship with processes regulating pigmentation patterns (Fig. 3). As PCA
529 however, RDA promotes useful graphical interpretation to contrast groups of individual
530 from each other (Fig. 1A; see Discussion below).

531 Results derived from RDA are strongly supported by functional evidence. This is
532 illustrated by the high number of RDA-defined CPLs associated to genes involved in
533 pigmentation at the developmental, tissular or cellular levels in other fish or vertebrates.
534 Of outstanding importance is that one hundred and twenty genes detected by RDA as
535 involved in pigmentation variation in the brown trout were effectively found differentially
536 expressed across life-stages in the skin transcriptome of the rainbow trout yellow mutant
537 (Wu *et al.*, 2022). While body colours differ between trout, these genes are thus involved
538 in the establishment of pigmentation phenotypes in close relatives. Functional evidence is
539 also supported by GO-terms for molecular or biological functions. For example, calcium
540 binding is important for melanogenesis (Bush and Simon, 2007; Bellono and Oancea, 2014;
541 Jia *et al.*, 2020), including regulation of spots on butterfly wings (Özsu and Monteiro, 2017).
542 Metal ion binding is necessary in colour patterning and at various steps of melanogenesis,
543 including transmembrane transport, melanocyte migration, or melanosome trafficking
544 within melanocytes (e.g., Denecker *et al.*, 2014; Logan *et al.*, 2006; Hong and Simon, 2007;
545 Wiriyasermkul *et al.*, 2020). Furthermore, the main biological functions highlighted in this
546 study (e.g., cellular adhesion processes, signal transduction and translation) are involved in
547 chromatophore development, interactions among chromatophores or between
548 melanocytes and other skin cell types (fibroblasts, keratinocytes) (Yamaguchi *et al.*, 2007;
549 Raposo and Marks, 2007; Patterson *et al.*, 2014; Wiriyasermkul *et al.*, 2020).

550 Interestingly, some RDA-defined CPLs were also found associated to genes known
551 to be involved in background colour adaptation (*GABRA2*, gamma-aminobutyric acid
552 receptor subunit alpha 2; Bertolesi *et al.*, 2016), or light-induced colour change (*FGFR1*,
553 fibroblast growth factor receptor 1; Czyz, 2019). This suggests a genomic basis to

554 environmentally-induced pigmentation variation in trout, and thus genotype x
555 environment interaction (Westley, Stanley and Fleming, 2013).

556

557 **4.2 - RDA IS NOT ENOUGH**

558 RDA, however, cannot be promoted unequivocally for several reasons. Indeed, Forester et
559 al. (2018) showed that RDA suffered less false positives in GEA, but this however does not
560 mean it is not affected. For example, linkage disequilibrium (LD) that promotes marker
561 inheritance in a block-like manner impacts the loading scores in PCA, and RDA suffers the
562 same bias (Lottherhos, 2019). If the 'true' CPL involved in the genotype-association is
563 effectively present, linked loci might be identified as false positives. For example, 86 CPLs
564 have been identified onto LG31 in this study ($n = 85$ by RDA; Fig. 4), LG31 effectively
565 contains relevant CPLs (Table 3), but 40 of 86 were not associated to any genes and false
566 positives are likely. Marker density has to be increased to reach better conclusion on the
567 nature of association. For example, Wang S. et al. (2020) showed that a window $<0,5\text{Mb}$
568 was necessary to detect the most likely variant involved in pigmentation variation in North
569 American warblers, one intronic variant effectively close to pigmentation genes. This
570 threshold is not reach in this study. Another source related to the detection of false
571 associations by RDA is the use of a ± 2.5 SD significance threshold to retain SNPs as CPLs.
572 In this case, one SNP associated to *Mitf1* may represent a false negative, ignored by RDA
573 despite its biological importance in fish pigmentation (e.g., Wang T. et al., 2020). Methods
574 aiming at capturing allele frequency variation differently than RDA or F_{ST} should possibly
575 be considered to correct for false positive/negative loci (e.g., Field *et al.*, 2016; Chen and
576 Narum, 2021), but are not under the scope of this study.

577 Furthermore, despite the low sample size considered in this study, GWAS also
578 provided relevant CPLs. The power of GWAS was undoubtedly improved by considering
579 population stratification based on admixture patterns of individuals defined by Leitwein et
580 al. (2018). Controlling for population stratification by admixture mapping (i.e., levels of
581 ancestry) is crucial in association studies (e.g., Zhu *et al.*, 2008; Lin *et al.*, 2021), and has
582 been already successfully used in pigmentation studies (Shriver *et al.*, 2003; Daya *et al.*,
583 2014; Brelsford *et al.*, 2017), including fish (Miller *et al.*, 2007; Malek *et al.*, 2012).
584 Furthermore, as trout presents low local effective population sizes (N_e) (e.g., Charlier *et al.*,
585 2012) and might be inbred (Magris *et al.*, 2022), strong LD may increase the power to detect
586 of large effect loci involved in phenotypic variation. This may favor GWAS that reported loci
587 involved in pigmentation and/or body colour variation in vertebrates (e.g., *SOX10* [Sex-
588 determining region Y-box 10], *PMEL* [Premelanosome protein], *SLC45A2* [Solute Carrier
589 Family 45 Member 2]) (e.g., Greenhill *et al.*, 2011; Moran *et al.*, 2022; Wu *et al.*, 2022;
590 Fukamachi, Shimada and Shima, 2001; Dooley *et al.*, 2013; Ahi and Sefc, 2017; Luo *et al.*,
591 2019). *SOX10* and *PMEL* were found differentially expressed in skin of trout belonging to
592 different lineages (Djurdjevič *et al.*, 2019). GWAS retrieved loci with F_{ST} values closer to the
593 background neutral estimation and sometimes nearly null F_{ST} values that RDA ignored. The
594 association of relevant variants to high F_{ST} values is not the rule (e.g., McCluskey *et al.*,
595 2021), and stabilizing or balancing selection have also been shown crucial in pigmentation
596 variation (Croucher *et al.*, 2011; Bourgeois *et al.*, 2016; Lindtke *et al.*, 2017; Schweizer *et*
597 *al.*, 2018). GWAS thus provided information not reached by RDA and the genomics
598 landscape of trout pigmentation was probably better assessed by their respective
599 performance.

600

601 **4.3 - A POLYGENIC ARCHITECTURE OF PIGMENTATION PATTERNS**

602 Pigmentation and/or colour associated loci are often hundreds (e.g., Bennett and
603 Lamoreux, 2003; Baxter *et al.*, 2019), and recent genomic studies revealed an ever-
604 increasing polygenic basis of pigmentation patterns (e.g., skin and hair in human: Crawford
605 *et al.*, 2017; Pavan and Sturm, 2019; structural colour variation or eyespot numbers in
606 butterflies: Brien *et al.*, 2019; Rivera-Colón *et al.*, 2020). Recently, Baxter *et al.* (2019)
607 reported 311 genes responsible for pigmentation in zebrafish, and RNA sequencing studies
608 supported a large molecular basis associated to pigmentation variation in, e.g., cichlids
609 (Henning *et al.*, 2013), tilapia (Zhu *et al.*, 2016), common carp (Li *et al.*, 2015), and other
610 salmonids (Wu *et al.*, 2022). Such a large molecular basis is expected because of the
611 diversity of skin pigment cells, their own interactions and interactions with other cell types
612 that affect their distribution, the increased understanding in the mechanisms of pigment
613 cell fates during development that mobilize numerous interacting molecular pathways to
614 express extensive pigmentation variation in fish (e.g., Nüsslein-Volhard and Singh, 2017;
615 Irion and Nüsslein-Volhard, 2019; Patterson and Parichy, 2019). In line with this complexity,
616 the combination of RDA and GWAS support a polygenic architecture of body pigmentation
617 in the brown trout based on dozens of polymorphic loci distributed in coding, non-coding
618 and regulatory regions over many LGs.

619 This observation contrasts with former knowledge. Indeed, models depending on
620 few major quantitative trait loci (QTLs) and simple inheritance mechanisms were often
621 found sufficient to explain pigmentation variation in salmonids (Blanc *et al.*, 1982, 1994;
622 Boulding *et al.*, 2008; Colihueque, 2010; Smith *et al.*, 2020), and other fish species (e.g.,
623 Barson *et al.*, 2007; Miller *et al.*, 2007; Greenwood *et al.*, 2011; Malek *et al.*, 2012; O'Quin
624 *et al.*, 2012, 2013; Henning *et al.*, 2014; Yong *et al.*, 2015). Only few exceptions have been

625 documented so far in fish (Tripathi *et al.*, 2009; Albertson *et al.*, 2014). It was shown that
626 increasing the number of pigmentation traits as done in this study translated into the
627 detection of more putative QTLs or variants distributed over more LGs.

628 CPLs detected in this study were found located in both gene body and upstream-
629 downstream regions close to genes. The distribution of SNPs in distinct genomic regions is
630 important in evolutionary studies, and now often reported in the literature (e.g., She and
631 Jarosz, 2018; Fruciano *et al.*, 2021). Within pigmentation studies, literature initially
632 emphasized both the relative roles of coding (Protas and Patel, 2008; Uy *et al.*, 2016), and
633 upstream-downstream regulatory variation (Larter *et al.*, 2018; Toomey *et al.*, 2018) –
634 more rarely their interactions (Vickrey *et al.*, 2018)— during colour pattern establishment
635 and evolution. The role of regulatory variation in observed pigmentation variation received
636 increased interest and support in many organisms in the past few years (Lewis and Van
637 Belleghem, 2020, for a review), including fish (zebrafish: Irion and Nüsslein-Volhard, 2019;
638 threespine stickleback: Miller *et al.*, 2007; cichlids: Roberts *et al.*, 2009; Santos *et al.*, 2014;
639 Urban *et al.*, 2021; 2020; fighting fish; Wang *et al.*, 2021). Data suggest that regulatory
640 variation by upstream-downstream sequences across genes might be important in trout
641 pigmentation phenotypes. However, it is difficult to fully appreciate their potential
642 contribution as ddRADseq provides a reduced genome representation that targets only a
643 tiny portion of the genome (discussed in Gauthier *et al.*, 2020, for pigmentation). While
644 quite few were detected in this study, lncRNAs might also be important in the regulation
645 of pigmentation and melanogenesis in fish (Luo *et al.*, 2019; Gan *et al.*, 2021).

646

647 **4.4— DYNAMICS OF PIGMENTATION VARIATION AND MANAGEMENT**

648 How differences in body pigmentation are related to admixture is still poorly studied in fish
649 yet (Boulding *et al.*, 2008; Egger *et al.*, 2012; Malek *et al.*, 2012; Meier *et al.*, 2018; Moran
650 *et al.*, 2022). RDA (Fig. 1B) illustrated shifts in pigmentation variation in hatchery and wild
651 trout with different levels of admixture (Leitwein *et al.*, 2018). While wild-caught F1's are
652 intermediate from Atlantic and Mediterranean fish for pigmentation, RDA showed that
653 'late-backcrossed' individuals clustered close to the local Mediterranean hatchery strain
654 and wild individuals of the Gravezon River that were used to seed this strain. Shift in trout
655 pigmentation patterns during introgression are thus transient, with individuals identified
656 as 'late backcrossed' by Leitwein *et al.* (2018) almost reaching the initial Mediterranean
657 local pigmentation after few dozens of generations (Atlantic introgression in these
658 Mediterranean rivers was estimated to range from 22 to 31 generations in average;
659 Leitwein *et al.*, 2018). Rapid trait evolution is often reported in salmonids (e.g., Hendry *et al.*
660 *et al.*, 2000; Jensen *et al.*, 2017), but this was not shown for pigmentation yet. The dispersion
661 of F2 and 'early backcrossed' trout *sensu* Leitwein *et al.* (2018) along RDA axis 1 showed
662 these individuals might be either more Atlantic- or Mediterranean-like. This very probably
663 reflects the diversity of ancestry tracts and thus CPLs they inherited through recombination
664 events that lead to large variation in expression of pigmentation. A similar pattern was also
665 shown in backcrosses of *Danio quagga* and *D. kyathit* by McCluskey *et al.* (2021) that were
666 shown much more diverse in backcrosses than in parents.

667 The shift to Mediterranean pigmentation pattern in late-backcrossed individuals
668 might be explained by not mutually exclusive hypotheses. First, counter selection of
669 Atlantic pigmentation alleles (or ancestry tracts) may have occurred in the Orb River
670 catchment. Counter-selection of Atlantic alleles was formerly suspected by Poteaux *et al.*
671 (1998, 1999) using allozymes, including the Orb River catchment. Second, successive

672 recombination events that decreased the number and shortened the length of Atlantic
673 ancestry tracts did not allow for specific regulatory mechanisms to maintain the expression
674 of an Atlantic-like pigmentation within a Mediterranean genomic background. Phenotypic
675 integration of 'pigmentation modules' could constrain possible combinations and force
676 certain phenotypic changes to coincide, leading to the expression of a Mediterranean-like
677 pigmentation once a threshold or dosage is reached, and depending on the restoration of
678 Mediterranean genome expression. Whatever the right hypothesis, Mediterranean
679 pigmentation appears to be adaptive as it converges to the Mediterranean wild type, but,
680 during this process, it is not related to the level of admixture of individual trout.

681 Indeed, the distribution of individuals along RDA axis 1 also illustrated that the
682 pigmentation patterns observed in trout are likely a poor proxy of introgression. For
683 example, 'pure' Mediterranean individuals fished in the Orb and Mare rivers showed
684 pigmentation patterns that are more like F1's (Orb) or Atlantic-like (Mare) regarding RDA
685 axis 1, while they present no or few Atlantic ancestry tracts within their genome (Leitwein
686 *et al.*, 2018). Thus decision-making on the 'genetic integrity' of local trout based on their
687 pigmentation phenotype is questionable (see Aparicio *et al.*, 2005). Partial convergence of
688 phenotypes in the Orb and Mare rivers to the Atlantic phenotype may reflect local selection
689 or drift. For example, intermediate F1-like plumage colouration observed in the North
690 American owl *Strix varia* was shown unrelated to plumage colouration introgression from
691 *S. occidentalis*, and was better explained by selection (Hanna *et al.*, 2018). However, as
692 genetic drift certainly predominates in trout because of small local N_e , it may have a critical
693 role in shaping the architecture of local adaptation for polygenic traits (Yeaman, 2015;
694 Stephan and Jones, 2020). We may hypothesize that changes in allele frequencies at CPLs
695 might promote the emergence of distinct phenotypes in local populations. This was

696 demonstrated for other complex traits in isolated populations (e.g., Southam *et al.*, 2017).
697 Local shifts in pigmentation patterns are common in trout (Skaala and Solberg, 1997;
698 Westley *et al.*, 2012), and the relative role of selection and drift has to be investigated
699 further.

700

701 **5 - CONCLUSIONS**

702 As suggested by Jombart *et al.* (2009), RDA mainly used in GEA studies was shown useful
703 and complimentary to GWAS to investigate a genotype-phenotype relationship for
704 pigmentation variation in trout. In the case of trout in which ancestry tracts were
705 accessible, RDA also documented the patterns of phenotypic evolution across generations
706 for a suite of pigmentation traits and suggested that Mediterranean body pigmentation is
707 locally adaptive. Replication of this study and data coming from more natural populations,
708 clades and/or subspecies within the *S. trutta* complex remain however necessary in order
709 to better investigate and elucidate both the proximate and ultimate causes of pigmentation
710 pattern variation in the polytypic brown trout. This necessitates to include (i) further
711 phenotypic (e.g., background color, hue, reflectance), and (ii) *-omic* data coming from or
712 associated to controlled settings (e.g., Gerwin *et al.*, 2021; McCluskey *et al.*, 2021). The
713 effect of age and size of fish should probably be investigated in more details. Innovative
714 pigmentation studies based on gene editing or improvement in reaction-diffusion and self-
715 organization models (Irion and Nüsslein-Völlard, 2019; Parichy, 2021; Wang *et al.*, 2021)
716 formerly used in salmonids (Miyazawa *et al.*, 2010) could also be a basis to future
717 multidisciplinary *in vivo* and *in silico* research aiming to explain flexibility and robustness of
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719

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727

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729 TV, ML, and BG analysed data and drafted early version the paper; all authors contributed
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731 acquired field data and JML produced phenotypic data to be analysed. ML and ED acquired
732 the genomic data. PB, ML, BG conceived and designed the study. All authors agreed with
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734

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- 1369

1370 **Table 1**

Phenotypic variables	Description	Nature* (coding)
<i>N. PR. Tot</i>	Total number of red spots over flanks	Q
<i>N. PN. Tot.</i>	Total number of black spots over flanks	Q
<i>Diam. PN</i>	Mean diameter of black spots	Q
<i>Oc. PN</i>	White ring around black spots	SQ (1= no ring; 2 = attenuated/thin ring, 3 = marked/thick ring)
<i>Fr. D</i>	Fringe on the dorsal fin	SQ (1= no fringe; 2 = white fringe, 3 = black & white fringe)
<i>Fr. An</i>	Fringe on the anal fin	SQ (1= no fringe; 2 = white fringe, 3 = black & white fringe)
<i>Fr. P</i>	Fringe on the pelvic fins	SQ (1= no fringe; 2 = white fringe, 3 = black & white fringe)
<i>L. Lat</i>	Visibility of the lateral line due to pigments	SQ (1 = not visible; 2 = partially visible; 3 = fully visible)
<i>Parr</i>	Presence of parr marks	SQ (1 = absence; 2 = presence)
<i>Macrost.</i>	Macrostigma spot (large black dot on the pre-opercular bone)	SQ (1 = absence; 2 = partially visible/diluted; 3 = fully visible)
<i>Zeb</i>	Zebras on flank	SQ (1 = absence; 2 = presence)

* : Q = quantitative variable; SQ = semi-quantitative variable (coding in brackets)

Table 1: Pigmentation variables retained in this study. The full list is provided in Suppl. Mat. Table S1.

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1373 **Table 2**

	RDA	Single GWAS		Multi-GWAS		Total
Number of pigmentation variables considered	8	1	1	2	7	-
Selected variables	<i>N. PR. Tot</i> <i>N. PN. Tot.</i> <i>Macrost</i> <i>Diam. PN</i> <i>Fr. An</i> <i>Fr. P</i> <i>L. Lat</i> <i>Zeb</i>	<i>N. PR. Tot</i>	<i>N. PN. Tot.</i>	<i>N. PR. Tot</i> <i>N. PN. Tot.</i>	<i>N. PR. Tot</i> <i>N. PN. Tot.</i> <i>Macrost</i> <i>Diam. PN</i> <i>Fr. An</i> <i>Fr. P</i> <i>L. Lat</i>	
Number of CPLs	320*	9	17	7	31	384 [#]
% of total SNPs	0.42%	0.012%	0.022%	0.009%	0.041%	0.51%
Number of different CPLs associated to an annotated gene (% of total CPLs)	192 (60.0%)	7 (77.7%)	15 (88.2%)	5 (71.4%)	23 (74.19%)	241 (62.7%)
Number of different annotated genes	175	7	13	3	22	218
Number of different annotated genes with at least one literature reference dedicated to skin pigmentation	160	7	11	3	18	197

* : RDA axis 1 only

[#] : corresponding to a total of 337 distinct RAD-loci**Table 2:** Summary of results obtained with RDA, single- and multi-trait GWAS. Phenotypic variables are abbreviated as in Table 1.

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Accession	L.O. # gene	L.O. # gene	Position on L.O. (kb)	Position on L.O. (kb)	Position on L.O. (kb)	Position on L.O. (kb)	Gene ID	GO term: Molecular function	GO term: Biological process	Associated reference(s)	Expressed in relative to wild type (RT, n=3)	Function
WU	1	10	100000	100000	100000	100000	WU001	cellular protein binding		Maroney and Burtstein (2002)	Normal	binds signaling molecules to regulate and coordinate transcription
WU001	1	10	100000	100000	100000	100000	WU001	RNA binding, nucleic acid binding	transcription process	Wang et al. (2002)	Y	transcription factor (TF) involved in regulation of transcription
WU002	2	10	110000	110000	110000	110000	WU002	RNA binding, nucleic acid binding	transcription process	Wang et al. (2002)	Y	transcription factor (TF) involved in regulation of transcription
WU003	3	10	120000	120000	120000	120000	WU003	RNA binding, nucleic acid binding	transcription process	Wang et al. (2002)	Y	transcription factor (TF) involved in regulation of transcription
WU004	4	10	130000	130000	130000	130000	WU004	RNA binding, nucleic acid binding	transcription process	Wang et al. (2002)	Y	transcription factor (TF) involved in regulation of transcription
WU005	5	10	140000	140000	140000	140000	WU005	RNA binding, nucleic acid binding	transcription process	Wang et al. (2002)	Y	transcription factor (TF) involved in regulation of transcription
WU006	6	10	150000	150000	150000	150000	WU006	RNA binding, nucleic acid binding	transcription process	Wang et al. (2002)	Y	transcription factor (TF) involved in regulation of transcription
WU007	7	10	160000	160000	160000	160000	WU007	RNA binding, nucleic acid binding	transcription process	Wang et al. (2002)	Y	transcription factor (TF) involved in regulation of transcription
WU008	8	10	170000	170000	170000	170000	WU008	RNA binding, nucleic acid binding	transcription process	Wang et al. (2002)	Y	transcription factor (TF) involved in regulation of transcription
WU009	9	10	180000	180000	180000	180000	WU009	RNA binding, nucleic acid binding	transcription process	Wang et al. (2002)	Y	transcription factor (TF) involved in regulation of transcription
WU010	10	10	190000	190000	190000	190000	WU010	RNA binding, nucleic acid binding	transcription process	Wang et al. (2002)	Y	transcription factor (TF) involved in regulation of transcription
WU011	11	10	200000	200000	200000	200000	WU011	RNA binding, nucleic acid binding	transcription process	Wang et al. (2002)	Y	transcription factor (TF) involved in regulation of transcription
WU012	12	10	210000	210000	210000	210000	WU012	RNA binding, nucleic acid binding	transcription process	Wang et al. (2002)	Y	transcription factor (TF) involved in regulation of transcription
WU013	13	10	220000	220000	220000	220000	WU013	RNA binding, nucleic acid binding	transcription process	Wang et al. (2002)	Y	transcription factor (TF) involved in regulation of transcription
WU014	14	10	230000	230000	230000	230000	WU014	RNA binding, nucleic acid binding	transcription process	Wang et al. (2002)	Y	transcription factor (TF) involved in regulation of transcription
WU015	15	10	240000	240000	240000	240000	WU015	RNA binding, nucleic acid binding	transcription process	Wang et al. (2002)	Y	transcription factor (TF) involved in regulation of transcription
WU016	16	10	250000	250000	250000	250000	WU016	RNA binding, nucleic acid binding	transcription process	Wang et al. (2002)	Y	transcription factor (TF) involved in regulation of transcription
WU017	17	10	260000	260000	260000	260000	WU017	RNA binding, nucleic acid binding	transcription process	Wang et al. (2002)	Y	transcription factor (TF) involved in regulation of transcription
WU018	18	10	270000	270000	270000	270000	WU018	RNA binding, nucleic acid binding	transcription process	Wang et al. (2002)	Y	transcription factor (TF) involved in regulation of transcription
WU019	19	10	280000	280000	280000	280000	WU019	RNA binding, nucleic acid binding	transcription process	Wang et al. (2002)	Y	transcription factor (TF) involved in regulation of transcription
WU020	20	10	290000	290000	290000	290000	WU020	RNA binding, nucleic acid binding	transcription process	Wang et al. (2002)	Y	transcription factor (TF) involved in regulation of transcription
WU021	21	10	300000	300000	300000	300000	WU021	RNA binding, nucleic acid binding	transcription process	Wang et al. (2002)	Y	transcription factor (TF) involved in regulation of transcription
WU022	22	10	310000	310000	310000	310000	WU022	RNA binding, nucleic acid binding	transcription process	Wang et al. (2002)	Y	transcription factor (TF) involved in regulation of transcription
WU023	23	10	320000	320000	320000	320000	WU023	RNA binding, nucleic acid binding	transcription process	Wang et al. (2002)	Y	transcription factor (TF) involved in regulation of transcription
WU024	24	10	330000	330000	330000	330000	WU024	RNA binding, nucleic acid binding	transcription process	Wang et al. (2002)	Y	transcription factor (TF) involved in regulation of transcription
WU025	25	10	340000	340000	340000	340000	WU025	RNA binding, nucleic acid binding	transcription process	Wang et al. (2002)	Y	transcription factor (TF) involved in regulation of transcription
WU026	26	10	350000	350000	350000	350000	WU026	RNA binding, nucleic acid binding	transcription process	Wang et al. (2002)	Y	transcription factor (TF) involved in regulation of transcription
WU027	27	10	360000	360000	360000	360000	WU027	RNA binding, nucleic acid binding	transcription process	Wang et al. (2002)	Y	transcription factor (TF) involved in regulation of transcription
WU028	28	10	370000	370000	370000	370000	WU028	RNA binding, nucleic acid binding	transcription process	Wang et al. (2002)	Y	transcription factor (TF) involved in regulation of transcription
WU029	29	10	380000	380000	380000	380000	WU029	RNA binding, nucleic acid binding	transcription process	Wang et al. (2002)	Y	transcription factor (TF) involved in regulation of transcription
WU030	30	10	390000	390000	390000	390000	WU030	RNA binding, nucleic acid binding	transcription process	Wang et al. (2002)	Y	transcription factor (TF) involved in regulation of transcription
WU031	31	10	400000	400000	400000	400000	WU031	RNA binding, nucleic acid binding	transcription process	Wang et al. (2002)	Y	transcription factor (TF) involved in regulation of transcription
WU032	32	10	410000	410000	410000	410000	WU032	RNA binding, nucleic acid binding	transcription process	Wang et al. (2002)	Y	transcription factor (TF) involved in regulation of transcription
WU033	33	10	420000	420000	420000	420000	WU033	RNA binding, nucleic acid binding	transcription process	Wang et al. (2002)	Y	transcription factor (TF) involved in regulation of transcription
WU034	34	10	430000	430000	430000	430000	WU034	RNA binding, nucleic acid binding	transcription process	Wang et al. (2002)	Y	transcription factor (TF) involved in regulation of transcription
WU035	35	10	440000	440000	440000	440000	WU035	RNA binding, nucleic acid binding	transcription process	Wang et al. (2002)	Y	transcription factor (TF) involved in regulation of transcription
WU036	36	10	450000	450000	450000	450000	WU036	RNA binding, nucleic acid binding	transcription process	Wang et al. (2002)	Y	transcription factor (TF) involved in regulation of transcription
WU037	37	10	460000	460000	460000	460000	WU037	RNA binding, nucleic acid binding	transcription process	Wang et al. (2002)	Y	transcription factor (TF) involved in regulation of transcription
WU038	38	10	470000	470000	470000	470000	WU038	RNA binding, nucleic acid binding	transcription process	Wang et al. (2002)	Y	transcription factor (TF) involved in regulation of transcription
WU039	39	10	480000	480000	480000	480000	WU039	RNA binding, nucleic acid binding	transcription process	Wang et al. (2002)	Y	transcription factor (TF) involved in regulation of transcription
WU040	40	10	490000	490000	490000	490000	WU040	RNA binding, nucleic acid binding	transcription process	Wang et al. (2002)	Y	transcription factor (TF) involved in regulation of transcription

Table 3. The regions of relative overexpression (ROE) identified in brain tissue by the three genotyping strategies: microarray (MGA), single and real-time RT-PCR. For the single and MGA, the results are listed in the main table as in Table 1. For the real-time RT-PCR, numbers in bold indicate the number of genes that were found to be overexpressed in brain tissue (see Table 1 and main text). The position of ROE on the brain tissue (L.O. (kb)) and on the relative reference genome (L.O. (kb)) are reported. Protein names and Gene ID are given, together with GO terms for molecular function and biological processes. Useful references and the function(s) associated to pigmentation are reported for each ROE. The reference is bold when directly associated to the ROE and underlined when not. For additional relative differential expression of ROE across developmental stages of the relative mouse cell lines (Wu et al., 2002) (Y = yes; N = no). The words indicate the gene of the same family was identified participating to the regulation of body pigmentation in rainbow trout, but not the molecule gene itself associated to the ROE in this study. The list of ROE located at previously reported in body pigmentation research in this study begins in body pigmentation information Table 4.

1378 **Captions of the Figures**

1379

1380 **Fig. 1:** RDA triplots for canonical axes 1 and 2, respectively. Pigmentation-related variables
1381 retained by forward modelling are represented by arrows (see Table 1 for abbreviations).
1382 Length of arrows is proportional to the strength of correlation of each variable with
1383 individual axis. Arrows pointing to different direction indicate negatively correlated
1384 variables, and distinct phenotypes. The percentage of variance associated to each axis is
1385 reported, as well as the eigenvalue graph for constrained axis. **(A):** Individual trout are
1386 positioned on the map with positioning of SNPs as a grey block to the centre of the factor
1387 map. Individuals are coloured according to nine ancestry groups (Leitwein et al 2018). *G*
1388 and *M* indicates released hatchery fish caught in the Gravezon ($n = 6$) and Mare ($n = 1$)
1389 rivers, that effectively clustered with Atlantic and Mediterranean hatchery fish, respectively.
1390 **(B):** Zoom on the centre of the map to illustrate the position of SNPs. SNPs ± 2.5 S.D. either
1391 on RDA axis 1 or axis 2 are coloured by the pigmentation-related variable they are the most
1392 associated with in the model, while SNPs in grey are within ± 2.5 S.D. and are not considered
1393 in the study. Percentages of variation explained by each RDA axis are reported.

1394

1395 **Figure 2:** Manhattan plots of SNPs found associated to pigmentation variables in GWAS
1396 models considered in this study. **(A):** Results for the multi-trait GWAS model with two
1397 variables (*N. PN.Tot* and *N.PR.Tot*). SNPs found significant in this model are represented by
1398 circles ($n = 7$ CPLs). Results from single-trait GWAS have been added to this panel to
1399 provide a full summary of results for the *N. PN.Tot* (17 associated CPLs; black arrows) and
1400 *N.PR.Tot*. (9 associated CPLs; red arrows) variables. An asterisk indicates two loci to close

1401 to each other to be indicated by distinct arrows. The CPL associated to the *GJD2* gene on
1402 LG9 also detected by the RDA is indicated by a longer arrow. **(B):** Results for the multi-trait
1403 GWAS based on the variables retained with the RDA, except *Zeb* (31 candidate SNPs). The
1404 significance of SNPs with each variable of the model is indicated by a different symbol. The
1405 full list of candidate SNPs detected using single and multi-trait GWAS are reported in Suppl.
1406 Mat. Table S4. Variables are labelled as in Table 1. The y -axis reports values of $-\log_{10}(P)$,
1407 with P being the probability of one association between a variable and a SNP. The red line
1408 indicates the 5×10^{-8} significance threshold retained in this study. Details in text.

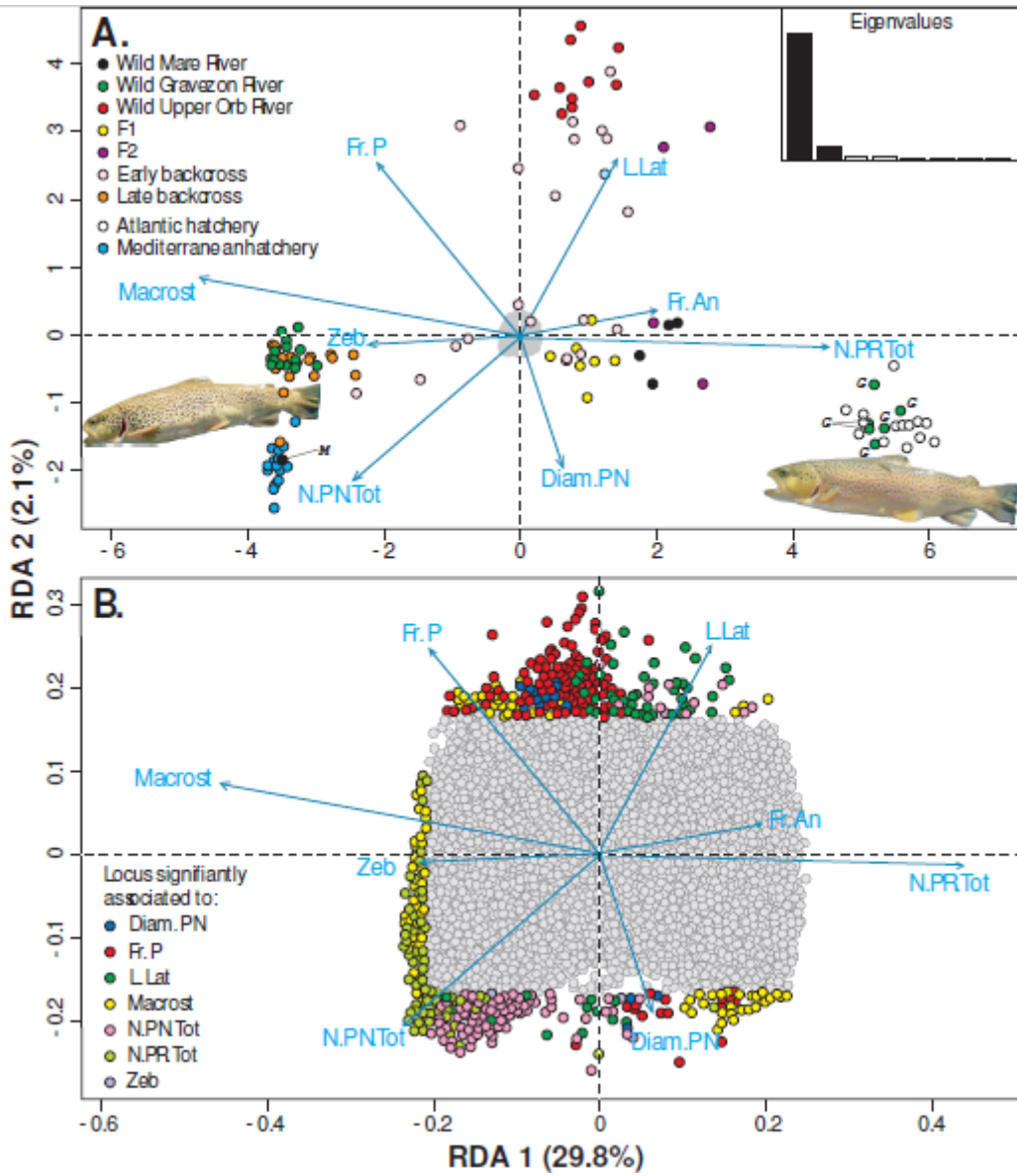
1409

1410 **Figure 3:** Distribution of F_{ST} values for the 40,519 RAD-loci (blue) and for CPLs reported
1411 in this study. The insert at the top right reports detail for CPLs associated to the first axis of
1412 the RDA. For each data set, mean F_{ST} values and confidence intervals are provided as full
1413 and hatched lines, respectively. Mean values for the single- and multi-trait GWAS
1414 approaches are illustrated by the green and the orange lines, respectively. No confidence
1415 intervals are reported for GWAS because of the low number of CPLs detected with these
1416 approaches. Details in text.

1417

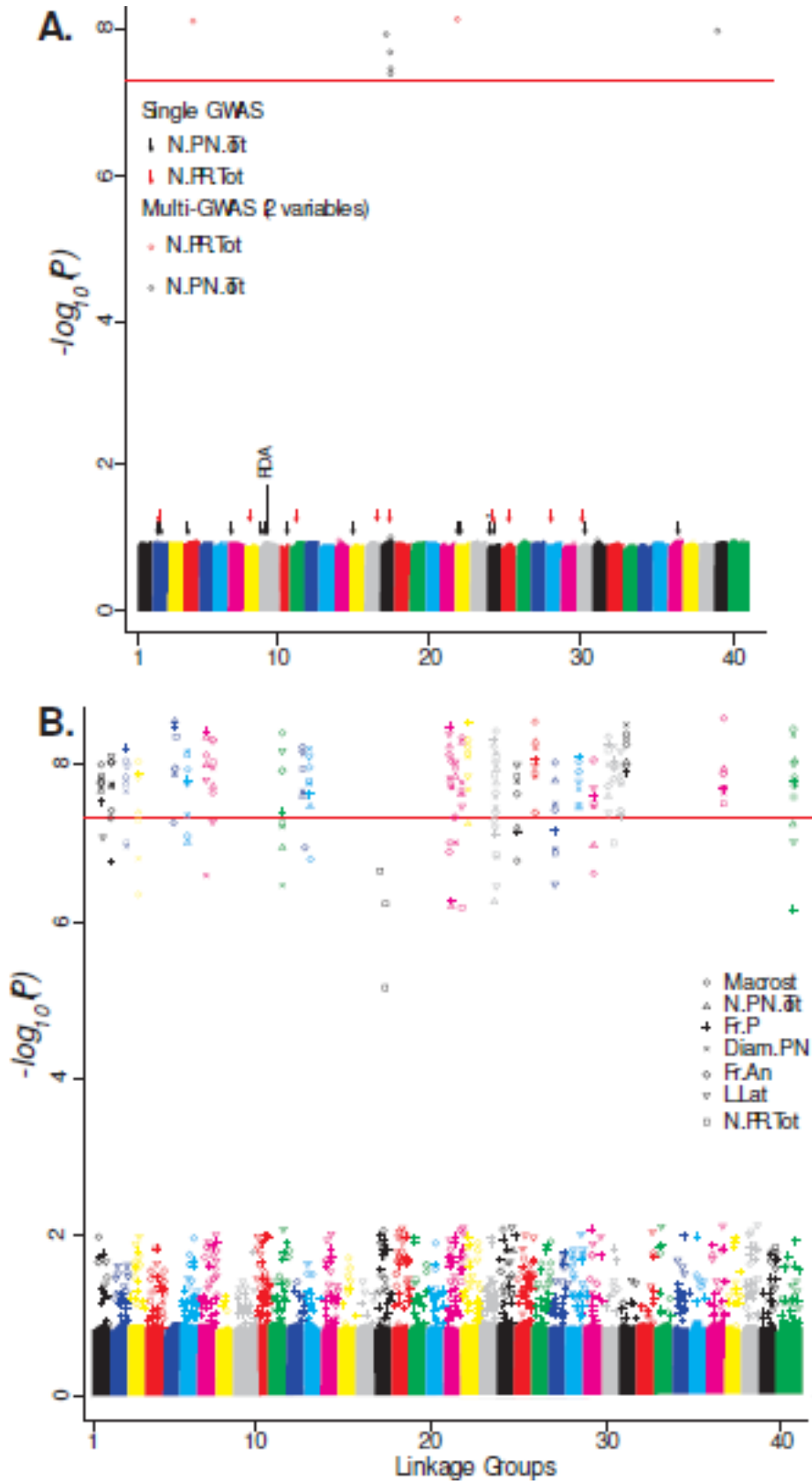
1418 **Figure 4:** Positioning of the 300 mapped RAD-loci CPLs detected in this study on the forty LGs of the
1419 brown trout. Loci found associated to at least one single phenotypic trait are indicated by green, red
1420 and yellow circles when detected with the RDA, single- or multi-GWAS analyses, respectively.
1421 Horizontal bars indicate the distribution of the full set of SNPs over LGs. The number of CPLs
1422 detected on each LG is indicated. As numerous loci are close to each other, symbols may overlap.
1423 Details in text.

1424 Figure 1



1425

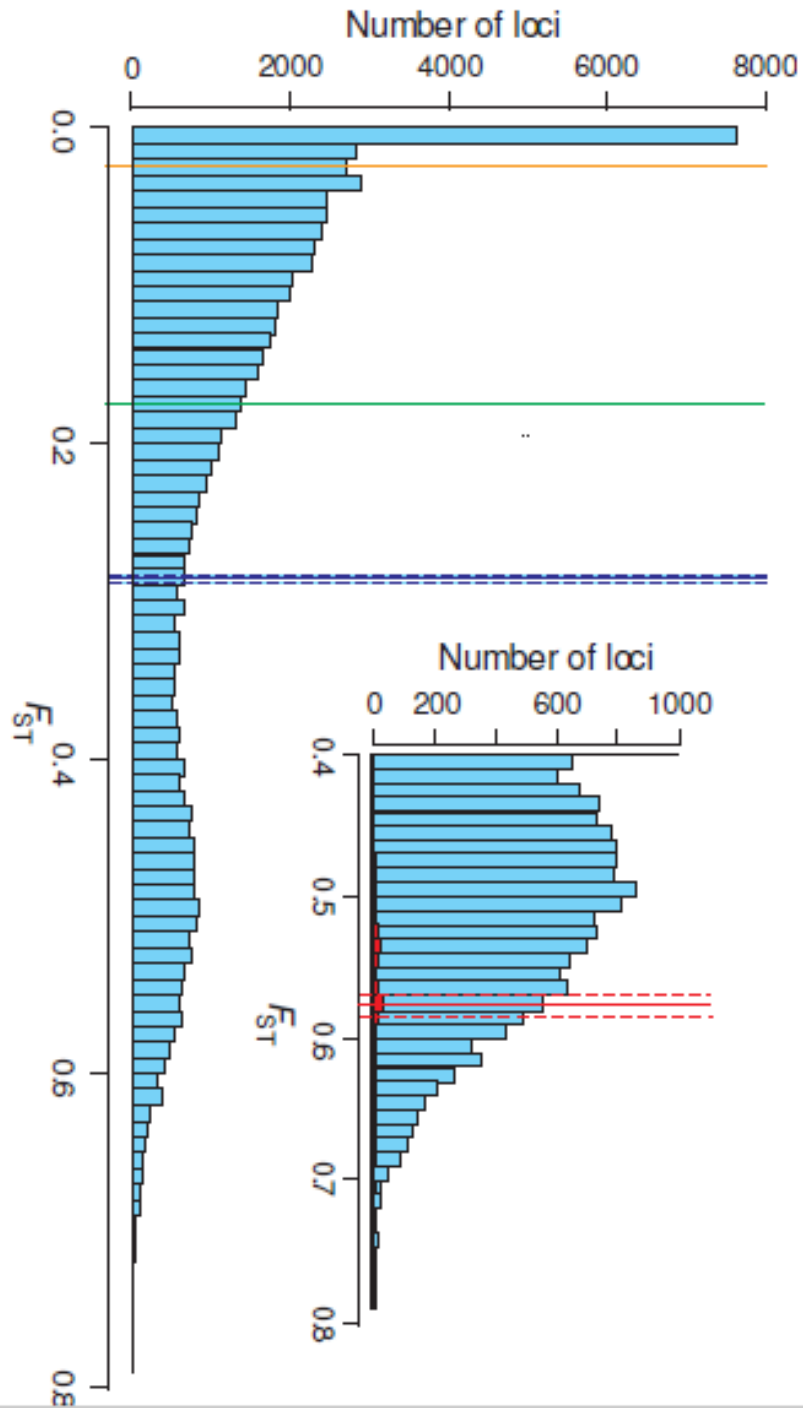
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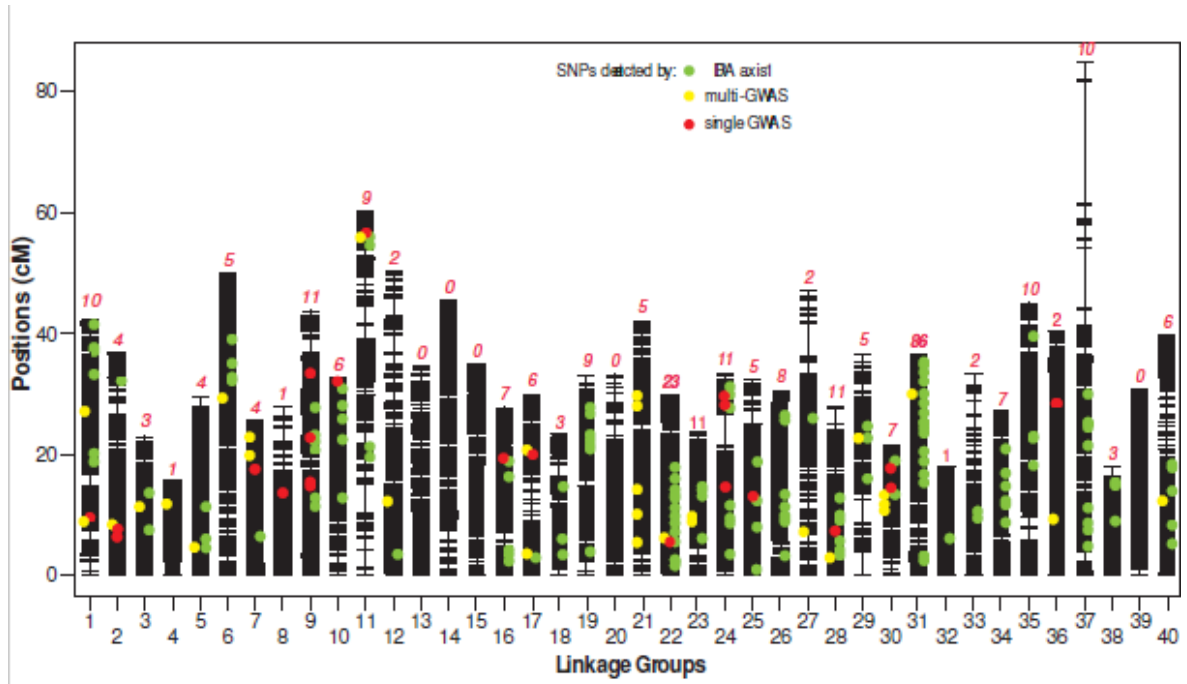
1430 Figure 3



1431

1432

1433 Figure 4



1434