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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

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

**Théo VALETTE^{1#}, Maëva LEITWEIN ^{1,4#}, Jean-Marc LASCAUX², Erick DESMARAIS¹,
Patrick BERREBI³ & Bruno GUINAND¹**

¹ : UMR ISEM - CNRS IRD UM EPHE – Montpellier, France

² : ECOGEA – Muret, France

³ : GENOME – RECHERCHE ET DIAGNOSTIC – Saint Just, France

⁴ : present address : UMR MARBEC - UM, Ifremer, CNRS, IRD - Palavas-les-Flots, France

Correspondence:

Bruno GUINAND

UMR ISEM – UM CNRS IRD EPHE – Place E. Bataillon, cc63, 34095 Montpellier Cedex 05,
France

Email: bruno.guinand@umontpellier.fr

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: TV and ML could be considered as joint first author

ABSTRACT

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

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

1— INTRODUCTION

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

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

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

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

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

2 - MATERIALS AND METHODS

2.1 - BROWN TROUT'S GENOMICS DATA

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

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

2.2 - PHENOTYPIC DATA

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

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

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

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

2.3 - GENOTYPE-PHENOTYPE ASSOCIATION

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

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

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

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

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

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

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

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

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

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

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

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

2.4 - MAPPING AND ANNOTATION

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

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

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

2.5 - GENOMIC DIFFERENTIATION

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

3 - RESULTS

3.1 - PIGMENTATION VARIABLES

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

3.2 - REDUNDANCY ANALYSIS

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

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

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

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

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

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

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

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

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

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

3.3 - SINGLE- AND MULTI-TRAIT GWAS

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

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

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

3.4 - GENOMIC DIFFERENTIATION

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

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

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

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

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

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

3.6 - GO TERMS

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

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

4 - DISCUSSION

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

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

4.1 - RDA: A POWERFUL GENOTYPE-PHENOTYPE ASSOCIATION METHOD

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

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

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

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

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

4.2 - RDA IS NOT ENOUGH

RDA, however, cannot be promoted unequivocally for several reasons. Indeed, Forester et al. (2018) showed that RDA suffered less false positives in GEA, but this however does not mean it is not affected. For example, linkage disequilibrium (LD) that promotes marker inheritance in a block-like manner impacts the loading scores in PCA, and RDA suffers the same bias (Lotterhos, 2019). If the 'true' CPL involved in the genotype-association is effectively present, linked loci might be identified as false positives. For example, 86 CPLs have been identified onto LG31 in this study ($n = 85$ by RDA; Fig. 4), LG31 effectively contains relevant CPLs (Table 3), but 40 of 86 were not associated to any genes and false positives are likely. Marker density has to be increased to reach better conclusion on the nature of association. For example, Wang S. et al. (2020) showed that a window $<0.5\text{Mb}$ was necessary to detect the most likely variant involved in pigmentation variation in North American warblers, one intronic variant effectively close to pigmentation genes. This threshold is not reach in this study. Another source related to the detection of false associations by RDA is the use of a ± 2.5 SD significance threshold to retain SNPs as CPLs. In this case, one SNP associated to *Mitf1* may represent a false negative, ignored by RDA despite its biological importance in fish pigmentation (e.g., Wang T. et al., 2020). Methods aiming at capturing allele frequency variation differently than RDA or F_{ST} should possibly be considered to correct for false positive/negative loci (e.g., Field et al., 2016; Chen and Narum, 2021), but are not under the scope of this study.

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

4.3 - A POLYGENIC ARCHITECTURE OF PIGMENTATION PATTERNS

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

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

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

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

4.4— DYNAMICS OF PIGMENTATION VARIATION AND MANAGEMENT

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

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

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

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

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

5 - CONCLUSIONS

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

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729 TV, ML, and BG analysed data and drafted early version the paper; all authors contributed
730 to later versions. TV and BG performed literature search and curation. ML, PB, ED, and BG
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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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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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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[illegible]

Captions of the Figures

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

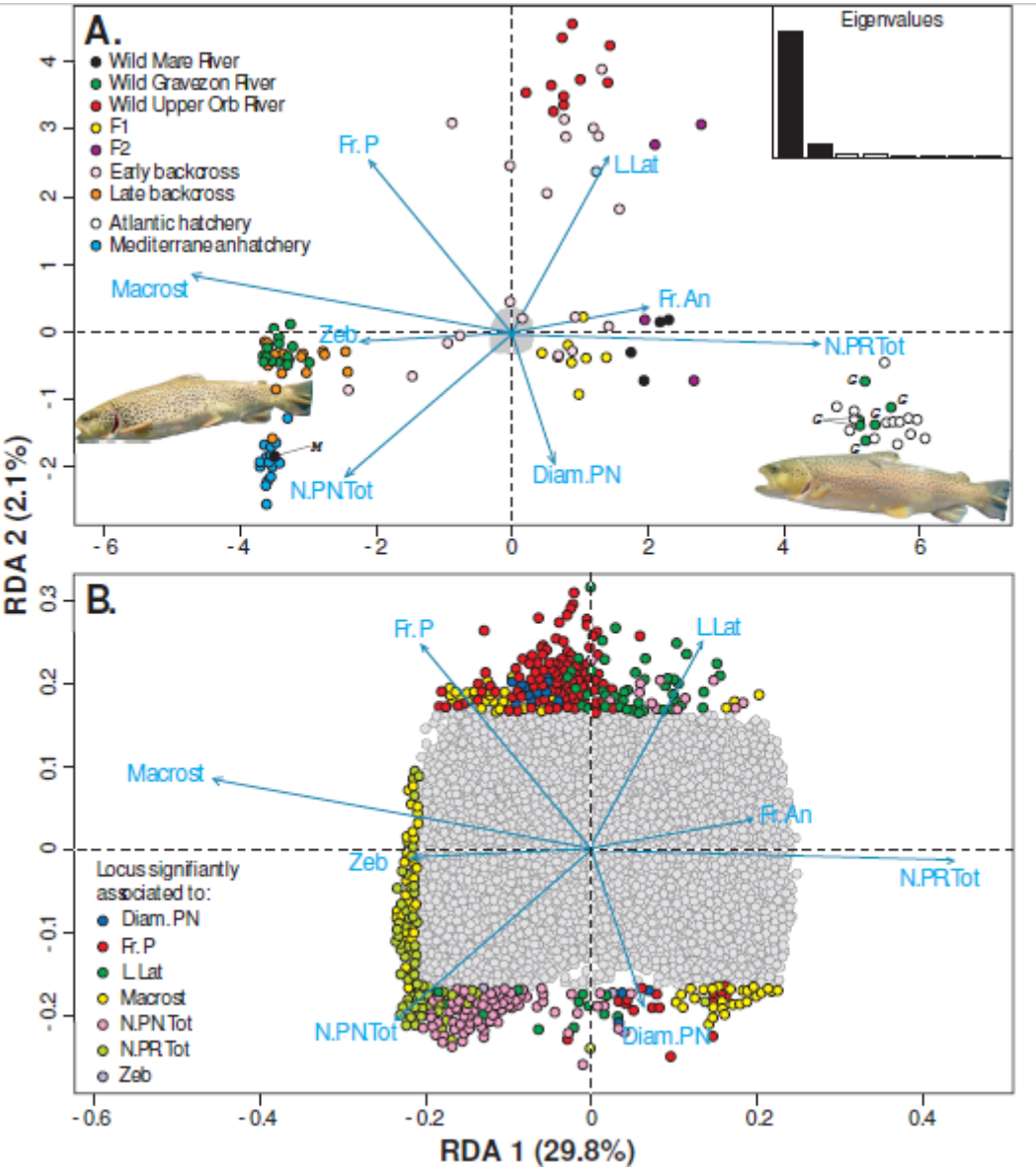
Figure 2: Manhattan plots of SNPs found associated to pigmentation variables in GWAS models considered in this study. **(A):** Results for the multi-trait GWAS model with two variables (*N. PN.Tot* and *N.PR.Tot*). SNPs found significant in this model are represented by circles ($n = 7$ CPLs). Results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

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

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

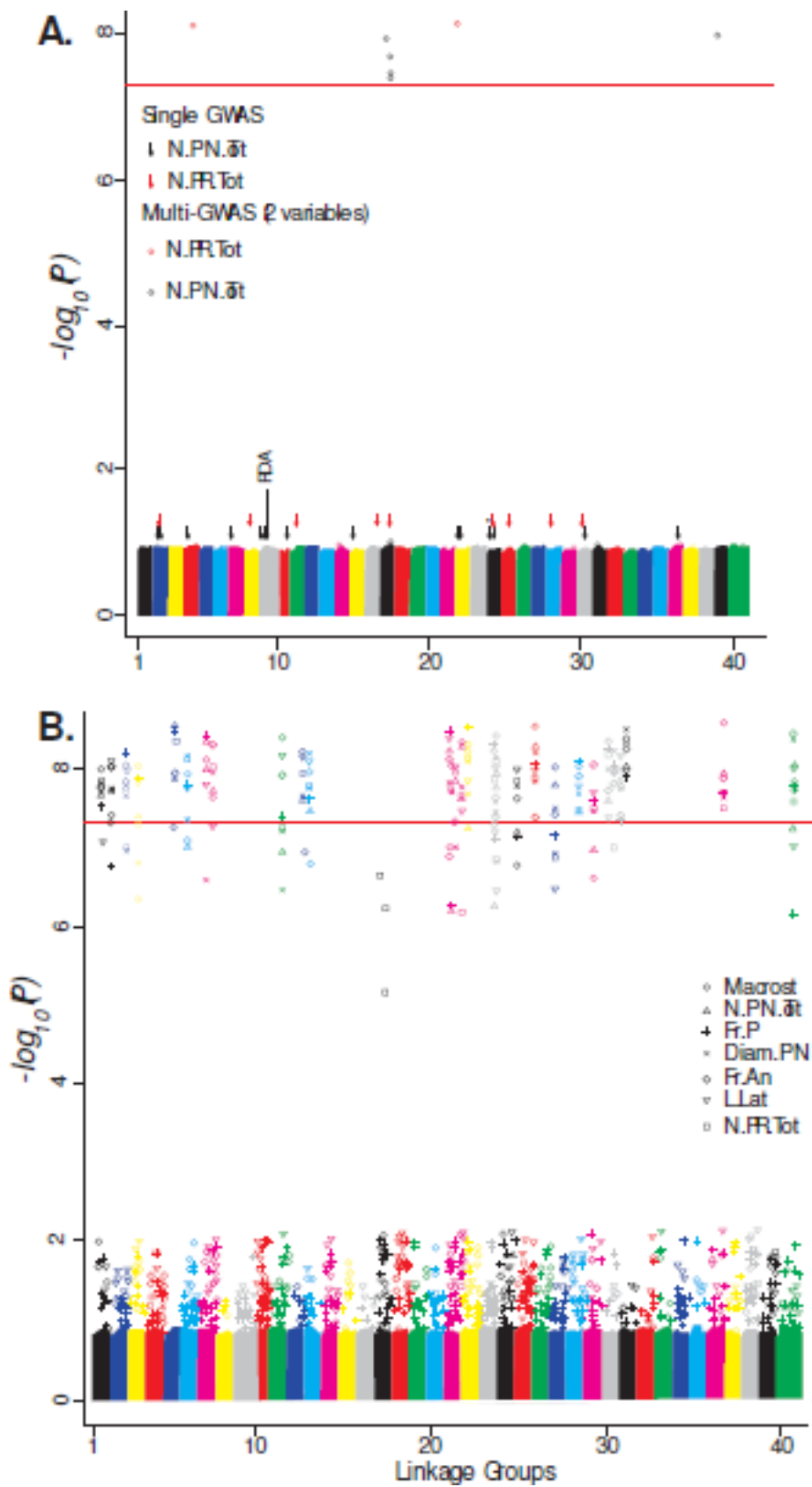
Figure 4: Positioning of the 300 mapped RAD-loci CPLs detected in this study on the forty LGs of the brown trout. Loci found associated to at least one single phenotypic trait are indicated by green, red and yellow circles when detected with the RDA, single- or multi-GWAS analyses, respectively. Horizontal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

1424 Figure 1

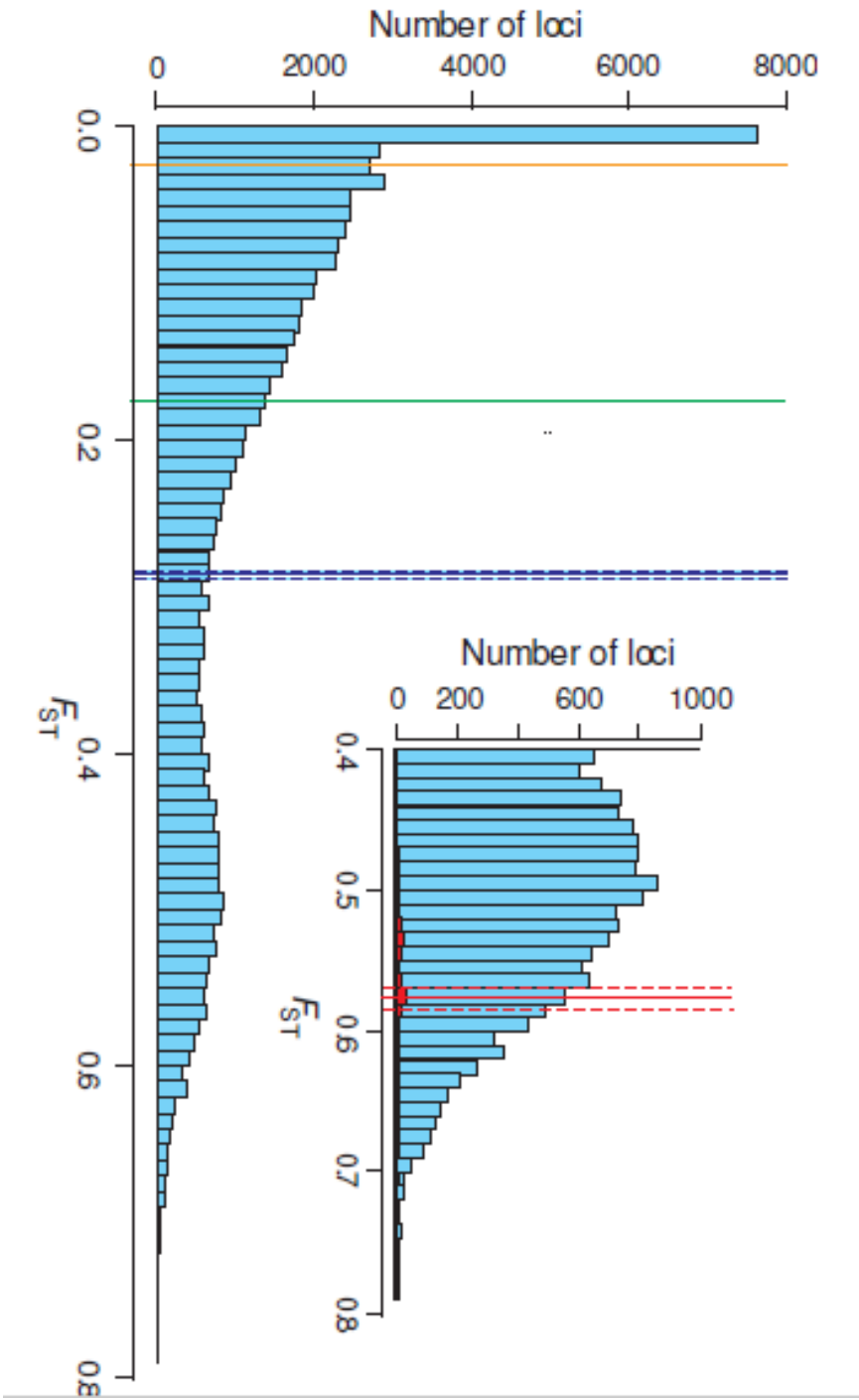


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1430 Figure 3



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