Tissue-specific metabolic enzyme levels covary with whole-animal metabolic rates and life-history loci via epistatic effects

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Summary

Metabolic rates, including standard (SMR) and maximum (MMR) metabolic rate have often been linked with life-history strategies. Variation in context- and tissue-level metabolism underlying SMR and MMR may thus provide a physiological basis for life-history variation. This raises a hypothesis that tissue-specific metabolism covaries with whole-animal metabolic rates and is genetically linked to life history. In Atlantic salmon (*Salmo salar*), variation in two loci, *vgll3* and *six6*, affects life history via age-at-maturity as well as MMR. Here, using individuals with known SMR and MMR with different *vgll3* and *six6* genotype combinations, we measured proxies of mitochondrial density and anaerobic metabolism, i.e., maximal activities of the mitochondrial citrate synthase (CS) and lactate dehydrogenase (LDH) enzymes, in four tissues (heart, intestine, liver, white muscle) across low- and high-food regimes. We found enzymatic activities were related to metabolic rates, mainly SMR, in the intestine and heart. Individual loci were not associated with the enzymatic activities,

but we found epistatic effects and genotype-by-environment interactions in CS activity in the heart and epistasis in LDH activity in the intestine. These effects suggest that mitochondrial density and anaerobic capacity in the heart and intestine may partly mediate variation in metabolic rates and life history via age-at-maturity.

Keywords: glycolysis, mitochondria, food restriction, salmonid, energy allocation, epistasis

Introduction

Species and populations can show substantial and adaptive variation in whole-animal metabolic rate [1–3], which can be an important target for selection, as metabolism affects the allocation of energy to fitness-related traits, potentially constraining life-history evolution [4]. Understanding the evolutionary origins of variation in metabolic rates, such as standard/basal (SMR/BMR) and maximum/active (MMR) metabolic rates is, however, challenging, due to the complexity of underlying mechanisms and plasticity in them [3,5–7]. For example, cellular metabolism exhibits temporal and spatial compartmentalisation in different cell types and tissues during development, in different environmental conditions, and across life stages [8], leading to differences in energy allocation and functions among tissues, and subsequently, ecology of the species. In principle, the contribution of different tissues on metabolic rate varies between resting and active conditions: organs that explain a relatively large proportion at rest include, for example, the heart, intestine, brain, and liver, while during exercise, muscles and heart increase their relative contribution above others [5]. Moreover, the extrinsic environment and food availability modulate how metabolic rates affect fitness-related traits, but the underlying (e.g., tissue-specific) mechanisms that determine plastic metabolic responses to food availability are not well established [9,10]. Therefore, insight into the context-dependence and cellular underpinnings of metabolism is central for understanding how variation in metabolic rates is maintained.

The functioning of the mitochondrial citric acid cycle and electron transport chain have received attention as potential mechanisms explaining (adaptive) variation in both SMR and MMR, given that metabolic rates are often measured as O_2 uptake, and the large majority of O_2 is used in electron transport chain in mitochondria [3,5,13–15]. Citrate synthase (CS), the first enzyme of the mitochondrial citric acid cycle, provides a useful proxy of mitochondrial density (i.e., aerobic activity and capacity) of the tissue [16]. Previous studies have found correlations between CS activity and aerobic performance [17,18], but not between CS activity and SMR [13,18]. Yet, it is not well known to what extent these associations are tissue specific. On the other hand, in anaerobic conditions, such as high-intensity exercise, lactate dehydrogenase

(LDH), found in almost all tissues, enables continued glycolysis and ATP production. I Its activity therefore reflects the anaerobic capacity of the tissue, but the association between anaerobic capacity and aerobic metabolic rates (SMR/MMR) has rarely been tested. A link between anaerobic capacity and whole-animal metabolism can, however, be hypothesized. It may be that the individuals having high MMR also have high anaerobic capacity for ATP production via LDH, which could enable faster recovery after exercise. However, it needs to be noted that recovery via LDH activity after exercise would depend on the availability of other intracellular carbon sources and can happen with a long delay, suggesting it may not be a strong association (e.g., [19–21]). Lactate is also produced routinely and either metabolized at site or shuttled between tissues to balance glucose supply and demand [22], which suggests that LDH also supports basal metabolism, and may thus influence SMR.

The advantages of studying CS and LDH activities as proxies of mitochondrial content (and therefore aerobic capacity) and anaerobic capacity, respectively, are that their maximal activities can be measured in high throughput from frozen tissue samples across tissues with different functional roles. For example, glycolysis fuels fast-twitch (white) muscle anaerobically during burst swimming [23], while cardiac muscle typically has high capacity for both anaerobic and aerobic metabolism to maintain energy supply during routine function [24–28]. Although metabolic enzymes can be produced in excess in relation to performance needs [29], maintaining high mitochondrial content and anaerobic capacity is costly, thus among-individual variation in tissue-specific metabolic activities reflects differences in energy allocation [30]. As such, allocation of energy into aerobic and anaerobic metabolism in tissues may contribute to life history via intermediate traits, e.g., by changing how nutrients can be acquired, absorbed or assimilated, and how somatic growth, maintenance, and reproductive functions are supported [31]. A greater understanding of the shared genetic basis of tissue-level metabolic activity and life-history variation may help to explain how ecological factors, such as nutrient availability, shape life-history strategies.

Atlantic salmon (*Salmo salar*) is an ideal species for testing the associations between adaptive life-history variation and metabolism at different levels of organisation. Two loci in Atlantic salmon, so-called *vestigial-like family member 3*

(*vgll3)* and *SIX homeobox 6* (*six6)* after the most prominent genes located in these genomic regions, explain a substantial part of variation in a key life-history trait, age-at-maturity [32–36]; their two alleles are named early (E) and late (L) after their associations with age-at-maturity. *Vgll3* is also associated with other life-history traits, such as number of reproduction events and early (precocious) maturation of male salmon [37,38]. Interestingly, the loci have also been linked to whole-animal metabolic rates as they exhibit physiological epistasis that explains *ca.* 5% of variation in MMR; in juvenile salmon, individuals that carry homozygous late-maturation genotypes in both loci (i.e., $vgll3_{LL}$ and $six6_{LL}$) have a lower MMR compared to the additive expectations of both genotypes [39], but there is no difference in SMR between the early and late maturation genotypes [39,40]. Based on their expression patterns, v*gll3* and *six6* may exert their effects on phenotypes for example via cell fate commitment [41,42], but it is not known which mechanisms induce the effects on MMR, or if the loci exert other tissue-specific effects on metabolism that could influence the timing of maturation. Therefore, Atlantic salmon provides a compelling system to study the genetic associations between life history and metabolism, and to explore tissue-specific allocation of metabolic activity in relation to whole-animal metabolism.

The aims of this study were threefold: firstly, to better understand the link between tissue level and organismal metabolic rates we explored if tissue-specific CS and LDH activities explained among-individual variation in metabolic rates, measured as oxygen uptake, across high and low food availability conditions. Secondly, we determined if variation in tissue-specific metabolism via CS and LDH activities is associated with adaptive life-history variation (at loci *six6* and *vgll3*) in Atlantic salmon. Thirdly, we assessed the potential role of genotype-by-environment interactions (GxE) in mediating the effects of the life history genotypes on metabolic functions in tissues. We hypothesised that: i) mitochondrial density, i.e., CS activity, is positively associated with metabolic rates due to higher capacity for oxidation, ii) higher LDH activity in the heart increases post-exercise MMR, and iii) the life-history genotypes show corresponding effects on CS and LDH activities as observed in MMR, including physiological epistasis and reduced activities in fish carrying late maturation genotypes [39], and finally, iv) that food limitation decreases the activities of the enzymes to reduce energy expenditure [43–45], and we tested if this occurs in a

genotype-dependent manner. GxE and epistatic effects are common in metabolic enzymes and in processes affecting metabolic rate, which was a further basis for testing these effects (e.g., [46–48]).

The four tissues included in this study (heart, liver, intestine, and white muscle) were selected based on their relevance for organismal energy metabolism. The cardiac muscle is central for delivering oxygen to tissues, and its mitochondrial density and anaerobic capacity often reflect cardiac performance [25–27], contributing to endurance swimming and coping with stressors (e.g., [49]). White muscle is an energy storage and is responsible for fast (burst) swimming, which is fuelled by anaerobic metabolism [23], and its mitochondrial content has been linked with MMR in brown trout (*Salmo trutta*) [15]. The liver is a detoxifying and metabolic organ also functioning as a glycogen storage, and its metabolic markers and efficiency of mitochondrial respiration are associated with SMR and growth in brown trout [13,15,50]. Finally, mitochondrial density and lactate synthesis in the intestine may influence growth rate [51] by fuelling metabolism in other tissues [22] and is therefore relevant with respect to SMR, even though this association has not been tested previously to our knowledge. The anaerobic capacity of the intestine also contributes to osmoregulation, e.g., in anadromous salmonids that must cope with a change in salinity between life stages [52]. By assessing proxies of mitochondrial density and anaerobic capacity across these four tissues our aim was to identify which of them are possibly mediating the effects of *vgll3* and *six6* on age-at-maturity and metabolism at high and low food availability.

Material and Methods

Experimental design

The experiments were conducted under a permit granted by the Finnish Project Authorisation Board (permit no. ESAVI/4511/2020). The details of the rearing of fish, the experimental design and the metabolic rate measurements are described elsewhere [39]. Briefly, we crossed four full-sib families from parents carrying heterozygous genotypes for *vgll3* and *six6*, originating from River Kymijoki broodstock maintained by Natural Resources Institute Finland (Luke) at the Laukaa

fish hatchery, Finland. The progeny thus had all *six6*-*vgll3* genotype combinations within a family, enabling quantification of the genotype effects among full-sib individuals with similar genetic backgrounds. The eggs were incubated at Viikki campus at the University of Helsinki (7°C) and the alevins were transferred to Lammi Biological Station, after which temperature followed the temperature of incoming lake water [39]. Individuals were reared in circular tanks, each family in a separate tank, and fed to satiation (feed Vita, Veronesi) using automatic feeders. In July 2020, individuals were tagged with 8 mm passive integrated transponder (PIT) tags, fin clipped, and their genotypes of *vgll3*, *six6*, and the genetic sex (using the *sdy* locus [53]) were identified using competitive allele specific PCR (KASP) assays [39]. Individuals carrying homozygous genotypes for early or late maturation in *vgll3* and *six6* ($vgll3_{EE}$ or $vgll3_{LL}$, and $six6_{EE}$ or $six6_{LL}$) in different combinations were then selected for the experiment from each family.

Approximately eight months after hatching, in August 2020, when individuals were at parr (juvenile) stage, they were divided into two treatments: high and low food availability. Fish in the high food treatment were fed to satiation daily using automatic feeders, and fish in the low food treatment were fed a whole daily ration of food (on average 2.6% of fish mass) every three days during a period of two hours.

Measurements of metabolic rates

After four weeks in each treatment, we placed a 16 individuals / day (representing an equal mix of all genotypes from the same family, the individuals of which were identified using their PIT-tags) individually in separated enclosures at 11°C, which corresponded to the incoming lake water temperature at the beginning of measurements. After two days of acclimation without feeding, we measured the SMR and MMR of each individual at 11°C, as part of another study (details in Online Supplemental Information of [39]). The SMR of each individual was measured during approximately 20h in the dark. The measurement was conducted with intermittent flow respirometry using cycles of 5 min flush, 2 min mixing period, and 13 min measurement period, of which 12 min were included in analysis. SMR data were filtered to retain only linear measurement periods, and fish-specific SMR calculated with the median of the lowest normal distribution (MLND) method [54]. The data

collected with this method represents SMR, since it retains data only from the periods of lowest metabolic rate during the trial.

The MMR of each fish was measured after SMR by chasing the fish for 3 min (at which point fish all fish had become exhausted) and measuring post-exercise O_2 uptake immediately after the chase. No air exposure was used. The slope of O_2 uptake related to MMR was calculated from the beginning of the measurement, excluding the first 20s, using the so-called spline method. This method fits a polynomial curve on each MMR slope with 10 degrees of freedom and extracts the slope from the beginning of the curve. Both SMR and MMR slopes were analysed with the package *FishResp* [55] in R. Background respiration was measured daily using empty chambers and found to be negligible, as the respirometers were cleaned every 5 d with bleach.

After MMR measurement, individuals were placed into 10-L buckets with aeration at 11°C until they were euthanized, and their tissues sampled (*ca*. 2 – 4 h after the completion of the MMR measurements). The fish were euthanized with an overdose of MS-222 (250 mg/L, sodium bicarbonate buffered), and immediately measured for fork length (to 1 mm) and weighed (to 0.1 g). A gill arch was cut to remove excess blood from the tissues, and samples collected from the liver, ventricle (hereafter heart), pyloric caeca (hereafter intestine) and a sample of the epaxial white muscle between the dorsal fin and the tail fin (hereafter muscle). The intestine samples were rinsed and cut up in phosphate-buffered saline to remove food residues. Samples were snap-frozen in liquid nitrogen and stored at –70°C until processing at the Viikki campus during August 2021 – July 2022. Each enzymatic assay per tissue was completed within a few weeks.

Enzymatic assays

The maximal enzymatic activities of CS and LDH, reflecting the abundance of each enzyme in the sampled tissue, were measured in tissue homogenates following protocols from [56,57] (for details see Online Supplementary Material and Table S1). Measurements were conducted at 25°C, which is higher than the fish rearing temperature, but activities are expected to increase consistently with temperature

(e.g., [58]), and temperatures at or above 20°C are often implemented when measuring maximal enzyme activities in Salmonids (e.g., [27,59]). To normalise the metabolic enzymatic activities to the total tissue mass, total protein concentrations were quantified using the Pierce BCA assay (ThermoFisher Scientific). Individuals from one family (F8) in the high food treatment were excluded from the analysis due to very low sample sizes (maximum of three individuals in each assay). In total, 96 fish from three families, and 168 fish from four families were analysed from high and low food treatments, respectively, with some variation between the assays: sample sizes within a group (i.e., per tissue, treatment, and genotype combination) ranged from 14 to 39 for LDH and from 17 to 42 for CS (Table S2).

Statistical analyses

The data were analysed in R v. 4.2.2 [60] and visualised using *ggplot2* v. 3.3.6 [61] and *interactions* [62] packages. Family and treatment replicates (four families in the low food treatment, three families in the high food treatment, all in different tanks) were accounted for in the models by including them as an interacting random effect (with seven levels), which is also confounded by the tank effects. This removed any random variation that is due to either family or tank effects.

To normalise enzymatic activities, we first divided the total activity of each enzyme in the sample with the total protein concentration, and second, used linear models with ln-transformed activity per mg protein as the response, and body mass and protein concentration, ln-transformed, as explanatory variables. This was necessary, as accurately measuring the tissue mass was not feasible in this study, and simply correcting activities with protein concentration did not account for non-linear relationships between activity, concentration, and body mass. Residuals from these models represent normalised activities of each enzyme (while the body mass associations of enzymes were tested in models 17 &18 (see below and Table S4)).

First, we tested if the tissue-specific enzymatic activities were associated with whole-animal metabolic rates using separate linear mixed models for each enzyme-by-tissue combination (models 1-16 in Table S4). Separate models had substantially higher sample sizes than the model would have had with all enzymes combined due

to the cumulative amount of independent missing observations in each assay. Before analysis, mass-normalised metabolic rates were obtained as the residuals from a linear model where the ln-transformed SMR or MMR (from [39], as mg O_2/h) was used as a response variable and ln-transformed body mass as an explanatory variable, as body mass is strongly and hypo-allometrically correlated to metabolic rates (shown in supplemental material in [39]). There was strong overlap in size distributions between the treatments (Table S3, range in body mass was 2.4 – 6.9 g in high food and 1.5 – 6.5 g in low food treatment), thus a single normalisation with body mass was used. The residual (mass normalised) SMR/MMR data were then used as response variables, and as explanatory variables we used normalised activity of CS or LDH, treatment (as a factor), and their interaction. The day of sampling (corresponding to the day of SMR and MMR measurement) and the treatment by family interaction were used as random effects (all models described in Table S4).

To assess the overall differences in enzymatic activity between tissues and treatments, we fitted linear mixed models on each of CS and LDH data (as µmol citrate (CS) or NADH (LDH)/mg protein/min) (models 17 & 18, Table S4). Here, enzyme activity was not normalised with body mass, but mass was included in the model as a covariate in interaction with tissue type, because the whole tissue was not always used in the analysis and therefore some tissues were expected to correlate more strongly with mass. Tissue and treatment (as factors), and their interaction were used as fixed effects in the model. The response and covariate were ln-transformed to meet the assumptions of the model.

The normalised activities of the enzymes were then analysed for genotype and GxE effects in separate models for each tissue (models 19-26, Table S4). Fixed effects in these models included food availability (treatment), *vgll3* and *six6* genotypes, and the pairwise interaction between the genotypes and between each genotype with treatment.

All models were fitted with maximum likelihood using the package *lme4* v. 1.1 [63]. P-values were obtained with Type III tests calculated with Satterthwaite's method (alpha = 0.05) with lmerTest v. 3.1-3 [64]. Outliers were detected by the *outlierTest* function from the package *car* [65] and removed from analysis. Overall, three and five

outliers in overall CS and LDH models, respectively, and none to two outliers in tissue-specific models were excluded from the analyses. Pairwise differences in estimated marginal means — between tissues for CS and LDH and between *vgll3* and *six6* genotypes in tissue-specific models — were tested with post hoc analysis using functions *emmeans, pairs,* and *test* from the package *emmeans* [66]. The associations between enzymatic activities and metabolic rate at each treatment level was assessed using the *joint_tests* function in *emmeans*[66], if the treatment and metabolic rate interaction was significant in the CS and LDH models. P-values for post hoc tests were adjusted with the Benjamini-Hochberg (B-H) method, where the four comparisons for *vgll3* or *six6* from each tissue-specific model formed one family of tests. Residuals of models were confirmed to be homoscedastic and normally distributed by visual inspection using package *DHARMa* [67]*.* Predicted, normalised means and confidence intervals for genotype-specific enzymatic activities were obtained with function *ggpredict* from package *ggeffects* [68]. To obtain predicted activities as µmol citrate or NADH/mg protein/min, these were added to the mean activity predicted for an average sized fish with an average protein concentration in each tissue.

Results

Associations of metabolic enzymes on organismal metabolic rates

Overall, whole-animal metabolic traits (SMR and MMR) were not associated with CS or LDH activities in the liver and muscle tissues (Table S5). CS activity in the heart and intestine was correlated with SMR, but in opposite directions at high and low food availability ($F_{1,197}$ = 5.78, p = 0.017 and $F_{1,215}$ = 4.68, p = 0.031 for the interaction effect in heart and intestine, respectively, Table S5, Fig. 1a-b); heart CS activity exhibited a negative association with SMR in the low food ($F_{1,204}$ = 3.98, p = 0.047) and a positive trend in the high food treatment ($F_{1,195}$ = 2.19, p = 0.14), and intestine CS activity was marginally and negatively associated with SMR in the high food treatment ($F_{1,216}$ = 3.50, p = 0.062) and had a positive trend in the low food treatment ($F_{1,220}$ = 1.21, p = 0.27). There was only one significant association between metabolic enzymes and MMR, whereby a positive effect of CS activity on

MMR was observed in the intestine ($F_{1,226}$ = 4.52, p = 0.03, Fig. 1c). LDH activity in the intestine had a positive effect on SMR ($F_{1,199}$ = 5.67, p = 0.02), but an interaction with food availability was not supported (Table S3, Fig. 1d). However, the above results were not adjusted for multiple testing, and should therefore be considered circumstantial.

Differences in enzymatic activities between tissues and treatments

Overall, maximal CS activity was highest in the heart, followed by the intestine, white muscle, and liver (all non-adjusted pairwise p-values <0.001, Fig. 2). CS activity was overall lower in the low food compared to the high food treatment ($F_{1,10}$ = 7.15, p = 0.022, Table S6, Fig. 2a).

LDH activity was highest in the (white) muscle, followed by heart, and finally at similar lower levels in the intestine and liver (all non-adjusted pairwise p-values <0.001 except intestine-liver 0.609, Fig. 2b). There was no overall effect of food availability on LDH ($F_{1,7}$ = 0.63, p = 0.453), but there was a significant interaction between treatment and tissue ($F_{1,840}$ = 3.5, p = 0.015, Table S6): LDH activity seemed to be lower in low food availability than high food availability in the muscle and liver but not in the intestine or heart, although none of the tissue-specific differences were significant between food regimes (Tables 2, S10-S12).

The effects of genotypes on CS activity

In the heart, CS activity was significantly affected by an interaction between the two loci (p = 0.024, Table 1). There was also a decreasing trend in CS activity with low food availability (p = 0.075), and an interaction between *six6* and food availability (p = 0.043, Table 1). $Six6_{LL}$ fish exhibited higher CS activity than the $six6_{EE}$ fish in the high food treatment when the fish also had $vgll3_{EE}$ genotype (Fig. 4). A contrasting effect was observed in the low food treatment, where CS activity was lower in the $six6_{LL}$ than $six6_{EE}$ within the $vgll3_{LL}$ genotype (Fig. 3), suggesting epistatic effects and genotype-by-environment interactions (Table 1).

In the intestine, muscle or liver, there were no significant effects by genotype or interaction between genotype and treatment on CS activity (Table S7-9, Fig S1a-c).

The effects of genotypes on LDH activity

In the intestine, there was a significant interaction effect between the loci on maximal LDH activity (Fig. 4, Table 2). *Six6*$_{LL}$ fish tended to have a higher LDH activity than *six6*$_{EE}$ fish, but only in *vgll3*$_{EE}$ fish in the high food treatment, whereas in the low food treatment and *vgll3*$_{LL}$ genotype, there was an opposite trend with *six6*. Furthermore, in the high food treatment and *six6*$_{LL}$ genotype, LDH activity was lower in the *vgll3*$_{LL}$ compared to *vgll3*$_{EE}$ genotype. A similar trend was also seen in the low food treatment, while there was an opposite trend between *vgll3* genotypes in fish with *six6*$_{EE}$ genotype (Fig. 4).

In the heart, muscle or liver, there were no significant effects by genotype or interaction between genotype and treatment (Table S10-12, Fig. S2a-c).

Discussion

The intraspecific variation in energy acquisition and allocation pathways and the energetic trade-offs between different processes (e.g., motility, reproduction, digestion) shape the life history and success of individuals across different environmental conditions [69]. Quantifying the tissue-specific energy landscape is therefore crucial to understanding variation in fitness-related traits, and such physiological investigations should be a significant part of ecological studies of wild populations [3]. In our results, mitochondrial density and anaerobic capacity (measured as maximal activities of CS and LDH respectively) of the heart and intestine tissues were associated with among-individual variation in organismal metabolic rate (SMR and MMR). Enzymatic activities in these tissues were also genetically linked to life history, particularly age-at-maturity, via *vgll3* and *six6* loci. Specifically, epistasis and genotype-by-environment interactions affected CS activity in the heart, seen as different effects of genotypes between the two food treatments (Fig. 3). Physiological epistasis also affected LDH activity in the intestine, where we found interacting effects between the loci but not with food availability (Fig. 4). These findings thus suggest digestion and circulation to be functional processes that may be genetically linked with or mediate the life-history effects of *vgll3* and *six6* loci in Atlantic salmon.

The energy used by a specific organ or tissue is a function of its mass and its 'metabolic intensity' (the amount of energy used per mass of tissue). Our experiment, which measured proxies of tissue-specific metabolic intensity (i.e., enzymatic activity per mg of protein), showed that the CS and/or LDH activities in the heart and intestine covary with metabolic rate (depending on food availability, Fig. 1). Heart and intestine are not commonly explored for their association to organismal SMR, but variation in cardiac performance explains variation in MMR [70,71]. Previous studies have found evidence that mitochondrial efficiency or cytochrome C oxidase enzyme activity in muscle and liver is correlated with variation in SMR in fish [13,15,72], yet these were not supported by our measures of mitochondrial density (CS activity). In contrast, there was a positive relationship between mitochondrial density in the heart and SMR in the high food treatment. This association between may be driven by the constant high demand for ATP in the heart [28], compared to the relatively lower demand in other tissues during the SMR measurement, when the use of energy for e.g., growth, motility and digestion is minimal. However, the negative relationship in low food conditions is surprising, and speculatively could reflect a redirection of resources via a reduction of aerobic metabolism in the heart to maintain capacity for other functions, such as digestion, which corresponds with a positive correlation between SMR and CS activity in the intestine. Further, the positive association between LDH activity and SMR could be explained by fuelling the function of the digestive tract itself [73], or by the role of LDH in producing lactate at the site of digestion to be transported to other tissues [22], where it could potentially support oxidative phosphorylation. Likewise, the association of CS activity and MMR could reflect the metabolic costs of maintaining capacity for digestion, which is logical given that metabolic rate during digestion of a large meal can approach that during exhaustive exercise [74]. In summary, despite the phenotypic correlations found between metabolic rates and enzymatic activities in the intestine and heart, overall the associations were weak for the tissues studied here.

The main result of this study is the finding of tissue-specific physiological epistasis between two life-history loci in proxies of mitochondrial density (CS activity) and anaerobic capacity (LDH activity), adding to the previously described physiological epistasis in MMR [39]. Physiological epistasis is thought to be common in wild populations [75] and helps maintain additive genetic variation of traits [76,77], including

metabolic rates [47]. Yet, the underlying biochemical and physiological mechanisms are rarely investigated in an ecological context, likely due to inherent challenges in quantifying epistasis across polygenic backgrounds [78,79]. Likely, a combination of developmental and biosynthetic pathways result in the epistatic effect between the loci, as previous studies have shown that *vgll3* and associated genes are expressed variably in the intestine (pyloric caeca) and heart of juvenile salmon [41,80] and that *six6* is expressed in several organs, including the intestine and heart, during development [42]. Associations between *vgll3* genotype and several additional traits of relevance to energetics have been reported, whereby the L allele has been associated with more aggressive behaviour [81] as well as lower body condition in juveniles in the spring but higher body condition in the autumn compared to the E allele [38,82]. Less is known about the traits linked to variation in *six6*, but it is a strong candidate locus for local adaptation [83] and one previous study on wild salmon found that $six6_{LL}$ individuals feed less frequently than $six6_{EE}$ individuals at sea, while there was no significant association with *vgll3* [84]. In summary, studies thus far indicate that the loci have multiple either independent or interacting effects on the phenotype, but the fitness consequences of the interactions are still unknown.

Currently, our results provide the first opportunity to develop hypotheses related to the ecological consequences of the genotype-dependent variation in CS and LDH activities. Firstly, CS activity in the heart usually follows cardiac performance, that is, capacity for sustained aerobic activities [27,85]. Thus, due to the epistatic effect and GxE in CS activity, it can be hypothesized that selection on performance can be a possible mechanism for maintaining genetic diversity in *vgll3* and *six6,* depending on the environment. On the other hand, if a higher mitochondrial density leads to higher cardiac output, this could provide a survival/fitness advantage for the $six6_{LL}$-$vgll3_{EE}$ individuals in some conditions, or on the other hand, limit the performance of $six6_{LL}$-$vgll3_{LL}$ individuals. Since the genotype effects on CS activity at low food conditions were nearly parallel to their effects on MMR [39], a possible scenario is that the genotypes affect MMR partly via mitochondrial density in the heart, which limits the aerobic scope of the $six6_{LL}$-$vgll3_{LL}$ individuals. This may lead to slower resource acquisition, thus delaying maturation. Interestingly, a somewhat similar epistatic effect was observed as delayed maturation of Atlantic salmon males with $six6_{LL}$-$vgll3_{LL}$ genotypes from historical samples [36]. The lack of such an effect in

contemporary samples may have been explained by intensified environmental effects on maturation (e.g., higher water temperature), and similarly, our results indicate that epistatic effects between the loci can depend on the environment.

Secondly, increased LDH activity (i.e., anaerobic capacity) in the intestine of, for example, fish with *six6*$_{LL}$ - *vgll3*$_{EE}$ genotype, could improve fish growth [51] or the ability for osmoregulation when entering the marine environment [52], if the difference is consistent in salmon at sea. However, if variation in *vgll3* and *six6* shapes the function of tissues via CS and LDH content, it appears to not affect SMR [39,40], thus suggesting a mechanism that is not related to maintaining growth potential. Previously, digestion and feeding have been linked to ecotype differences in salmonids; in rainbow trout (*Oncorhynchus mykiss*), fast-growing piscivorous ecotypes maintain an active digestive tract and higher assimilation efficiency than slow-growing insectivorous ecotypes [86]. Thus, another important next step would be to test whether variation in *vgll3* and *six6* is related to costs or assimilation efficiency of digestion in salmon. On the other hand, it is not clear whether the associations between metabolic enzymes and the genomic regions are adaptive or causally linked to life-history variation. Both loci are likely to have broad pleiotropic effects in different tissues, and across life-stages, making causal inferences challenging. Despite these challenges, Atlantic salmon is emerging as an intriguing model system for understanding physiological epistasis and the dynamics of how trait variation is maintained in the wild.

Overall, the patterns of CS and LDH activities are consistent with our expectations given the role of the tissues in ecology of salmon, e.g., high LDH activity in white muscle, high CS activity in the heart, low activities in the liver [24,27]. Considering the small effect sizes from the genotypes and the large among-individual variation in metabolic processes, as also shown here, the genotype effects would not be feasible to detect without a balanced experimental design with a large sample size (still uncommon in physiological investigations). On the other hand, food availability had a limited and no effect on CS and LDH activities, respectively, even with a relatively high statistical power, which is consistent with a lack of an effect on SMR and MMR in [39]. If the fish had required an energy saving strategy to cope with food availability, we would have expected a stronger decrease in the enzymatic activities across

tissues. However, the intermittent feeding regime could favour the maintenance of metabolic capacity in tissues to maximise food intake and absorption when feeding occurred, rather than an energy saving strategy which could have been favoured under constant, low food availability. Alternatively, the weak response of CS and lacking response of LDH activity to food availability, may indicate that salmon juveniles have limited potential for phenotypic plasticity in enzymatic activity and in metabolic rates [39] in response to food availability, even if such strategy may be beneficial [87]. Disentangling these mechanisms would require testing the limits and benefits of plasticity under different types of food restriction treatments.

In line with previous studies associating *vgll3* and *six6* with growth and energy metabolism proxies in juvenile salmon [34,38,39,82] and evolution of body size in wild salmon [88], our findings contribute to the emerging view that these loci can shape resource allocation and acquisition. Our results posit the intestine and the heart as tissues in which energy metabolism is fine-tuned by adaptive genetic variation in these loci via physiological epistasis. These genetic effects do not, however, correspond with changes in SMR, and only partly in MMR. In addition, we demonstrated phenotypic correlations between organismal metabolic rate and metabolic intensity, particularly in the intestine. These results, while providing only a glimpse into the environmental and genetic underpinnings of metabolic rate in animals, suggest that large-effect loci in salmon can interact with resource availability at the tissue level, and may therefore mediate allocation trade-offs between different and potentially competing functions. Deciphering the links between cellular mechanisms and adaptation remains an exciting challenge, requiring comparative molecular approaches that account for genetic variation in both loci.

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Data availability

The compiled datasets for enzyme activities with R codes for mixed models are available in Zenodo ([89], https://doi.org/10.5281/zenodo.8014314). The raw data and R codes for data processing are available in another repository ([90], https://doi.org/10.5281/zenodo.10209401).

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Table 1. The results of the linear mixed model with Type III test for CS activity in the heart. CS activity (in µmol citrate/mg protein/min) was ln-transformed and normalised by ln(body mass) and ln(protein concentration) before the analysis. Intercept is shown with t-test value. Significant p-values shown with asterisks. See Table S7-9 for the results of other tissues. Estimates show high vs. low food (Treatment) or EE vs. LL genotype (*vgll3* and *six6*).

Fixed effect	**Estimate**	**95% C.I.**	**SSq**	**Den df**	**F**	**p**
Intercept	0.002	-0.081, 0.085		23	0.05	0.96
Treatment	-0.033	-0.14, 0.073	0.118	7	4.34	0.075
Vgll3	0.053	-0.032, 0.14	0.0001	223	0.002	0.96
Six6	0.11	0.025, 0.19	0.005	223	0.20	0.66
Treatment x Vgll3	-0.008	-0.097, 0.082	0.001	223	0.03	0.87
Treatment x Six6	-0.093	-0.18, -0.003	0.11	223	4.16	0.043*
Vgll3 x Six6	-0.10	-0.19, -0.014	0.14	223	5.20	0.024*
Random effect	**Var**	**C.I.low**	**C.I.high**			
Treatment x Family	0.002	0.0002	0.009			
Residual	0.027					

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Table 2. The results of linear mixed model with Type III test for LDH activity in the intestine. LDH activity (in µmol NADH/mg protein/min) was ln-transformed and normalised by ln(body mass) and ln(protein concentration) before the analysis. Intercept is shown with t-test value. Significant p-values shown with asterisks. See Table S10-12 for the results of other tissues. Estimates show high vs. low food (Treatment) or EE vs. LL genotype (*vgll3* and *six6*).

Fixed effect	**Estimate**	**95% C.I.**	**SSq**	**Den df**	**F**	**p**
Intercept	-0.094	-0.27, 0.08		9	-1.19	0.26
Treatment	0.034	-0.20, 0.26	0.006	6	0.13	0.73
Vgll3	0.035	-0.069, 0.14	0.034	227	0.77	0.38
Six6	0.13	0.03, 0.24	0.014	227	0.31	0.58
Treatment x Vgll3	0.057	-0.055, 0.17	0.045	227	1.01	0.32
Treatment x Six6	-0.056	-0.17, 0.056	0.044	227	0.98	0.32
Vgll3 x Six6	-0.18	-0.29, -0.068	0.459	227	10.26	0.0016**
Random effect	**Var**	**C.I.low**	**C.I.high**			
Treatment x Family	0.014	0.004	0.055			
Residual	0.045					

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Figure captions

Fig. 1. Regression lines (with 95% confidence intervals) for the tissue-specific enzymatic activities vs. SMR or MMR in juvenile Atlantic salmon (all with significant main effect or interaction with enzyme activity, p-values indicated in the plots). a) Heart CS activity vs. SMR, b) Intestine CS activity vs. SMR. c) Intestine CS activity vs. MMR, d) Intestine LDH activity vs. SMR. The values were normalised for body mass (SMR and MMR) or body mass and protein concentration (CS and LDH) before the analysis. See all results in Table S5.

Fig. 2. Enzymatic activities for citrate synthase (CS) (a) and lactate dehydrogenase (LDH) (b) across tissues and food availability treatments in juvenile salmon. Legend for both figures shown in (a). All pairwise differences of tissues in each enzyme were significant apart from intestine-liver difference in LDH. Significances for the main effects of tissue and treatment and their interaction shown in the plots (full models in Table S6). Boxes indicate interquartile range, whiskers reach to the last points within the 1.5 x interquartile range from the boxes (individual points are outside this range), and horizonal lines indicate medians. The *Y*-axis is on the logarithmic scale.

Fig 3. Epistatic effects and GxE observed in CS activity in the heart of juvenile salmon. Predicted means (± 95% confidence intervals) of CS activity across four different genotype combinations in two different food availability treatments (Table 1). Significant B-H corrected p-values for pairwise genotype differences (posthoc analysis) of *six6* are shown in the plot. N = 17-40 (Table S2).

Fig. 4. Epistatic effects on LDH activity in the intestine of juvenile salmon. Predicted means (± 95% confidence intervals) of LDH activity across four different genotype combinations in two different food availability treatments (Table 2). Significant/marginal B-H corrected p-values for pairwise genotype differences (posthoc analysis) are shown in the plot. N = 20-39 (Table S2).