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### ► To cite this version:

H. Lagarde, Florence Phocas, Simon Pouil, Lionel Goardon, M. Bideau, et al.. Are resistances to acute hyperthermia or hypoxia stress similar and consistent between early and late ages in rainbow trout using isogenic lines?. *Aquaculture*, 2023, 562, pp.738800. 10.1016/j.aquaculture.2022.738800 . hal-03778854

**HAL Id: hal-03778854**

**<https://hal.inrae.fr/hal-03778854>**

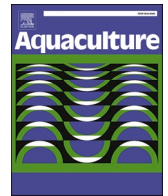
Submitted on 16 Sep 2022

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# Are resistances to acute hyperthermia or hypoxia stress similar and consistent between early and late ages in rainbow trout using isogenic lines?

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## ARTICLE INFO

### Keywords:

Temperature  
Oxygen  
Water quality  
Climate change  
Selection

## ABSTRACT

Global warming is expected to increase the frequency and intensity of heatwaves, resulting in more common combined acute hyperthermia and hypoxia conditions in fish farms. Such poor thermal and oxygenation conditions induce problems, including growth losses, increased pathogens pressure and mortality. Selective breeding is a promising solution to improve resistance to non-optimal water quality. Indeed, genetic variability to survive in acute hyperthermia or hypoxia conditions has been proved in fish. However, the characterization of these traits is not yet detailed enough to include them in a selection program. Here, we investigated the ranking stability of genotypes for acute hyperthermia or hypoxia resistances over age and between acute hyperthermia and acute hypoxia resistances. To this end, we established rankings of six isogenic lines of rainbow trout (*Oncorhynchus mykiss*) for their resistance to acute hyperthermia and hypoxia stress factors at 6 and 15 months. The experimental design was robust with more than a hundred fish per line and age. There were statistically significant resistance variations among lines confirming the potential of genetic selection for these traits. Hyperthermia and hypoxia resistance rankings were found stable 1 year apart for most genotypes. Therefore, it would be possible to select for resistance to hyperthermia and hypoxia at an early stage. No overall relationship was found between acute hyperthermia and hypoxia resistance traits: some lines were resistant to both stress factors while others were resistant to one but sensitive to the other. This indicates no strong antagonistic genetic effects between acute hyperthermia and hypoxia resistance traits in rainbow trout.

## 1. Introduction

Global surface temperature average is predicted to rise by 1–6 °C by 2100 compared to 1850–1900 (IPCC, 2021). By a causal relationship, extreme climate events are expected to increase in intensity and frequency (IPCC, 2021). Among these, heat waves affect water quality by increasing temperature and decreasing dissolved oxygen. For example, Jankowski et al. (2006) reported a mean increase of water temperature of 2 °C and a mean dissolved oxygen depletion of 1.7 mg/L compared to the long-term summer mean in the upper water layer of a Switzerland

lake during the 2003 European heat wave. Fish farms will be directly affected as ponds, raceways, cages and other standard rearing devices are generally shallow and therefore only marginally buffer acute atmospheric variations, impairing fish productions (e.g. in salmon sea-cages, see Wade et al., 2019; Gamperl et al., 2021). These crises remain limited as they are still geographically and temporally circumscribed. However, considering IPCC predictions, the risk of heatwaves will expand and could jeopardize the rearing of fish in vulnerable places.

Rainbow trout (*Oncorhynchus mykiss*) is a cold-water salmonid of major economic importance with 850,000 tons produced worldwide in

**Abbreviations:** EC, Early Challenge, phenotyping resistance of fish at 6 months; LC, Late Challenge, phenotyping resistance of fish at 15 months; LOE, Loss Of Equilibrium, resistance phenotyping endpoint; CTE, Cumulative Thermal Exposure, the acute hyperthermia resistance phenotype measured in degree-minutes; CHE, Cumulative Hypoxia Exposure, the acute hypoxia resistance phenotype measured in saturation-minutes.

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<https://doi.org/10.1016/j.aquaculture.2022.738800>

Received 2 June 2022; Received in revised form 25 August 2022; Accepted 5 September 2022

Available online 8 September 2022

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2018 (FAO, 2020). Rainbow trout farming requires cold and oxygenated water with maximal growth rate observed for a water temperature between 13 and 17 °C (Hokanson et al., 1977; Bear et al., 2007) and for an oxygen concentration between 8 and 10 mg/L (Svobodová et al., 1993) with variations between strains and individuals. High thermal and low oxygenation water conditions have deleterious effects: it can induce feeding reduction, growth losses, increased pathogens pressure and reduced survival, impairing production efficiency (Aksakal and Ekinci, 2021; Jiang et al., 2021). Numerous solutions exist to reduce the adverse effects of heat waves on aquaculture productions including nutrition, genetic selection, engineering and management (Reid et al., 2019). Pettinau et al. (2022) also demonstrated that it was possible to increase cardiac hyperthermia resistance of fish with aerobic exercise training. Given the extent of the problem, it is likely that no solution will be enough by itself and that the best option will probably consist in combining different approaches. Selective breeding is an interesting option to improve fish tolerance to non-optimal water quality (Vandeputte and Prunet, 2002). The main advantages of this solution are that genetic progress is cumulative from one generation to another and can be easily disseminated in all fish farms.

Improving resilience to climate change will require strengthening the resistance to chronic hyperthermia and hypoxia to cope with the constant water temperature increase and deoxygenation (Shepherd et al., 2017; IPCC, 2021) and strengthening the resistance to acute hyperthermia and hypoxia to face extreme and sudden heatwaves (Janowski et al., 2006). However, the few papers on the relationship between acute and chronic hyperthermia resistance in fish have shown that these two traits are genetically distinct (Dupont-Nivet et al., 2014; Bartlett et al., 2022). The underlying mechanisms of resistance to acute hypoxia and chronic hypoxia were also shown to be different in various fish species (O'Connor et al., 2011; Rimoldi et al., 2012; Li et al., 2018) suggesting that these traits are also distinct. In the present study, we focus on acute stress and the results are therefore not extendable to chronic stresses.

For acute hyperthermia resistance, several studies have highlighted the potential for genetic selection in rainbow trout. Acute hyperthermia resistance trait was formally demonstrated to be heritable:  $h^2 = 0.41 \pm 0.07$  (Perry et al., 2005) and quantitative trait loci (QTL) associated with this trait were found in rainbow trout (Jackson et al., 1998; Perry et al., 2001). Evidence of this genetic basis has also been demonstrated in practice. A strain of rainbow trout selected for acute hyperthermia resistance for at least 14 generations in Japan had a higher hyperthermia resistance compared to a hyperthermia naive rainbow trout strain (Ineno et al., 2005, 2018). Similar results were found in Australia for a rainbow trout line which was potentially naturally selected for acute hyperthermia resistance during two successive massive summer heat waves mortality events (Molony et al., 2004). In comparison, the genetic basis of acute hypoxia resistance has been much less investigated in rainbow trout and more generally in fish. In common carp (*Cyprinus carpio*), the heritability of acute hypoxia resistance was calculated to be 0.51 (Nagy et al., 1980 cited by Vandeputte, 2003). In Atlantic salmon (*Salmo salar*), significant differences in acute hypoxia resistance were found between families (Anttila et al., 2013). Even though this is not formal evidence, significant between-strains differences were reported for acute hypoxia resistance in rainbow trout (Scott et al., 2014; Fetherman et al., 2016), suggesting genetic basis. Acute hyperthermia resistance being heritable in rainbow trout, and most likely resistance to acute hypoxia, selective breeding could positively increase the average resistance of a population over generations for both traits.

It is established that acute hyperthermia resistance level in most fish species varies with life stage: thermal windows, i.e. the range of tolerated temperature, are tight in early life stages of fish, then widen in juveniles and young adults and narrow at spawning (Dahlke et al., 2020). A more essential information for assessing the relevance of selection for a trait is the stability of the ranking of genotypes for this trait with age. This information is crucial to determine the age at which fish

should be phenotyped for this trait in a breeding program. Indeed, if resistance rankings of genotypes fluctuated with age, acute hyperthermia or hypoxia resistance of selected genotypes will only be improved at specific ages. The relevance of selecting for these traits could then be questioned. Stability of the ranking of genotypes for acute hyperthermia or hypoxia resistances can be assessed by repeating challenges on the same individuals, each individual corresponding to a genotype. However, this method presents limitations as the repetition of acute hyperthermia and hypoxia challenges potentially influenced resistance in rainbow trout. Indeed, Strowbridge et al. (2021) found that acute hypoxia resistance of individuals challenged for acute hyperthermia 3 weeks before was significantly decreased and Blair and Glover (2019) found that exposition to high temperature during 96 h can reduce acute hyperthermia resistance for at least 2 months. According to Williams et al. (2019), individuals exposed periodically to low O<sub>2</sub> concentrations increase their acute hypoxia resistance. Similar results were found in various fish species including zebrafish and killifish (Morgan et al., 2018; Rees and Matute, 2018; Borowiec et al., 2020).

Some previous studies avoided this issue by comparing resistances of different strains or families of fish using different individuals between challenges (e. g. Anttila et al., 2013; Strowbridge et al., 2021), each strain corresponding to a relatively homogenous genetic group. However, within strain or family, various genotypes are present which can bias the differences observed between the groups, especially if group size is low.

Over short periods of time (1 week to 1 month), resistance rankings of individuals were shown to be repeatable in various fish species for both acute hyperthermia (Claireaux et al., 2013; Mauduit et al., 2016; Morgan et al., 2018; Joyce and Perry, 2020; Nelson et al., 2021), and acute hypoxia (Claireaux et al., 2013; Mauduit et al., 2016; Rees and Matute, 2018). However, data on the long-term (i.e. over 6 months) ranking stability of genotypes are scarce for acute hyperthermia and hypoxia resistance traits. In brook (*Salvelinus fontinalis*) and rainbow trout, the long-term rankings of genotypes for hyperthermia were found stable with age (O'Donnell et al., 2020; Strowbridge et al., 2021) while it was not the case for sea bass (Mauduit et al., 2016). For hypoxia, resistance rankings of genotypes were found stable with age in three fish species: in European seabass (*Dicentrarchus labrax*), in striped bass (*Morone saxatilis*) and in rainbow trout (Joyce et al., 2016; Mauduit et al., 2016; Nelson et al., 2019; Strowbridge et al., 2021).

However, most of these studies looked at the resistance stability within a life stage (e.g. within the adult or juvenile stage) and not between different life stages. In selective breeding, the aim is to select as early as possible to reduce selection costs, but on the condition that the resistance ranking is stable throughout the animal's life. Therefore, an additional study on the ranking stability of genotypes for acute hyperthermia and hypoxia resistance traits between juvenile and adult life stages in rainbow trout was necessary.

Acute hyperthermia and hypoxic conditions frequently occur in combination in aquatic environment. Considering the simultaneous introduction of these two traits into breeding programs therefore makes sense. However, depending on the genetic correlation between the two traits, selection for these traits will be more or less effective. There is no consensus in the literature: several studies found a positive association between the two traits in fish (Anttila et al., 2013; Zhang et al., 2018; Strowbridge et al., 2021), while other studies did not find any (Healy et al., 2018; Jung et al., 2019; Joyce and Perry, 2020). Complementing current knowledge on such association in rainbow trout is also a fundamental question.

To address these issues, we used an original and powerful genetic resource, the rainbow trout isogenic lines (Quillet et al., 2007). Within each line, all fish share the same genotype while the different lines represent a sample of the genetic variability of the population from which they were derived. Thanks to genetic uniformity within isogenic line, it was possible to repeat hyperthermia and hypoxia challenges on the same genotypes, but using systematically different individuals. Fish

from the same isogenic line could be considered as repetitions of the same genotype, avoiding the potential influence of repeated challenges on the ranking stability of genotypes for hyperthermia or hypoxia resistance. Previous experiments have revealed contrasting levels of resistance to hyperthermia between lines (Dupont-Nivet et al., 2014). Six isogenic lines were chosen among the ones showing contrasted resistance to hyperthermia. Both acute hyperthermia and hypoxia resistances were assessed at 6 months and 15 months using systematically different fish at the different ages and between hyperthermia and hypoxia challenges.

The objectives of the study were to: i) confirm the existence of genetic variability for acute hyperthermia resistance and acute hypoxia resistance in rainbow trout; ii) investigate the ranking stability of genotypes at different ages for each resistance trait to acute challenges; iii) determinate the ranking stability of genotypes between acute hyperthermia and acute hypoxia resistances.

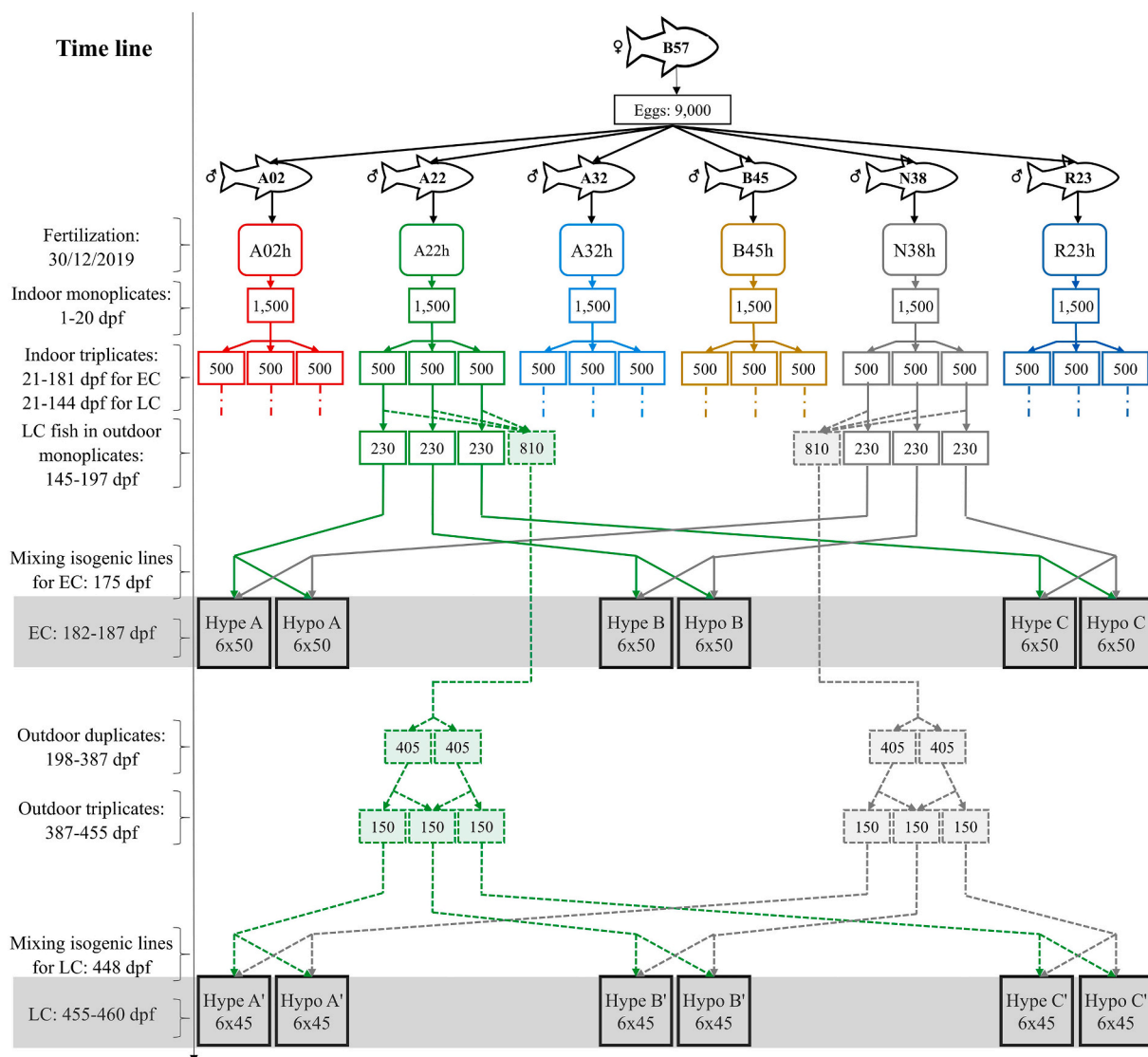
## 2. Material and methods

### 2.1. Ethical statement

All the experiments were carried out at the INRAE experimental facilities (PEIMA, INRAE, 2021, Fish Farming systems Experimental Facility, doi: [10.15454/1.5572329612068406E12](https://doi.org/10.15454/1.5572329612068406E12), Sizon, France) authorized for animal experimentation under the French regulation C29-277-02. The experiment was carried out according to the European guidelines; the protocols were evaluated and approved by the ethical committee CEFEA No 74 and authorized by the French ministry of higher education and research (APAFIS#24784\_2020032510486957 and APAFIS#28807\_2020122314543943).

### 2.2. Isogenic lines

Six rainbow trout heterozygous isogenic lines were used in this study. Within each line, all fish share the same genotype while the



**Fig. 1.** Rearing history from fertilization to acute hyperthermia and hypoxia phenotyping challenges. Each isogenic line is represented by a different color. Full rearing history was only represented for lines A22h and N38h. The four other lines were not represented so as not to overload the figure but they had a similar life history as A22h and N38h. Rectangulars represent rearing tanks with a distinction between indoor tanks (white-filled and solid borders) and outdoor tanks (colored-filled and dotted borders). The theoretical number of fish is indicated inside each rectangular. Movements of fish are represented by the lines, solid for early challenges (EC) fish and dotted for late challenges (LC) fish once separated from EC fish. Black squares highlighted by the grey outlines represent challenges, “Hype” stands for hyperthermia challenge and “Hypo” for hypoxia challenge.



different lines represent a sample of the genetic variability of the population from which they were derived. The six isogenic lines can therefore be considered as six different genotypes. Isogenic lines were obtained and bred in Sizun, France at PEIMA experimental fish farm. They were produced by crossing several females from the B57 homozygous isogenic line with neomales (sex-reversed XX males) from six other homozygous isogenic lines called A02, A22, A32, B45, N38 and R23. All homozygous isogenic lines used in the present study were originally established and maintained at INRAE (Quillet et al., 2007). Males were chosen among homozygous lines whose hybrids, from crosses with B57 line, have shown different resistance levels to hyperthermia acute stress in a previous experiment (Dupont-Nivet et al., 2014). Eggs were collected among eleven same-day naturally spawning B57 females. Eggs were mixed and equally distributed in six batches. These steps were intended to minimize inter-line maternal and environmental effects. Each batch was fertilized by a unique male from the six homozygous isogenic lines mentioned above. Thus, the only source of variations between heterozygous lines at this stage was expected to be the paternal isogenic line genetic effect. We preferred to use heterozygous isogenic fish rather than homozygous isogenic fish to avoid strong inbreeding depression effects and to ensure same fertilization date for all isogenic lines, spawning periods being different between isogenic lines. Heterozygous isogenic lines obtained were named A02h, A22h, A32h, B45h, N38h and R23h.

## 2.3. Rearing conditions

### 2.3.1. Rearing conditions till separation of fish for early and late challenges

The fish rearing conditions from fertilization to challenges are summarized in Fig. 1. For the sake of clarity, only rearing conditions of isogenic lines A22h and N38h were fully represented on Fig. 1 but the four unrepresented isogenic lines followed similar life histories. From fertilization to 20 days post-fertilization (dpf), eggs from each isogenic line were incubated in one hatching tray per line, supplied with open circuit natural spring water with small thermal amplitude over the concerned period (11.5 °C - 12.4 °C). At 21 dpf, ~1500 eyed eggs were collected from each hatching tray and were randomly divided into three new hatching trays per isogenic line (500 eyed eggs per hatching tray, 3 × 6 hatching trays). Hatching occurred progressively between 26 dpf and 33 dpf. At 43 dpf, prior to first feeding, larvae were transferred into 0.12 m<sup>3</sup> indoor tanks with no mixing between replicates (500 larvae per tank, 3 × 6 tanks) supplied with the same spring water and lit by artificial lights from 8:00 am to 8:00 pm (Fig. 1). At 145 dpf, fish used for early challenges (EC) were separated from those used in late challenges (LC). For LC, 270 fries were randomly collected in each of the 18 indoor tanks and transferred according to their isogenic line in outdoor tanks (810 fries per tank, 6 tanks, one tank for each isogenic line). Outdoor tanks were 1.8 m<sup>3</sup> and were supplied with lake dam water with normal seasonal temperature variations throughout the concerned period (daily mean temperature: 6.0 °C - 18.4 °C) and lit by natural light. The remaining fish (230 fish per indoor tank, 3 × 6 tanks) stayed in indoor tanks for EC (Fig. 1). In both EC and LC, fish were fed daily to satiation with a commercial diet from Le Gouessant's company.

### 2.3.2. Early challenges – rearing conditions

At 150 dpf, the density of the 18 indoor rearing tanks was reduced from 7.0 to 4.5 kg/m<sup>3</sup> by randomly keeping 160 fish per tank. On the same day, 100 individuals among the 160 kept in each tank were individually anaesthetized, PIT-tagged (Biolog-id, pit-tag length: 12 mm, diameter: 2 mm, weight: 0.1 g) and placed back in their respective tanks. Pit-tags were injected horizontally in the dorsal muscle just behind the head. Mean fish body weight at tagging was 7.5 g. Untagged fish were kept in their respective tanks and used in experiments not presented in this study. At 175 dpf, as shown in Fig. 1, replicates for early hyperthermia and hypoxia challenges were formed: 50 PIT-tagged fish from each isogenic line were distributed into 6 tanks corresponding to the 6

EC replicates (3 for acute hyperthermia EC and 3 for acute hypoxia EC). Each EC replicate contained approximately 50 fish from each of the six lines, making ~300 fish per EC replicate. Furthermore, there was no mixing between rearing tanks within EC replicate: in each EC replicate, fish from each line originated from a unique rearing tank. Thus, rearing tanks' and EC challenge replicates' effects were confounded in our experimental design. During all rearing processes of fish for EC, the maximum rearing density was 8.7 kg/m<sup>3</sup>.

### 2.3.3. Late challenges – rearing conditions

At 198 dpf, the 6 outdoor tanks were duplicated, making 12 tanks with 405 fish in each (Fig. 1). At 287 dpf, density was reduced in tanks by randomly keeping 280 fish per tank and removing extra fish. At 387 dpf, duplicates were turned into triplicates by transferring 75 fish from each isogenic line duplicates in a third tank; the number of fish in each triplicate was then 150 (Fig. 1). At 436 dpf, 90 fish were collected from each of the 18 tanks and individually PIT-tagged following the same procedure as the one used in early challenge. Mean fish body weight at tagging was 216.0 g. Untagged fish were kept in their respective tanks and used in experiments not presented in this study. At 448 dpf, replicates for LC were formed: approximately 45 PIT-tagged fish from each isogenic line were distributed into 6 tanks corresponding to the 6 LC replicates (3 for acute hyperthermia and 3 for acute hypoxia). Each replicate contained 45 fish per line, making ~270 fish per LC replicate. As in EC, there was no mixing between rearing tanks within LC replicate: the 45 fish of each line in each replicate originated from the same rearing tank. The number of fish per LC replicate was reduced compared to the number of fish per EC replicate for logistical reasons. The maximum rearing density reached was 25.5 kg/m<sup>3</sup> during the rearing process of fish for LC.

## 2.4. Acute hyperthermia and hypoxia resistance phenotyping methods

Early hyperthermia and hypoxia challenges were performed in June 2020 (182 dpf to 187 dpf ~ 6 months, mean body weight of 15.5 g) and late hyperthermia and hypoxia challenges in March 2021 (from 455 dpf to 460 dpf ~ 15 months, mean body weight of 236 g) at the rate of one replicate of hyperthermia or hypoxia challenge per day. Thermal acclimations were different between EC and LC as the sources of water (spring water in EC and dam water in LC) and season of challenges were different (summer in EC and spring in LC). Prior to challenges, water temperature was 12.4 °C in EC and 9.7 °C in LC. The four challenges (early hyperthermia, early hypoxia, late hyperthermia and late hypoxia) were done in triplicates. Animals were starved 3 days before challenges. The evening prior to each challenge, fish of the challenged replicate were moved to the indoor challenge tank (0.12 m<sup>3</sup> for EC, 2 m<sup>3</sup> for LC) supplied with the same water source as the one used in the respective rearing tanks and left alone for the night for acclimation. Temperature and O<sub>2</sub> concentration and saturation were recorded every 5 min during acute hyperthermia and hypoxia challenges using electronic probes (HQ40d, Hach Company, Loveland, CO, USA). During the challenges, NH<sub>4</sub><sup>+</sup> concentration was checked at precise time points (peak of loss of equilibrium and end of challenge) using a commercial colorimetric kit (LCK 304, Hach Company, USA) and CO<sub>2</sub> concentration was measured continuously with a CO<sub>2</sub> analyzer (Oxyguard, Denmark).

### 2.4.1. Early and late hyperthermia challenges

Temperature was gradually increased from 12.4 °C in EC and 9.7 °C in LC up to 27.5 °C by renewing challenge tank water with heated water from a buffer tank of volume 0.2 m<sup>3</sup> in EC and 0.75 m<sup>3</sup> in LC. As temperature increased, fish were gradually losing equilibrium. Temperature increase rate was not constant during the course of the challenges. Temperature was first quickly increased at a rate of 3.6 °C/h during 3 h in EC and 2.7 °C/h during 5 h in LC. This period was followed by a slower temperature increase at a rate of 0.6 °C/h in EC and 0.7 °C/h in LC until all fish have lost equilibrium (Fig. S1-A). This slower

temperature increase was intended to increase the between-fish variability of acute hyperthermia resistance phenotypes. Temperature increase profiles were very similar between EC or LC replicates, but slightly lower in LC than in EC (Fig. S1-A). Indeed, we reduced temperature increase rate in the beginning of LC because we expected fish to be more sensitive to hyperthermia in LC than in EC as acclimation temperature was lower by 2.7 °C in late challenges. Oxygen saturation was maintained above 65% by softly bubbling pure O<sub>2</sub> (Fig. S1-B). Water renewal flow was approximately 48 L/h in EC and 300 L/h in LC. Water inlets were turned towards the sides of the tank to reduce the water flow inside the tank to a minimum. When a fish lost equilibrium, it was removed from the tank, its isogenic line origin was identified using the PIT-tag and the exact time of loss of equilibrium (LOE) was recorded. Fish were then softly anaesthetized (Tricaine MS222, 50 mg/L), weighed using digital scales (CP2202S, Sartorius, Germany in EC; i20 R1DLS, Precia-Molen, France in LC), and euthanized by an overdose of anaesthetic (Tricaine MS222, 150 mg/L). Challenges ended when the last fish lost its equilibrium.

#### 2.4.2. Early and late hypoxia challenges

Dissolved oxygen saturation was quickly decreased from nearly 90% to 20% in 3 h. When 20% of oxygen saturation was reached, oxygen saturation decrease was slowed down and 4.5 h after the beginning of the challenge, saturation stabilized at around 15% for the rest of the challenge (Fig. S1-D). Dissolved oxygen was decreased by bubbling dinitrogen gas into a saturation column. Temperature was not controlled and increased on average from 12.7 °C to 13.5 °C in EC and from 9.7 °C to 14 °C in LC because of heat exchange with air (Fig. S1-C). At the beginning of hypoxia challenges, water renewal flow was not recorded in EC and was 400 L/h in LC. Water inlets were turned towards the sides of the tank to keep the tank's water flow to a minimum. As fish were losing equilibrium, the amount of consumed O<sub>2</sub> in the tank was decreasing. We therefore had to gradually reduce and then stop the water supply in the course of the experiment to maintain an O<sub>2</sub> saturation close to 15%. After LOE, fish were removed from the tank, identified with their PIT-tag, anaesthetized, weighed, and euthanized as in temperature challenges. Hypoxia challenges ended when the last fish lost equilibrium or less than ten fish remained in the tank and none of the remaining fish lost equilibrium for 30 min. In the latter case, challenge was stopped and fish were given the end time of the challenge (it was the case in all replicates of EC and in replicate A of LC).

#### 2.5. Data and statistical analysis

Acute hyperthermia resistance was quantified as the cumulative thermal exposure (CTE) in degree-minutes (deg-min) as defined in Perry et al. (2005). This indicator is calculated as a combination between time and temperature: the difference between the temperature measured in the challenge tank and a reference temperature of 12.5 °C was cumulated each minute from the beginning of the challenge to the time of LOE. The reference temperature was 12.5 °C because it is the maximal initial temperature in EC and LC acute hyperthermia challenges. This method enables us to consider slight differences in thermal profiles between replicates in both EC and LC as described in O'Donnell et al. (2020). By similar reasoning, the cumulative hypoxia exposure (CHE) in saturation-minutes (sat-min) was calculated as a combination between time and O<sub>2</sub> saturation: the difference between a reference O<sub>2</sub> saturation of 90% and the O<sub>2</sub> saturation measured in the challenge tank was cumulated every minute from the beginning of the challenge to the time of LOE. The reference O<sub>2</sub> saturation was 90% because it corresponds to the maximal initial O<sub>2</sub> saturation in EC and LC hypoxia challenges. Note that high CTE or CHE indicates high hyperthermia and hypoxia resistance respectively. In the rest of the present study, both hyperthermia and hypoxia resistance phenotypes will refer to CTE and CHE.

All statistics were performed with R software 4.0.3 (R Core Team, 2019). Mean body weight (BW) differences among isogenic lines were

analyzed by analysis of variance (ANOVA) and Tukey's test for multiple pairwise comparisons. ANOVA assumptions of normality and homoscedasticity were verified by visual inspection of residual-fit plots. Coefficients of variations, the ratio of the SD to the mean, were used to quantify the within-line variability of BW and phenotypic resistances.

A linear mixed model was computed to examine the effect of isogenic line on resistant phenotypes (response to early or late acute hyperthermia challenges quantified as CTE and response to early or late acute hypoxia challenges quantified as CHE).

All the resistance phenotypes were described by the following model:

$$y_{ijk} = \mu + \alpha_i + (\beta + \delta i) \times BW_{ijk} + r_j + e_{ijk}$$

Where  $y_{ijk}$  is the resistance phenotype of individual  $k$  of isogenic line  $i$  in replicate  $j$ ,  $\mu$  the mean resistance,  $\alpha_i$  the fixed effect of the isogenic line  $i$ ,  $\beta$  the slope of the regression of  $y$  on  $BW$ ,  $\delta i$  the intra-line  $i$  slope of the regression of  $y$  on  $BW$ ,  $r_j \sim N(0, \sigma_r^2)$  the replicate random effect and  $e_{ijk} \sim N(0, \sigma_e^2)$  the residual effect.

Assumptions of linearity, normality and homoscedasticity were checked by visual inspections of residuals against fitted values. Assumptions were validated for hyperthermia EC data and hyperthermia LC data. However, residuals of the hypoxia EC and late hypoxia LC data showed heterogeneity in the variances among replicates and isogenic lines. Therefore, a heteroscedastic model was applied to CHE performances with different residual variance estimates for each combination replicate: isogenic line. Function `lme` in package `nlme` 3.1-153 (Pinheiro et al., 2021) was used to fit linear mixed models. The best model for fixed effects was chosen with Akaike information criteria (AIC) and F-tests using package `lmerTest` 3.1-3 (Kuznetsova et al., 2017). The marginal  $r^2$  ( $r_m^2$ ) and the conditional  $r^2$  ( $r_c^2$ ) were calculated using the `MuMin` 1.43.17 R package (Barton, 2020).  $r_m^2$  is the proportion of variance explained by fixed factors and  $r_c^2$  is the proportion of variance explained by both fixed and random factors (Nakagawa and Schielzeth, 2013). Differences among isogenic lines mean resistance were compared by Tukey's test using the `lsmeans` 2.30-0 package (Lenth and Love, 2015) with Type 1 error risk set at 0.05. Throughout the manuscript values are given as mean  $\pm$  SD.

### 3. Results

#### 3.1. Conduct of the experiments

All replicates of the four challenges were successfully managed except for replicate B of hypoxia EC where fish were not properly fasted. As a result, in the first hour, fish released faeces, dirtying water in the challenge tank and dissolved oxygen depletion was much faster compared to the other hypoxia EC (e.g. initial O<sub>2</sub> saturation was 61.5% in replicate B compared to 86.5% and 79.7% in A and C). These different experimental conditions had an impact on the resistance to hypoxia of isogenic lines in replicate B: lines A02h, A22h, A32h, B45h and N38h showed no significant variation in hypoxia resistance while it was the case in replicates A and C (Supplementary Fig. S2). For these reasons, we decided to remove replicate B of hypoxia EC from the study.

NH<sub>4</sub><sup>+</sup> concentration and pH measured at the end of challenges were similar between replicates of each challenge. In hyperthermia, the respective mean NH<sub>4</sub><sup>+</sup> concentration and pH were 2.5  $\pm$  0.6 mg/L and 6.6  $\pm$  0.03 in EC and 1.4  $\pm$  0.2 mg/L and 6.5  $\pm$  0.1 in LC. In hypoxia, NH<sub>4</sub><sup>+</sup> concentration was lower than 0.3 mg/L with a pH of 6.8 in both EC and LC.

CO<sub>2</sub> concentration gradually increased but did not exceed 17 mg/L and 22 mg/L in hyperthermia EC and LC and 10 mg/L and 12 mg/L in hypoxia EC and LC.

On average, hyperthermia challenges lasted 696  $\pm$  41 min in EC, 719  $\pm$  4 min in LC and hypoxia challenges lasted 768  $\pm$  19 min in EC, 748  $\pm$  60 min in LC. In hypoxia challenges, very few fish did not lose equilibrium during the challenges (14 out of 577 fish in EC and 2 out of 807 in LC; see Table 1 for isogenic lines details) and estimations of lines'

**Table 1**  
Phenotypes agglomerated by isogenic line.

Challenge	Variable	A02h	A22h	A32h	B45h	N38h	R23h
Early hyperthermia	Sample size	147	149	147	133	148	144
	BW (g)	14.3 ± 2.7 <sup>b</sup>	14.2 ± 2.6 <sup>b</sup>	12.2 ± 2.5 <sup>a</sup>	13.9 ± 2.7 <sup>b</sup>	18.1 ± 2.9 <sup>c</sup>	17.2 ± 3.7 <sup>c</sup>
	Resistance time (h)	9.8 ± 0.6	9.5 ± 0.7	10.7 ± 0.7	10.1 ± 0.7	9.2 ± 0.5	9.7 ± 0.5
	CTE x10 <sup>3</sup> (deg.min)	6.4 ± 0.6	6.1 ± 0.6	7.2 ± 0.7	6.7 ± 0.7	5.8 ± 0.5	6.3 ± 0.5
Late hyperthermia	Sample size	131	133	133	133	134	135
	BW (g)	222 ± 24 <sup>a</sup>	271 ± 30 <sup>d</sup>	218 ± 30 <sup>a</sup>	234 ± 27 <sup>b</sup>	232 ± 26 <sup>b</sup>	254 ± 3 <sup>c</sup>
	Resistance time (h)	10.1 ± 0.6	9.6 ± 0.6	10.6 ± 0.9	10.1 ± 0.6	10.7 ± 0.5	9.6 ± 0.5
	CTE x10 <sup>3</sup> (deg.min)	5.7 ± 0.5	5.3 ± 0.5	6.1 ± 0.8	5.7 ± 0.5	6.3 ± 0.4	5.3 ± 0.4
Early hypoxia	Sample size <sup>1,2</sup>	100 (4)	95 (4)	100 (0)	85 (1)	99 (1)	98 (4)
	BW (g)	15.2 ± 2.8 <sup>b</sup>	14.8 ± 2.7 <sup>b</sup>	12.9 ± 2.6 <sup>a</sup>	15.8 ± 2.7 <sup>b</sup>	18.4 ± 2.8 <sup>c</sup>	17.6 ± 3.7 <sup>c</sup>
	Resistance time (h)	7.5 ± 2.3	7 ± 2.2	5.6 ± 1.6	5.6 ± 1.7	6.6 ± 1.5	7.6 ± 2
	CHE x10 <sup>3</sup> (sat.min)	30 ± 10	28 ± 10	21 ± 7	21 ± 8	26 ± 6	31 ± 10
Late hypoxia	Sample size <sup>1</sup>	134 (0)	135 (1)	135 (0)	135 (0)	135 (0)	133 (1)
	Mean BW (g)	222 ± 28 <sup>b</sup>	261 ± 26 <sup>c</sup>	209 ± 28 <sup>a</sup>	234 ± 26 <sup>c</sup>	231 ± 29 <sup>bc</sup>	250 ± 29 <sup>d</sup>
	Resistance time (h)	7.9 ± 1.5	7.5 ± 1.9	5.2 ± 1.7	7.1 ± 1.9	8.2 ± 1.9	7.9 ± 2.1
	CHE x10 <sup>3</sup> (sat.min)	31.7 ± 6.9	29.8 ± 8.5	19.7 ± 7.4	27.9 ± 8.4	33 ± 8.6	31.7 ± 8.9

Variables are indicated as means ± SD. BW means with same letters within rows are not significantly different. CTE stands for cumulative thermal exposure and CHE for cumulative hypoxia exposure.

<sup>1</sup> Numbers in brackets next to sample size indicate the number of fish that resisted the whole challenge (e.g. did not lose equilibrium). All fish lost equilibrium in all hyperthermia challenges.

<sup>2</sup> In hypoxia EC, sample size is low compared to other challenges because replicate B was removed.

mean hypoxia resistances were therefore only slightly impacted. These fish were given the final CHE of the respective replicates. The dynamics of LOE in hyperthermia and hypoxia challenges were different. The first LOE happened much quicker in hypoxia challenges (220 ± 50 min in EC and 212 ± 19 min in LC) compared to temperature challenges (498 ± 20 min in EC and 399 ± 34 min in LC). Moreover, in hypoxia challenges, LOE were scattered as 90% of the fish lost equilibrium within 424 min in EC and 391 min in LC. In hyperthermia challenges, fish lost equilibrium within 144 min in EC and 136 min in LC. Kinetics of LOE are presented in Supplementary Fig. S3 for each of the four challenges, showing that, for each challenge, the rankings of isogenic lines were repeatable between the replicates.

### 3.2. Body weight and isogenic line effects influence on resistance phenotypes

Mean BW of fish was 15.5 ± 3.5 g in EC and 236 ± 33 g in LC all isogenic lines confounded. There were significant differences in BW between isogenic lines (Table 1). In EC, the biggest average BW difference was between lines A32h and N38h (average BW difference of 5.9 g in hyperthermia challenge and 5.5 g in hypoxia challenge). In LC, the biggest average BW difference was between lines A32h and A22h (average BW difference of 53 g in hyperthermia challenge and 52 g in hypoxia challenge). Despite significant mean BW differences between the lightest and the heaviest lines, BW ranges were overlapping in all challenges enabling us to disentangle the body weight effect and the isogenic line effect (Supplementary Fig. S4).

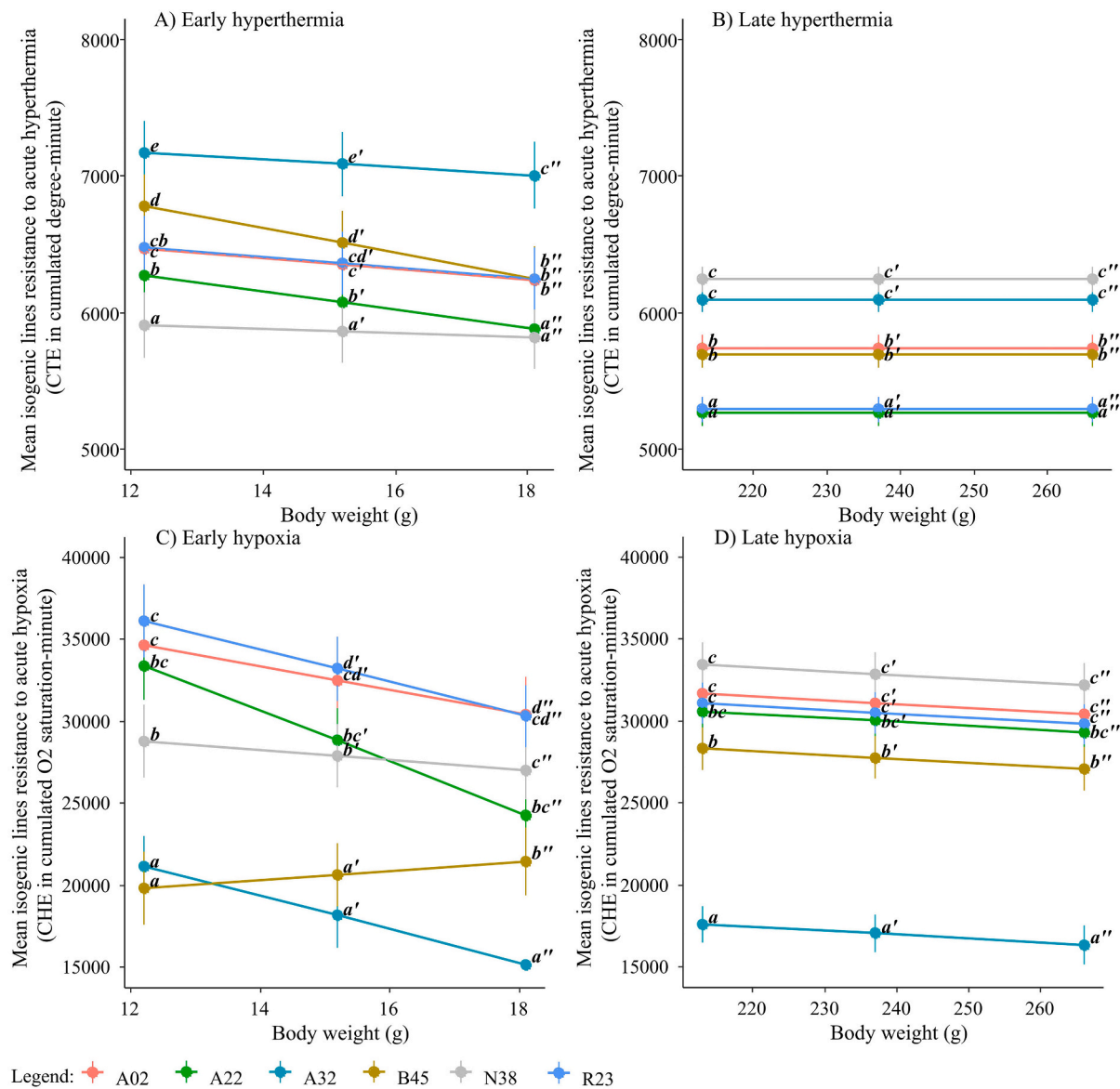
Significance of the isogenic line fixed effect, the BW covariate and of their interaction are provided in Table 2 for all resistance traits. Line effect was significant in all challenges while BW effect was only significant in early hyperthermia challenge and in the two hypoxia challenges (Table 2). In late hypoxia challenge, the best model (model 1) fitted a unique BW coefficient of regression for all lines (homogenous slopes). In contrast, in hyperthermia EC and hypoxia EC, the best models (model 2) fitted different BW coefficients of regression for each line (i.e. heterogeneous slopes: effect of BW on resistance is different between lines). Estimates of isogenic line effects and BW regression coefficients are given in supplementary Table S1. BW had a negative effect on resistance trait in EC hyperthermia, in EC hypoxia except for line B45h and in LC hypoxia. In LC hyperthermia, BW regression coefficients were not estimated because BW effect was not significant (Table 2).

Ranking of isogenic lines resistance phenotypes was varying with BW in EC because of heterogeneous BW regression coefficients (Fig. 2A&C).

**Table 2**  
Selection of variables to model resistance phenotypes.

Challenge	Effect	F-test	AIC	r <sup>2</sup> <sub>(m)</sub>	r <sup>2</sup> <sub>(c)</sub>
Hyperthermia early	Model 1	BW F <sub>(1,856)</sub> = 68.6*	12,976	0.37	0.66
	Line	F <sub>(5,856)</sub> = 253.5*			
	Model 2	BW F <sub>(1,851)</sub> = 169.3*	12,965	0.38	0.66
	Line	F <sub>(5,851)</sub> = 260.2*			
Hyperthermia late	Model 1	BW: Line F <sub>(1,789)</sub> = 0.9 F <sub>(5,789)</sub> = 573.2*	12,383	0.29	0.33
	Model 2	BW Line BW: Line n/a <sup>1</sup>		n/a <sup>1</sup>	
Hypoxia early	Model 1	BW Line F <sub>(1,569)</sub> = 95.5* F <sub>(5,569)</sub> = 64.1*	12,018	0.27	0.32
	Model 2	BW Line F <sub>(1,564)</sub> = 85.7* F <sub>(5,564)</sub> = 64.4* BW: Line F <sub>(5,564)</sub> = 4.2*	12,008	0.31	0.37
Hypoxia late	Model 1	BW Line F <sub>(1,798)</sub> = 537.0* F <sub>(5,798)</sub> = 161.6*	16,612	0.26	0.29
	Model 2	BW Line F <sub>(1,793)</sub> = 520.7* F <sub>(5,793)</sub> = 162.3* BW: Line F <sub>(5,793)</sub> = 1.4	16,616	0.26	0.30

All models included replicate as a random intercept effect. BW is body weight covariate with homogenous slope, Line is isogenic line fixed effect and BW:Line is body weight covariate with heterogeneous slopes for isogenic lines. In F-test, “\*” means the P-value is inferior to 0.05 significant threshold. r<sup>2</sup><sub>(m)</sub> is model marginal r<sup>2</sup> and r<sup>2</sup><sub>(c)</sub> is model conditional r<sup>2</sup>. <sup>1</sup>In late hyperthermia challenge, model 2 was not computed because BW covariate was found insignificant in model 1.



**Fig. 2.** Predicted resistance of the different isogenic lines by best models in: A) early hyperthermia challenge, B) late hyperthermia challenge, C) early hypoxia challenge and D) late hypoxia challenge. Full lines are final mixed linear models' fits. BW intervals are bounded by extreme mean BW isogenic lines: [12.6 g – 18.3 g] in EC and [210 g – 266 g] in LC. Isogenic lines mean resistances were compared with Tukey tests at three distinct BW in EC and LC: at the mean BW of all fish (15.2 g in EC and 237 g in LC) and at the bounds of the BW intervals. In LC, rankings did not change with BW because of homogenous slopes. CTE stands for cumulative thermal exposure and CHE for cumulative hypoxia exposure. Resistance means with similar letters and apostrophe (') are not significantly different. Vertical bars represent the standard error.

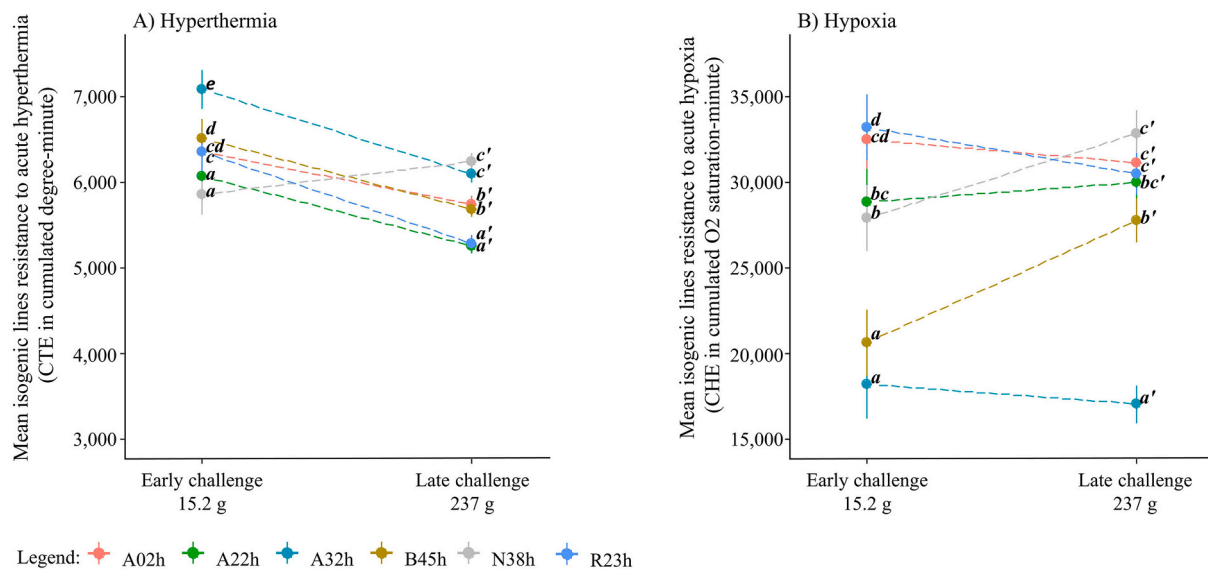
However, this effect remained marginal in both hyperthermia and hypoxia EC as shown by the small gain of  $r^2_{(m)}$  between model 1 and model 2 (Table 2). Accordingly, re-rankings remained minor over the BW range considered (Fig. 2A&C). Moreover, in hyperthermia EC, heterogenous slopes appeared to be driven by lines A22h and B45h that have different regression slopes while other lines have similar slopes (Fig. 2A). Regardless of BW, in hyperthermia EC, line A32h was significantly the most resistant line and N38h and A22h the most sensitive ones. In EC hypoxia, the most resistant lines were A02h, A22h, N38h and R23h and the most sensible ones were A32h and B45h. In the rest of the publication, we consider the ranking of isogenic lines at 15.2 g, the mean BW in EC, all lines and challenges included. On the other hand, rankings of resistance phenotypes of isogenic lines were stable with BW in LC because of homogenous slopes (Fig. 2B&D). In LC hyperthermia, A32h and N38h were significantly the most resistant lines and A22h and R23h the most sensitive lines. In LC hypoxia, the most resistant lines were

A02h, A22h, N38h and R23h and the most sensible was A32h. In the rest of the publication, we consider the ranking of isogenic lines at 237 g, the mean BW in LC.

### 3.3. Resistance stability between early and late challenges

In order to investigate the ranking stability of genotypes at different ages for hyperthermia and hypoxia resistances, performances of the six rainbow trout isogenic lines were compared between fish of 15.2 g (mean BW in EC) and fish of 236 g (mean BW in LC) (Fig. 3). In acute hyperthermia challenges, ranking of all lines between EC and LC was relatively well maintained except for line N38h which shifted from the most sensitive to the most resistant genotype (Fig. 3A). In acute hypoxia challenges, little changes in resistance ranking of lines were observed between EC and LC: A02h, A22h, N38h and R23h stayed the four most resistant genotypes, line B45h the intermediate resistant genotype and





**Fig. 3.** Isogenic lines re-rankings between early and late resistance to A) hyperthermia and B) hypoxia as estimated by best models. Predictions are estimated at a BW of 15.2 g in EC and of 237 g in LC. Resistance phenotypes with common letters within challenges are not significantly different. CTE stands for cumulative thermal exposure and CHE for cumulative hypoxia exposure.

A32h the most sensitive genotype (Fig. 3B).

### 3.4. Line response consistency across acute hyperthermia and hypoxia challenges

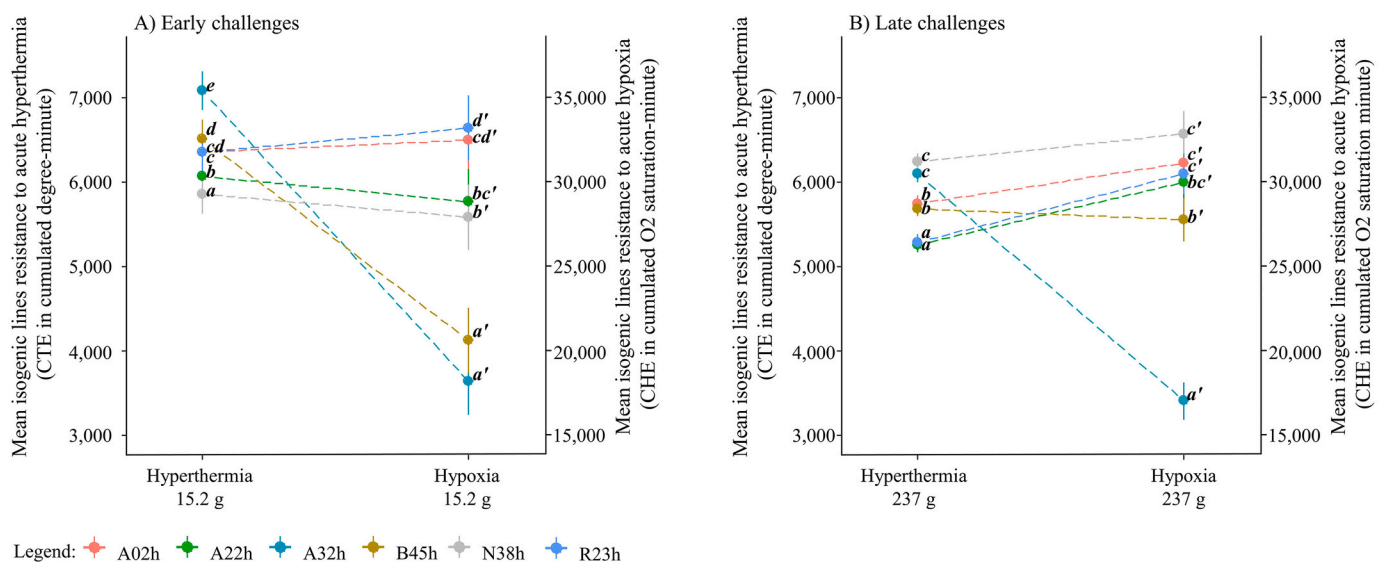
In order to determine the ranking stability of genotypes between hyperthermia resistance and hypoxia resistance, performances of the six rainbow trout isogenic lines were compared for both EC (Fig. 4A) and LC (Fig. 4B). No apparent association between acute hyperthermia resistance and acute hypoxia resistance emerged. In EC, A32h and B45h were the most resistant lines for hyperthermia but the most sensitive lines for hypoxia. On the other end, the resistance rankings between lines R23h, A02h, A22h and N38h did not change between hyperthermia and hypoxia in EC (Fig. 4A). In LC, N38h was among the most resistant lines in hyperthermia and hypoxia challenges. A32h was also one of the most resistant lines in LC hyperthermia but was the most sensitive in LC

hypoxia (Fig. 4B). These results suggest that resistance to acute hyperthermia and resistance to acute hypoxia are two different traits.

## 4. Discussion

The main asset of the present study is to rely on an original biological material, the rainbow trout isogenic lines, which allows us to directly investigate the genetic determinism of acute hyperthermia and hypoxia resistance by looking at the between-line phenotypic variations. Isogenic lines enabled to repeat acute hyperthermia or hypoxia challenges at two different ages on the same genotypes but systematically using different individuals, which is a novelty compared to the literature. Indeed, repetitions of acute hyperthermia and hypoxia challenges on the same individuals were found to significantly impact resistance in various fish species (e.g. Williams et al., 2019; Strowbridge et al., 2021).

Taking advantage of this experimental model, we sought to answer



**Fig. 4.** Isogenic lines re-rankings between hyperthermia resistance and hypoxia resistance in A) early challenges and B) late challenges as estimated by best models. Predictions are estimated at a BW of 15.2 g in EC and of 237 g in LC. Resistance phenotypes with common letters within challenges are not significantly different. CTE stands for cumulative thermal exposure and CHE for cumulative hypoxia exposure.

three key questions for introducing resistance traits to acute hyperthermia and acute hypoxia into rainbow trout breeding programs: i) confirm the existence of genetic variability for acute hyperthermia resistance and acute hypoxia resistance, ii) assess the ranking stability of genotypes to acute hyperthermia resistance or acute hypoxia resistance at different ages and iii) assess the ranking stability of genotypes between acute hyperthermia resistance and acute hypoxia resistance. While not specifically the objective of this study, we also studied the effect of BW on acute hyperthermia or hypoxia resistances and potential interaction with genotype at two different ages.

#### 4.1. Rearing of fish and acute hyperthermia and hypoxia resistance phenotyping

Currently, life stage, body size, phenotypic plasticity and genetics are the four main factors known to influence intraspecific fish hyperthermia resistance (reviewed in McKenzie et al., 2021) or hypoxia resistance (e.g. Ishibashi et al., 2007; Nilsson and Östlund-Nilsson, 2008; Borowiec et al., 2020). In the present study, environmental factors that could influence fish resistance to acute hyperthermia or hypoxia were carefully controlled in the experimental design. Fish were all the same age preventing a life stage effect influence. Potential maternal effects were controlled by using only one maternal line and mixing eggs obtained from different B57 females before separating them into six batches for fertilization. Isogenic lines were bred in homogenous conditions to limit the influence of environmental factors that could lead to phenotypic plasticity and hence bias our phenotyping methods. Fish were also most of the time bred in triplicates to avoid confusion between isogenic line and breeding tank effects on hyperthermia or hypoxia resistance. For EC, fish were bred in triplicates from 20 dpf. For LC, fish from each isogenic line were also intended to be reared in triplicates from 20 dpf but due to the initial Covid lockdown (March–April 2020) not enough tanks could be released on the experimental farm. As a result, each isogenic line was reared in a unique tank between 145 and 197 dpf. Therefore, there was a confusion of effect between rearing tank and isogenic line during this short period. However, this confounding effect remains low given that fish in each line were reared in triplicates or duplicates 84% of the time (Fig. 1) and the triplicates rearing periods of LC fish were distributed in the two key stages: in early life (dpf 21–144), when fish are the most sensitive to environmental variations and the period prior to phenotyping challenges (dpf 387–455). Last but not least, significant differences in BW were observed between isogenic lines in both EC and LC (Table 1) because of genetic differences between isogenic lines. However, isogenic line effect and BW were not confounded as even BW ranges of extreme isogenic lines were overlapping (Supplementary Fig. S4). The BW effect was therefore tested in statistical models and kept when significant. This will be discussed further.

Phenotyping methods used in acute hyperthermia and hypoxia challenges were chosen to maximize phenotypic variation between fish compared to classical phenotyping methods. For acute hyperthermia resistance, the classical phenotyping method is the critical thermal methodology (Beitinger et al., 2000): temperature is increased linearly and the CT<sub>max</sub>, the temperature at which fish lose equilibrium, is considered as the resistance trait. This methodology has been extensively tested in numerous fish species, but an identified limit is the lack of intra-specific phenotypic variations. For example, several studies compared CT<sub>max</sub> of strains of rainbow trout and between-strains mean differences were most of the time ranging between 0.2 °C and 0.7 °C (Carline and Machung, 2001; Hartman and Porto, 2014; Ineno et al., 2005, 2018; Chen et al., 2015; Strowbridge et al., 2021) and exceptionally above 1 °C (Zhang et al., 2018; Crichigno and Cussac, 2019). Such slight variations make CT<sub>max</sub> a poorly informative acute hyperthermia resistance phenotype, unable to discriminate acute hyperthermia sensitive strains from resistant strains. In comparison, time to loss of equilibrium and its derivative CTE were reported to produce more phenotypic variations: Ineno et al. (2019) found, in rainbow trout, a

coefficient of variation for time to loss of equilibrium of minimum 16% while it was, for CT<sub>max</sub>, of maximum 1.4%. We therefore preferred to use CTE rather than CT<sub>max</sub> in the present study. In addition, we decided to progressively reduce the rate of temperature increase in the course of each challenge (Supplementary Fig. S1) to obtain the finest possible resistance phenotype while in the critical thermal methodology temperature increase is recommended to remain constant and linear (Becker and Genoway, 1979). These two modifications from the standard protocol (CTE and non-linear temperature increase rate) effectively produced important phenotypic variations for acute hyperthermia resistance as coefficients of variations were  $9.7 \pm 0.5\%$  in EC and  $11.3 \pm 0.9\%$  in LC. In hypoxia challenges, the protocol based on the same principles (CHE and non-linear O<sub>2</sub> depletion rate) successfully achieved the same objective: coefficients of variation of CHE were  $35.5 \pm 0.5\%$  in EC and  $31.3 \pm 1.7\%$  in LC.

Despite a significant effect of replicate on resistance in the four challenges, our methodology was found repeatable as rankings of the six isogenic lines reduced and centred by replicates were very similar from one replicate to another within each of the four challenges (Supplementary Fig. S3). We can therefore reasonably assume that the slight variations in temperature or O<sub>2</sub> saturation observed between replicates (Supplementary Fig. S1) had negligible effects on ranking of genotypes within challenges. We also carefully controlled ammonia and CO<sub>2</sub> concentrations by renewing water in tanks during challenges. NH<sub>4</sub><sup>+</sup> concentrations were relatively high during hyperthermia challenges (EC:  $2.5 \pm 0.6$  mg/L; LC:  $1.4 \pm 0.2$  mg/L) but given the measured pH (EC:  $6.6 \pm 0.03$ ; LC:  $6.5 \pm 0.1$ ), ammonia was not toxic at this concentration (Randall and Tsui, 2002). In hypoxia challenges, NH<sub>4</sub><sup>+</sup> concentration was also below the toxicity threshold (NH<sub>4</sub><sup>+</sup> concentration lower than 0.3 mg/L; pH lower than 6.8). The highest measured CO<sub>2</sub> concentration in this study was in LC hyperthermia (22 mg/L). A 6-month exposure to an equivalent CO<sub>2</sub> concentration ( $24 \pm 1$  mg/L) was shown to have no significant impact on growth and survival of rainbow trout (Good et al., 2010). We can therefore assume that the effect of CO<sub>2</sub> on resistance phenotypes remained small in our experiment. Last but not least, in late acute hyperthermia challenges, oxygen saturation reached a minimum of 65% which may reduce the acute hyperthermia resistance of fish as it was shown in estuarine red drum (*Sciaenops ocellatus*) and the marine lumpfish (*Cyclopterus lumpus*) (Ern et al., 2016). However, the oxygen saturation minimum of 65% was reached at the beginning of late challenges, when the water temperature in the challenge tank was not yet lethal (below or equal to 24 °C). In the following 7.5 h of the challenges, O<sub>2</sub> saturation was quickly increased and maintained above 80% (Supplementary Fig. S1-B), limiting the effect of low oxygen saturation on acute hyperthermia resistance.

#### 4.2. Effect of body weight on acute hyperthermia and hypoxia resistance at different ages

Significant differences in mean BW were observed between isogenic lines (Table 1) despite important values for the phenotypic coefficients of within-line variation of  $19 \pm 2\%$  in EC and  $12 \pm 1\%$  in LC, which are consistent with previous experiments on the same isogenic lines (Lallias et al., 2017). Taking advantage of these large intra-line variations in BW, we examined the effects of BW on hyperthermia and hypoxia resistances in rainbow trout.

We can highlight two points concerning BW effect on acute hyperthermia resistance. Firstly, effect of BW was found different between the two age groups. In EC, BW had a negative influence on acute hyperthermia resistance, i.e. lighter fish were found more resistant to hyperthermia. In LC, BW was found to have no significant influence on hyperthermia resistance. These results are consistent with the current overall scientific consensus: body size (BW or body length) has an either absent or negative effect on hyperthermia resistance in fish (McKenzie et al., 2021). Concerning rainbow trout, results vary from one study to another. Several studies found no significant effect of body size on

hyperthermia resistance (Carline and Machung, 2001; Galbreath et al., 2004; Molony et al., 2004; Recsetar et al., 2012; Zhang et al., 2018). In contrast, Jackson et al. (1998) and Roze et al. (2013) found a significant negative effect of body size on temperature resistance. Only Perry et al. (2001) found a significant positive effect. Variation in results between studies probably depends on numerous factors among which experimental protocols (resistance phenotype measured as CT<sub>max</sub> or effective time to LOE, temperature increase rate, acclimation temperature), fish (age, health conditions, body weight range, strain), size indicator (body weight or body length) and power of the experiment (number of individuals).

Secondly, in EC, slopes of the regression of acute hyperthermia resistance on BW were found heterogeneous between the six isogenic lines meaning that BW effect was different depending on fish's genotypes. However, the magnitude of this biological interaction remained weak as the statistical model accounting for heterogeneous slopes among lines only marginally improved the  $r^2_{(m)}$  of the model compared to the model accounting for a unique BW regression slope (Table 2). Similarly, Hartman and Porto (2014) reported a small but significant interaction between body length, which is >0.9 correlated with BW in rainbow trout (Haffray et al., 2013), and strain effect on hyperthermia resistance in three strains of rainbow trout. The age of fish used in Hartman and Porto (2014) is not specified but given the mean body weights of the three strains (respectively 21 g, 27 g and 40 g), fish seem to be closer to the life stage of fish we used in EC than the ones used in LC. This example, combined with our results, show the potential existence of a differential effect of body size on hyperthermia resistance depending on the fish genotype at early ages in rainbow trout.

BW effect on hypoxia resistance was found negative in both EC and LC, i.e. lighter fish were found more resistant to hypoxia. This result was unexpected as Roze et al. (2013) found a significant positive correlation between BW and acute hypoxia resistance in rainbow trout. Moreover, two reviews (Nilsson and Östlund-Nilsson, 2008; Rogers et al., 2016) stated that, in general, BW has a null or positive effect on hypoxia resistance in fish. Few studies obtained a result similar to ours, in red drum (*Sciaenops ocellatus*) (Pan et al., 2016) and Atlantic menhaden (*Brevoortia tyrannus*) (Shimps et al., 2005). A possible explanation for this unexpected result is that in previous hypoxia resistance experiments, experimenters did not have access to repeated acute hypoxia resistance phenotypes of identical genotypes measured on different individuals as we have in this experiment thanks to isogenic lines. When isogenic line effect is removed from the model (keeping the BW effect and replicate random intercept), then BW effect becomes non-significant in both EC hypoxia and LC hypoxia. Phenotyping hundreds of fish from six different isogenic lines gives a new insight into BW effect on hypoxia resistance which should be confirmed in further experiments.

Furthermore, as in hyperthermia EC, effect of BW was found significantly different between isogenic lines in hypoxia EC. Similarly, Wang et al. (2017) reported a different effect of BW on acute hypoxia resistance in six strains of channel catfish (*Ictalurus punctatus*). These interactions between isogenic line and BW on acute hyperthermia and hypoxia resistances in EC are likely to blur the identification of resistant fish in a hypothetical breeding program if fish are phenotyped too early. Indeed, in the present study, it is possible to estimate the interaction between genotypes (each genotype being an isogenic line) and BW on resistance phenotypes as tens of individuals were phenotyped per isogenic line. In our particular case, it is possible to adjust resistance phenotypes for the BW effect and its interaction with genotypes. In a classic breeding program, each individual represents a unique genotype. It is therefore not possible to estimate the interaction between genotype and BW on resistance phenotypes and thus to correct it. This statement must however be tempered by the fact that although significant, the interaction between isogenic line and BW remained weak as shown by the marginal improvement in  $r^2_{(m)}$  between the models with and without the interaction term (Table 2) and therefore only slightly correct the resistance phenotypes.

The negative relationships between growth and resistance to acute hyperthermia in EC or hypoxia in EC and LC can potentially result from a trade-off which is a usual feature of fitness-determinant traits. For instance, in rainbow trout, trade-offs were reported between growth and various robustness traits such as aerobic scope and maximum metabolic rates (Allen et al., 2016), immunity (Gallardo-Hidalgo et al., 2021) or acute hyperthermia resistance (Roze et al., 2013). Conversely, Roze et al. (2013) found no trade-off between acute hypoxia resistance and growth in rainbow trout as the fast-growing strain was significantly more resistant to acute hypoxia. However, if the negative phenotypic relationship we found between hypoxia resistance and BW also exists at the genetic level and at harvest age, this could have a negative impact on fish resistance to hypoxia, as growth is one of the main selected traits in fish breeding programs (Chavanne et al., 2016). Further studies are needed to estimate the genetic correlations between BW at harvest and acute hyperthermia and hypoxia resistance in commercial populations of rainbow trout.

#### 4.3. Genotypic resistance stability to acute hyperthermia or acute hypoxia challenges at different ages

Isogenic line effect was found significant in the four challenges (Table 2). Given the control on other factors influencing temperature and hypoxia resistances, it is most probably due to heritable genetic differences between lines. This result is consistent with the literature. Heritable genetic basis for acute hyperthermia resistance was demonstrated in several fish species (reviewed in McKenzie et al., 2021) and more specifically in rainbow trout where heritability was found to be significant (Perry et al., 2005). Although less studied, there is also some evidence for the existence of a genetic basis for hypoxia resistance in salmonids as significant differences were found between families of Atlantic salmon (Anttila et al., 2013) and between strains of rainbow trout (Scott et al., 2014; Zhang et al., 2018; Strowbridge et al., 2021).

These significant differences in resistance between isogenic lines at these two ages enabled us to study the stability of these traits over time by comparing the resistance of the six lines to hyperthermia and hypoxia in EC and LC. A major advantage of using isogenic lines was that phenotyped individuals were different between EC and LC. Therefore, there was no habituation of fish to acute hyperthermia or hypoxia challenges and therefore no repetition bias, which is not the case in most studies already published on the subject. In EC, we considered resistance rankings at 15.2 g, the mean BW in EC, given the slightly heterogeneous BW coefficients of regression on resistance between isogenic lines. In LC, resistance rankings of isogenic lines were found independent of BW but to remain consistent with EC, we considered resistance rankings at 237 g, the mean BW in LC.

For acute hyperthermia resistance, ranking of isogenic lines was relatively well maintained between EC and LC with the notable exception of N38h which shifted from the most sensitive to the most resistant isogenic line. This shift in resistance was not random as N38h rankings (most sensitive line in EC, most resistant line in LC) were repeated in the three replicates in both EC and LC (Supplementary Fig. S3). Therefore, we can assume that, in general, ranking remained stable between ages for acute hyperthermia resistance, except for a few genotypes. We can only speculate on the causes of the shift in resistance of line N38h. A possible assumption would be that identical farming conditions have led to differential thermal plasticity between N38h and other isogenic lines: environmental conditions before EC or before LC have respectively weakened or strengthened N38h compared to other lines. This option seems likely as isogenic lines experienced a summer period before LC with water in rearing tanks reaching temperatures up to 20.5 °C. An alternative hypothesis would be the difference in development or maturity between N38h and other lines which changes between EC and LC. However, further investigations with more genotypes are required to conclude formally. Our results are in line with the literature. In adult brook trout, O'Donnell et al. (2020) found that hyperthermia resistance



ranking of genotypes was significantly stable by repeating successive hyperthermia challenges on 22 individuals at 2 and 3 years old. Strowbridge et al. (2021) also found a stable hyperthermia resistance ranking between three strains of rainbow trout at the fry and yearling stage, 6 months apart. In contrast, Mauduit et al. (2016) found no repeatability over 11 months of acute hyperthermia resistance in sea bass at the individual level. This result could be explained by the fact that fish were reared in semi-natural uncontrolled ponds between the repeated challenges, with potential strong genotypes  $\times$  environment interactions on acute hyperthermia resistance.

For hypoxia resistance, ranking of genotypes was also well maintained between EC and LC suggesting that the resistance to hypoxia remains stable between 6 and 15 months in rainbow trout. Once again, this result is consistent with the literature. In the same experiment, Strowbridge et al. (2021) found a stable hypoxia resistance ranking between three strains of rainbow trout at the fry and yearling stage. Joyce et al. (2016) showed that hypoxia resistance, measured twice on 16 individuals, is a stable trait in adult European seabass over a period of 15 months (Pearson's correlation coefficients,  $r = 0.62$ ,  $P < 0.0001$ ). Nelson et al. (2019) and Mauduit et al. (2016) came to the same conclusion with 13 of juveniles striped bass over a period of 6 months and with ~190 seabass over a period of 11 months, respectively. The stability of resistance observed between the early and late stages for acute hyperthermia and hypoxia resistances means that it would be possible to select at early stages for these traits and thus limit costs of potential breeding programs.

#### 4.4. Stability between acute hyperthermia resistance and acute hypoxia resistance

Hyperthermia and hypoxia resistance in fish species are often compared together because, in aquatic environments, acute hyperthermia and hypoxia stresses frequently occur in combination explaining the interest in selecting for both traits simultaneously. Therefore, it is essential to understand the link between resistance to hyperthermia and hypoxia.

Association between hyperthermia and hypoxia resistance traits was studied in various fish species and at various life stages but no consensus was reached. Indeed, the two traits were found independent in killifish (*Fundulus heteroclitus*) with no shared genetic markers associated with hyperthermia resistance and hypoxia resistance (Healy et al., 2018), in sheepshead minnow (*Cyprinodon variegatus*) where two subspecies presented similar hyperthermia resistance but very different hypoxia resistance (Jung et al., 2019) and in zebrafish with no significant correlation between hyperthermia and hypoxia resistances at the individual level but with only 12 individuals (Joyce and Perry, 2020). On the other hand, Anttila et al. (2013) found a significant and strong positive correlation of 0.69 between hyperthermia and hypoxia resistance in 41 families of Atlantic salmon. In rainbow trout, Zhang et al. (2018) found that resistance ranking was similar in hyperthermia and hypoxia challenges in three strains suggesting a possible positive association between the two traits. Still in rainbow trout, Strowbridge et al. (2021) estimated phenotypic correlations between hyperthermia and hypoxia resistances at the individual level using a robust experimental design composed of four strains, at fry and/or yearling ages, with more than one hundred individuals per strain and age combinations. Within all strain and age combinations, fish resistant to hyperthermia were found resistant to hypoxia and correlations between the two traits were significant in four age and strain combinations out of six. However, contrary to Anttila et al. (2013), in Strowbridge et al. (2021) correlations were very weak (0.12–0.23).

In the present study, we also investigated this question with the main advantage of using isogenic lines: fish were different between hyperthermia and hypoxia challenges avoiding interference between the repeated challenges while keeping fish with the same genotype within the different lines. We observed significant changes in ranking between

acute hyperthermia resistance and acute hypoxia resistance in EC and LC (Fig. 4A&B). In EC, A32h and B45h, the most resistant lines for hyperthermia, were the most sensitive lines for hypoxia. Comparably, in LC, A32h was the second most resistant line for hyperthermia and the most sensitive line for hypoxia. B45h was also reclassified downwards between hyperthermia and hypoxia challenges but less dramatically than in LC. However, for the four other lines, rankings were stable between hyperthermia and hypoxia resistances in EC and LC (Fig. 4A&B). The best example is N38h, which was the most resistant line in hyperthermia and hypoxia LC. Lines A32h and B45h appear to be genetically more distant from the other four for hyperthermia and hypoxia resistance traits. This suggests that the relationship between the hyperthermia and hypoxia resistance traits may differ according to the genotype. Thus, there would be no general antagonism between hyperthermia and hypoxia resistances traits. Therefore, it seems possible to select genotypes that are resistant to both stress factors. Our results, consistent between the two studied ages, indicated that resistances to hyperthermia and hypoxia are, at least partially, based on different physiological mechanisms in rainbow trout which confirm results of Healy et al. (2018); Joyce and Perry (2020); Jung et al. (2019); and Strowbridge et al. (2021).

According to this result, knowing an individual's hyperthermia resistance does not accurately predict its resistance to hypoxia and vice versa. Therefore, to select the two traits simultaneously, both challenges must be completed. Some genotypes were very resistant to both hyperthermia and hypoxia (e.g. N38h in LC). These genotypes are of great interest for the simultaneous genetic selection of these traits.

## 5. Conclusion

In conclusion, by comparing resistance to hyperthermia and hypoxia challenges at two different ages in six distinct heterozygous isogenic lines of rainbow trout, our results tend to show several essential points of relevance for introducing both traits into rainbow trout breeding programs:

- 1) Significant between-lines variations in resistance were found for both hyperthermia and hypoxia in EC and LC, providing further evidence of the possibility of selecting for these traits. BW effect on hyperthermia resistance was significantly negative in EC and non-significant in LC and significantly negative on hypoxia resistance in EC and LC.
- 2) Overall, resistances to acute hyperthermia and hypoxia were found stable between 6 months and 15 months in rainbow trout. However, this does not seem to be true for all genotypes as some may exceptionally reclassify significantly. Therefore, it would be possible to select for resistance to hyperthermia and hypoxia at an early stage.
- 3) No overall relationship was found between hyperthermia resistance and hypoxia resistance in EC and LC as some lines were resistant to both challenges while other lines were resistant to one but sensitive to the other. This suggests that there are no strong antagonistic genetic effects between the two traits, meaning that both traits could be selected simultaneously in rainbow trout breeding programs.

## CRedit authorship contribution statement

**H. Lagarde:** Investigation, Resources, Methodology, Software, Formal analysis, Data curation, Validation, Visualization, Writing - original draft, Writing - review & editing. **F. Phocas:** Methodology, Software, Writing - review & editing. **S. Pouil:** Investigation, Resources, Writing - review & editing. **L. Goardon:** Investigation, Resources. **M. Bideau:** Investigation, Resources. **F. Guyvarc'h:** Investigation, Resources. **L. Labbé:** Resources. **N. Dechamp:** Resources. **M. Prchal:** Investigation, Resources, Writing - review & editing. **M. Dupont-Nivet:** Supervision, Conceptualization, Methodology, Investigation, Resources, Formal analysis, Funding acquisition, Project administration,



Validation, Writing - review & editing. **D. Lallias**: Supervision, Conceptualization, Methodology, Investigation, Resources, Formal analysis, Funding acquisition, Project administration, Validation, Writing - review & editing.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data will be made available on request.

## Acknowledgements

This study was supported by the European Maritime and Fisheries Fund and FranceAgrimer (Hypotemp project, n° P FEA470019FA1000016) and ANR PIA funding: ANR-20-IDEE-0002.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.aquaculture.2022.738800>.

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