Cold temperature during embryonic development and its influence on responses to acute confinement and hyperthermia challenges in juvenile rainbow trout

Simon Pouil, Elodie Harté, Michaël Marchand, Lionel Goardon, Tina Terrenne, Laurent Labbé, Mathilde Dupont-Nivet, Isabelle Leguen, Delphine Lallias

Cold temperature during embryonic development and its influence on responses to acute confinement and hyperthermia challenges in juvenile rainbow trout

Simon Pouil[1], Elodie Harté[1], Michaël Marchand[1], Lionel Goardon[2], Tina Terrenne[2], Laurent Labbé[2], Mathilde Dupont-Nivet[1], Isabelle Leguen[3], Delphine Lallias[1]

[1] Université Paris-Saclay, INRAE, AgroParisTech, GABI, Jouy-en-Josas, France

[2] INRAE, UE 0937 PEIMA (Pisciculture Expérimentale INRAE des Monts d'Arrée), Sizun, France

[3] MARBEC, Univ. Montpellier, CNRS, Ifremer, IRD, INRAE, 34250, Palavas-Les-Flots, France

* Corresponding author: Simon Pouil

INRAE – UMR GABI

Domaine de Vilvert, Jouy-en-Josas, France

E-mail: simon.pouil@inrae.fr

Abstract

To expand the availability of marketable eyed eggs, a common practice in rainbow trout aquaculture is to store eyed eggs at low temperatures (2-4°C) for periods of up to 2-3 weeks. Early exposure to environmental stimuli such as temperature can impact fish physiology, growth, metabolism, and nutrition at mid- or long-term questioning about the potential effects of such breeder practice. This study aimed to test the impact of incubating eyed eggs at low temperatures (3°C instead of 12°C) for 15 days on resistance to later stresses using two experimental rainbow trout lines divergent for fat content. Plasma parameters (*i.e.* cortisol, glucose, lactate, and chloride ions) have been measured after an acute confinement challenge. For the hyperthermia challenge, time at the loss of equilibrium was compared between lines and incubation temperatures. Our results showed that deformities rate and early survival were not affected by cold temperature storage. Furthermore, plasma parameters measured after the confinement were not impacted by temperature. The two lines differently responded to the hyperthermia challenges with the fat line showing significantly higher resistance to temperature elevation. These findings suggest that the incubation temperature did not impair the responses to stress at the juvenile stage, indicating that the practice of cold storage of eyed eggs does not appear to be detrimental to subsequent rearing.

Keywords: Cold; Incubation; Eyed-eggs; Salmonids; Stress

1. Introduction

Rainbow trout (*Oncorhynchus mykiss*) is the predominant species in French aquaculture and globally significant in salmonid production, contributing to 953,000 tonnes in 2021, valued at approximately 4.37 billion US dollars (FAO, 2023). Rainbow trout reproduction is governed by photoperiodic cues (Whitehead and Bromage, 1978; Bromage et al., 1982), allowing for the manipulation of spawning times in aquaculture. Protocols enable the advancement or delay of spawning by up to 6 months (Bromage et al., 1993), facilitating year-round spawning for selective breeding. Additionally, breeding companies commonly store eyed eggs at cold temperatures to extend their market availability (Maddock, 1974; Richardson et al., 2002). However, while cold temperature manipulation slows embryonic development and delays hatching (Piper et al., 1982), its long-term consequences remain poorly investigated.

Chilled storage effects on unfertilised rainbow trout eggs have been extensively studied (Niksirat et al., 2007; Ubilla et al., 2016), establishing storage conditions that preserve their fertilising capacity (Komrakova and Holtz, 2009). However, the consequences of chilling fertilised eggs are less understood. Studies have shown that early incubation at low temperatures post-fertilisation affects embryonic development and reduces survival until the eyed stage (Stonecypher et al., 1994; Babiak and Dabrowski, 2003). Rainbow trout embryos' tolerance to chilling increases as they develop (Leveroni Calvi and Maisse, 1998). Richardson et al. (2002) extended the storage time of chilled rainbow trout eyed eggs by 35 days at 1 °C using perfluorochemicals to facilitate oxygenation. While most studies focus on short-term consequences such as hatching percentage, malformations, and survival of young stages, the long-term effects on later stages (*i.e.* juveniles and adults) remain largely unknown.

This study aimed to assess the impact of cold temperature storage of rainbow trout eyed eggs on their response to two acute challenges during the juvenile stage: confinement and hyperthermia. Two experimental lines of rainbow trout, selectively bred for either low or high

muscle fat content, were used (Quillet et al., 2005). Given the observed differences in energy utilisation and intermediate metabolism between these lines (Kolditz et al., 2008a, 2008b, 2010; Kamalam et al., 2012), we hypothesised that their responses to cold temperatures during incubation would differ.

2. Materials and methods

2.1. Ethics statement

All the experiments were conducted at the INRAE experimental facilities (PEIMA, INRAE, 2021, Fish Farming systems Experimental Facility, doi: 10.15454/1.5572329612068406E12, Sizun, France) authorised 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 authorised by the French Ministry of Higher Education and Research (APAFIS #31861-2021060117041206 v4).

2.2. Fish and rearing conditions

The study was conducted with two INRAE experimental lines of rainbow trout (*Oncorhynchus mykiss*), designated as Fat line (FL) and Lean line (LL), obtained after seven generations of divergent selection for respectively high or low muscle fat content using a non-destructive method (Distell Fish Fat Meter ®) as detailed by Quillet et al. (2007). Muscle fat content was found to be more than 3 times higher in the FL line (8.0%) than in the LL line (2.3%) in 200 g-trout after five generations of selection (Jin et al., 2014).

For each of the two lines, clutches from 20 females among those available on the same day were selected (*i.e.* homogeneity of the size of the oocytes between clutches, absence of blood, fatty deposits, and degraded oocytes). To obtain approximately 500 eggs per female, we estimated the weight needed from each clutch based on the average weight of an egg. The eggs from the 20 females have been pooled and coelomic liquid was added to homogenise the

pool. The eggs were then drained and redistributed into 20 cups for fertilisation with sperm from 20 males. All fertilisations were carried out on the same day. Fertilised eggs from each line were then incubated in hatching trays supplied with spring water at 12.0°C.

At 17 days post fertilisation (dpf), eyed eggs from the two experimental lines were distributed into small incubators installed in two separate 200-L tanks supplied with natural spring water at either 12.0°C (control) or chilled at 3.0°C (cold storage condition) for 15 days to mimic selective breeders' practices. For each line, three incubators containing 500 eyed eggs were used for each incubation temperature condition (2 lines x 2 incubation temperatures x 3 incubators). For the cold storage condition, the water temperature was gradually lowered at a rate of 3.0°C h^{-1} and then maintained at 3.0 ± 0.5°C using a water chiller (Cooling Plus Energy system, Hitema). After 15 days of cold storage, water temperature was increased at a rate of 3.0°C h^{-1}, by allowing a controlled stream of water at the targeted temperature to flow, reaching a final temperature of 12.0°C. The temperature was recorded continuously while the water flow was kept at 20 L h^{-1}.

Fry were kept in incubators and individuals with deformities were counted and removed regularly. Before the first feeding only viable individuals were transferred into 0.3 m^3 indoor tanks supplied with natural spring water at 12.0°C and lit by artificial neon lights from 8:00 AM to 8:00 PM, without mixing between lines and incubation temperature conditions (n = 3 tanks per condition and ~270 individuals per tank). At the age of 78 dpf (average weight: 0.6-1.0 g), the fry were transferred first into covered 0.3 m^3 tanks under a greenhouse and supplied with lake dam water and then, at 155 dpf, into covered 1.8-m^3 outdoor fiberglass tanks. From 78 dpf and up to the challenges, fish were subjected to normal seasonal temperature variations throughout the concerned period (daily mean temperature: 8.0 to 18.0°C) and lit by natural light. Until the beginning of the experiment, the fish were fed daily to satiation using automatic feeders with a commercial diet from Le Gouessant's company.

The growth was followed regularly by weighing a random subsample of 50 fish from each tank while the fish individual body weight was measured at 190 dpf (n = 353-459 per condition). Mortality has been checked daily throughout the experiment. Over the rearing period, O_2 concentrations in the outlet water ranged from 8.5 to 9.9 mg L^{-1} (*i.e.* 85-99 % of saturation level) while NH_4^+ and NO_2^- concentrations ranged from 0.2 to 0.7 mg L^{-1} and <0.01 to 0.8 mg L^{-1}, respectively (assessed on a bi-weekly basis on average).

The course of the different rearing stages and challenges performed is shown in Figure 1.

2.3. Confinement challenge

Acute confinement challenges were performed over three days at 217-219 dpf. About one month before the challenges, fish from each line and condition were transferred into 0.3 m^3 tanks still supplied with lake dam water and kept separate with 50 fish in each tank (n = 3 tanks per condition). Before the start of the confinement challenge, all the fish were starved for 24h and eight of them were euthanised by an overdose of anaesthetic (Tricaïne MS-222, 100 mg L^{-1}), weighed using digital scales (± 0.1 g), and immediately sampled for blood. Blood was taken from the caudal vein using heparinised syringes (heparin lithium, Sigma-Aldrich, USA). Blood samples were kept on ice until plasma was separated from whole blood by centrifugation at 2,500 g for 10 min at 4°C. Plasma samples were stored at -20°C before analysis. These fish constituted the time-zero control group before the confinement challenge. Then, 8 fish from each tank were transferred into a bucket filled with water according to the average weight per tank to reach a fish density of 200 kg m^{-3} and kept for 4 minutes with no water renewal or oxygen supplementation. Challenged fish were transferred under flow-through water conditions into a 0.3 m^3 recovery tank for 1h and then euthanised as described above, weighed, and sampled for blood according to the same procedure as for fish before confinement.

Glucose and lactate were measured in plasma (5 µL per measurement) using a portable digital blood glucose meter ACCU-CHEK ® Active (Roche Diagnostic Systems, Herts, UK) and a THE EDGE ® blood lactate analyser (APEXBIO, Taiwan), respectively.

Plasma cortisol was extracted by adding 1 mL diethyl ether to 100 µL of plasma in a 5 mL glass tube. The sample and solvent were vortex-mixed and frozen at -20°C for at least 1h to allow the phases to separate. The supernatant was then transferred into a 2 mL glass vial and evaporated at room temperature under a stream of nitrogen. The residue was dissolved in 100 µL of extraction buffer provided in the commercial ELISA kit (Cortisol ELISA KIT; Neogen ® Corporation) used for plasma cortisol measurements and performed according to the manufacturer's instructions.

Chloride ions were measured in plasma using a commercial kit (Kit Chlorures, Biolabo ®). Plasma (3 µL), diluted at ½ in deionised water, reacted with 300 µL of Hg(II) thiocyanate for 5 min at room temperature, and then absorbance was read at 500 nm. For both cortisol and chloride ions, measurements have been performed in triplicates, and values were calculated from reference standard curves.

2.4. Hyperthermia challenges

Acute hyperthermia challenges were performed at 224-226 dpf. At 191 dpf, fish were individually PIT-tagged (Biolog-id ®) and grouped into 3 separate 0.3 m^3 tanks containing each 200 fish, *i.e*. 50 fish per line and incubation temperature. PIT tags (pit-tag length: 12 mm, diameter: 2 mm, weight: 0.092 g) were injected horizontally into the dorsal muscle just behind the head. Animals were starved for three days before challenges according to Lagarde et al. (2023b). The evening before each challenge, fish were transferred into the challenge tank (0.3 m^3), supplied with the same lake dam water as the one used in the rearing tanks, and left alone for the night for acclimation. Temperature was gradually increased from 8.5 °C to 27.5 °C by renewing challenge tank water with heated water from a buffer tank. Temperature

was first quickly increased at a rate of 0.7°C every 10 minutes until 22°C. This period was followed by a slower temperature increase at a rate of 0.1°C every 15 minutes until all fish have lost equilibrium. This slower temperature increase was intended to increase the between-fish variability of acute hyperthermia resistance phenotypes (Lagarde et al., 2023b). Water was oxygenated to keep oxygen levels near saturation (Figure S1). Temperature and O_2 concentration and saturation were recorded every 5 min during challenges using electronic probes (HQ30d, Hach Company, Loveland, CO, USA), while NH_4^+ concentration was checked twice, *i.e.* at peak of loss of equilibrium - determined as the sudden increase in the number of fish losing equilibrium - and at the end of the challenge, using a commercial colorimetric kit (LCK 304, Hach Company, USA). CO_2 concentration was measured continuously with a CO_2 analyzer (Oxyguard, Denmark). The maximum concentrations measured were 2.6 mg L^{-1} and 9 mg L^{-1} for NH_4^+ and CO_2, respectively.

As the temperature increased, fish were gradually losing equilibrium. When a fish lost equilibrium, it was removed from the tank, its origin was identified using the PIT tag and the exact time of loss of equilibrium was recorded ($Time_{loss}$) as the phenotype of interest (see Section 4 for details). Fish were then softly anesthetised (Tricaïne MS-222, 50 mg L^{-1}), weighed using digital scales (± 0.1 g), and euthanised by an overdose of anaesthetic (Tricaïne MS-222, 100 mg L^{-1}). Challenges ended when the last fish lost its equilibrium.

2.5. Data analysis

For the deformity rate and survival rates, the assumptions of normality and homoscedasticity were not met. Hence, non-parametric Kruskal-Wallis tests followed by Dunn's tests with Bonferroni adjustment for p-values were used to analyse the differences between experimental conditions. Body weight differences among experimental conditions at 190 dpf, *i.e.* before the confinement and acute hyperthermia challenges, were analysed 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.

Concerning the confinement challenges, the plasma parameters were measured for four out of the eight sampled fish (*i.e.*, 12 fish per experimental condition and sampling time). A linear mixed model was computed with "line", "temperature" and "sampling time" (before or after the confinement challenge) as fixed effects and "replicates" as a random effect.

Acute hyperthermia resistance was quantified as the time at the loss of equilibrium ($Time_{loss}$; expressed in min). Differences in $Time_{loss}$ were compared between lines and incubation temperatures using individuals as the experimental unit. A linear mixed model was computed with "line", "temperature" and "weight" - given the differences in size between the two lines for this challenge (see Section 3) - as fixed effects, and "replicates" as a random effect.

Models were fitted using the *lme4* and *nlme* packages, and contrasts were analysed using the *emmeans* package. The interaction terms were not significant and not included in the final models. The best models for fixed effects were chosen with Akaike information criteria (AIC) and F-tests using the *lmerTest* package. The marginal r^2 (r^2_m) and the conditional r^2 (r^2_c) were calculated using the *MuMin* package (Barton, 2020). r^2_m is the proportion of variance explained by fixed factors and r^2_c is the proportion of variance explained by both fixed and random factors (Nakagawa and Schielzeth, 2013). Assumptions of normality and homoscedasticity were checked by visual inspections of residual-fit plots and log and square-root transformations were operated on lactate and cortisol data, respectively.

The significance level for statistical analyses was set to $\alpha = 0.05$. All statistics were performed using R freeware version 4.2.2 (R Development Core Team, 2022). Throughout the manuscript, values are given as mean ± standard deviation.

3. Results

The incubation temperature exhibited no discernible impact on the fry's deformity rate from hatching to the first feeding, with an average value of 6% across all experimental conditions ($H_{(3)}$ = 7.21, P = 0.07). Similarly, the survival rate during this phase remained unaffected by the incubation temperature ($H_{(3)}$ = 2.13, P = 0.55), with an average of 90% observed across all conditions during the indoor rearing phase (Table 1). In the outdoor rearing phase, survival rates slightly differed according to experimental conditions ($H_{(3)}$ = 8.44, P = 0.04) with survival rates significantly lower in LL kept at 3°C (80.8 ± 3.3 %) than FL under control temperature (94.4 ± 1.2 %). Interestingly, within the same line, we did not find significant effects of incubation temperature on survival rate (Table 1). As anticipated, the average individual weight, measured at 190 dpf one month before the confinement and hyperthermia challenges, was affected by both line ($F_{(1)}$ = 103.76, P < 0.001) and incubation temperature ($F_{(1)}$ = 107.23, P < 0.001). Fish from the LL line surpassed those from the FL line in size (40.4 ± 11.5 g vs 35.3 ± 11.1 g in normal temperature conditions), and those subjected to cold storage during incubation exhibited smaller sizes (35.2 ± 12.0 g and 29.2 ± 9.8 g for LL and FL, respectively) (Table 1). Basal blood parameters (i.e. chloride ions, cortisol, glucose, and lactate) were similar between both lines (Figure 2).

Following the confinement challenge, there was a significant increase in plasma cortisol levels ($F_{(1)}$ = 925.47, P < 0.001) with values ranging from 2.6 ± 2.8 to 80.0 ± 24.0 ng mL^{-1}, before and after the challenge respectively. On the other hand, neither the genetic lines nor incubation temperature at the eyed eggs stage affected the cortisol levels in both control and challenged fish (Figure 2). The same trend was observed for glucose ($F_{(1)}$ = 184.86, P < 0.001) and lactate concentrations ($F_{(1)}$ = 327.42, P < 0.001) with significant increases in concentrations after the confinement challenge for both lines but no significant difference between lines and incubation temperatures. Chloride ion concentrations were similar between

231 the experimental conditions and remained constant over the challenge (143 ± 10 mM; Figure
232 2).

233 The kinetics of loss of equilibrium over the acute hyperthermia challenge are presented in
234 Figure 3. Even when accounting for the significant effect of the weight ($F_{(1)}$ = 34.75, P <
235 0.001; Table 2), we found significant differences between both experimental lines ($F_{(1)}$ =
236 22.79, P < 0.001) with LL being less resistant ($Time_{loss}$: 547 ± 59 min) than FL ($Time_{loss}$: 581
237 ± 64 min). The incubation temperature did not significantly affect the resistance to
238 hyperthermia ($F_{(1)}$ = 0.23, P = 0.628).

239 **4. Discussion**

240 To our knowledge, this is the first study to investigate how cold temperature storage of
241 rainbow trout eyed eggs affects the response to subsequent juvenile challenges. Previous
242 research suggests that early environmental stimuli, such as temperature, can have medium to
243 long-term effects on fish physiology (Auperin and Geslin, 2008; Scott and Johnston, 2012;
244 Mateus et al., 2017). Considering the potential impact of cold storage on early-stage
245 zootechnical performances (Stonecypher et al., 1994; Richardson et al., 2002; Babiak and
246 Dabrowski, 2003), we examined its effects on deformities and early survival before first
247 feeding. Our findings indicate that cold storage of eyed eggs did not significantly affect these
248 parameters, consistent with Richardson et al.'s observations (2002).

249 In subsequent rearing phases, the growth disparities observed at 190 dpf were attributed to the
250 earlier hatching of animals kept at 12°C during incubation (see Figure 1). This aligns with
251 expectations, as cold storage slows down embryonic development and delays hatching
252 (Stonecypher et al., 1994; Babiak and Dabrowski, 2003). However, to accurately assess the
253 impact of cold storage on growth compared to control condition, mean body weight should
254 have been measured at a constant number of days post-hatching, which was not done in this
255 study. The effects of low incubation temperature were further evaluated through blood

parameter assessments in 7-month-old fish. Measurements of chloride ions, cortisol, glucose, and lactate obtained before the challenges showed no significant differences between the incubation temperatures. To further investigate the effects of cold storage, the fish were subjected to challenges involving two acute stressors.

Stress responses in teleost fishes involve three phases: primary, secondary, and tertiary. The primary response includes the release of catecholamines and cortisol, triggering secondary responses such as increased plasma glucose, lactate, and heart rate, as well as decreased plasma chloride, sodium, potassium, liver glycogen, and muscle protein (Pickering, 1981; Mommsen et al., 1999; Barton, 2002). These secondary responses can lead to tertiary responses, including reduced growth rate, metabolic scope, disease resistance, reproductive capacity, and altered behaviour and survivability (Wedemeyer et al., 1990; Barton and Iwama, 1991; Mommsen et al., 1999). Given the intensity and duration of the stressor, the challenges in this study can be considered as acute (Schreck and Tort, 2016). The hyperthermia challenge induced a loss of equilibrium, indicating a phenotype of resistance (Fry, 1971). In contrast, the confinement challenge focused on primary stress responses, measuring cortisol synthesis, and secondary responses through investigations on energy metabolism (glucose and lactate) and hydromineral balance (chloride ions).

Several studies have investigated the effects of confinement on physiological stress indicators in salmonids. Cortisol levels typically increase in response to confinement stress lasting between 2 minutes and 4 hours (Pickering et al., 1991; Jentoft et al., 2005; Sadoul et al., 2015; Magnoni et al., 2019). For example, Magnoni et al. (2019) observed a cortisol peak 1 hour after subjecting trout to confinement stress for 2 minutes at 200 kg m^{-3} before returning them to tanks. Gesto et al. (2015) made similar observations after a 3 min-disturbance stress.

Although we did not observe an effect of genetic background on the post-confinement increase in cortisol in this study, it is interesting to note that Pottinger and Carrick (1999)

demonstrated the feasibility of divergent selection on the intensity of the response to 3h-confinement stress repeated at monthly intervals for 5 months based on plasma cortisol measurements in rainbow trout performed right after the challenge. The same authors estimated a relatively high heritability for this trait ($h^2 \approx 0.4$) which suggests a strong genetic component in the cortisol responsiveness to stress in this species even if the underlying mechanisms remained unclear (Trenzado et al., 2003).

In our experiment, we found that acute stress led to a comparable release of plasma cortisol in juvenile fish, regardless of whether they were exposed to cold temperature storage at the eyed eggs stage. This pattern was consistent for both FL and LL fish lines, suggesting a uniform regulation of cortisol synthesis and release in both lines. Our confinement protocol, which involves a shorter stress duration than the protocol used by Pottinger and Carrick (1999) and a 1-hour recovery period, has proven effective in revealing genetic influences on cortisol response, as shown in preliminary investigations with other rainbow trout genetic lines (unpublished data). This suggests that, even after seven generations of divergent selection for fat content, the two experimental lines still exhibit a similar stress response after 1h of recovery.

Concerning the other physiological stress indicators measured in plasma, the increase in glucose and lactate observed indicates an establishment of the secondary response to confinement stress. Jentoft et al. (2005) observed that the glucose peak occurred 3 hours post-confinement. Here, we measured glucose and lactate at 1-hour post-stress, which agrees with previous studies performing stress of similar duration. Indeed, in a 3-min disturbance test, Gesto et al. (2015) demonstrated in rainbow trout that plasma glucose and lactate reached their maximum levels 3 min after the disturbance challenge while these levels were maintained for at least 45 min. It is interesting to note that this secondary stress response was similar regardless of cold stress carried out at the eyed eggs stage and genetic background. In

addition, the osmoregulatory ability of fish can also be disrupted by stress, inducing a change in blood osmolarity and ion contents (see review of Seibel et al. (2021)). In our experiment, however, plasma chloride ion concentrations were similar 1 hour after acute stress in all conditions. This result suggests that all fish, regardless of their life history or genetic background, could cope with acute stress.

The hyperthermia challenges conducted in this study over three days were highly repeatable, with consistent temperature rises and oxygen saturation levels across all three tests (Figure S1). Due to the consistent starting temperature (8.5°C) and the high repeatability of temperature rise profiles, we did not calculate cumulative thermal exposure (CTE) in degree-minutes, as defined by Perry et al. (2005) and used by Lagarde et al. (2023a). Currently, there is no consensus on the mechanisms behind the loss of equilibrium in acute hyperthermia conditions in fish, with differences observed between species (Lefevre et al., 2021; Desforges et al., 2023; Ern et al., 2023). Factors such as life stage, body size, phenotypic plasticity, and genetic background influence intraspecific fish hyperthermia resistance (McKenzie et al., 2021). This resistance phenotype, as measured here, has been demonstrated to be heritable (h^2 ≈ 0.3-0.4; Perry et al., 2005; Lagarde et al., 2023a), with strong variability observed between rainbow trout isogenic lines (Lagarde et al., 2023b). These results indicate a significant genetic component in hyperthermia stress resistance, explaining the differing resistance observed between the two experimental lines in this study.

Explaining the higher resistance of the FL compared to the LL to hyperthermia is challenging due to the diversity of mechanisms involved. The major phenotypic difference between these lines is their contrasting adiposity. Despite observing significant differences in average weight at the time of hyperthermia challenges, we accounted for this effect in our statistical model. Subcutaneous adiposity can act as an insulating layer, protecting organisms from temperature fluctuations and this ancient mechanism can be exploited by fish to thrive in cold oceanic

environments (Alexander et al., 2015). This could explain why the FL showed greater resistance to hyperthermia in our experiment. However, such mechanisms primarily protect organisms in low-temperature conditions rather than warm environments. Fish acclimated to high temperatures tend to have reduced lipid content in different body compartments, as observed in rainbow trout (Ingemansson et al., 1993) and Atlantic salmon (Jobling and Bendiksen, 2003). Additionally, no genetic correlation was found between resistance to acute hyperthermia and fat meter measurements in a commercial population of rainbow trout (Lagarde et al., 2023a). These findings suggest that increased resistance to hyperthermia in the FL due to physical insulation is unlikely.

The LL and FL lines also differ in their energy metabolism. Several studies showed that the FL had a higher capability to use glucose than the LL, linked to the enhancement of hepatic glycolysis, glycogen storage, and lipogenesis (Kolditz et al., 2008a; Skiba-Cassy et al., 2009; Kamalam et al., 2012; Jin et al., 2014). It is, therefore, possible that this ability to rapidly mobilise energy improves the resistance of this line to acute hyperthermia. However, additional studies that monitor plasma parameters during hyperthermia stress are necessary to test this hypothesis.

4. Conclusion

The cold storage of rainbow trout eyed eggs for 15 days did not result in drastic changes in early zootechnical performance. The responses to the acute confinement challenge after a 1-hour recovery period and to the hyperthermia challenge were not significantly influenced by the incubation temperature. Additionally, we demonstrated that the two lines exhibited differential responses to hyperthermia challenges. Based on this information, the practice of cold storage of eyed eggs does not appear to be detrimental to subsequent rearing, at least up to the juvenile stage.

Acknowledgments

356 This study was supported by the European Maritime and Fisheries Fund and France Agrimer
357 (EpiCOOL project, n° PFEA470018FA1000015) and the INRAE Animal Genetics Division.

References

Alexander, C.M., Kasza, I., Yen, C.L.E., Reeder, S.B., Hernando, D., Gallo, R.L., Jahoda, C.A.B., Horsley, V., MacDougald, O.A., 2015. Dermal white adipose tissue: A new component of the thermogenic response. J. Lipid Res. 56, 2061–2069. https://doi.org/10.1194/jlr.R062893

Auperin, B., Geslin, M., 2008. Plasma cortisol response to stress in juvenile rainbow trout is influenced by their life history during early development and by egg cortisol content. Gen. Comp. Endocrinol. 158(3), 234–239. https://doi.org/10.1016/j.ygcen.2008.07.002

Babiak, I., Dabrowski, K., 2003. Refrigeration of rainbow trout gametes and embryos. J. Exp. Zool. Part A Comp. Exp. Biol. 300, 140–151. https://doi.org/10.1002/jez.a.10319

Barton, K., 2020. MuMIn: Multi-model inference. version 1.43.17. https://CRAN.R-project.org/package=MuMIn

Barton, B.A., 2002. Stress in fishes: A diversity of responses with particular reference to changes in circulating corticosteroids. Integr. Comp. Biol. 42, 517–525. https://doi.org/10.1093/icb/42.3.517

Barton, B.A., Iwama, G.K., 1991. Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids. Annu. Rev. Fish Dis. 1, 3–26. https://doi.org/10.1016/0959-8030(91)90019-G

Bromage, N., Randall, C., Davies, B., Thrush, M., Duston, J., Carillo, M., Zanuy, S., 1993. Photoperiodism and the control of reproduction and development in farmed fish, in: Lahlou, B., Vitiello, P. (Eds.), Aquaculture: Fundamental and Applied Research. American Geophysical Union, Washington, pp. 81–102.

Bromage, N., Whitehead, C., Elliott, J., Breton, B., Matty, A., 1982. Investigations in the importance of daylength on the photoperiodic control of reproduction in the female rainbow trout, in: Richter, C.J.J., Goos, H.J.T. (Eds.), Proceedings of the International Symposium on Reproductive Physiology of Fish. Centre for Agricultural Publishing and Documentation, Wageningen, pp. 233–236.

Desforges, J.E., Birnie-Gauvin, K., Jutfelt, F., Gilmour, K.M., Eliason, E.J., Dressler, T.L., McKenzie, D.J., Bates, A.E., Lawrence, M.J., Fangue, N., Cooke, S.J., 2023. The ecological relevance of critical thermal maxima methodology for fishes. J. Fish Biol. 1000–1016. https://doi.org/10.1111/jfb.15368

Ern, R., Andreassen, A.H., Jutfelt, F., 2023. Physiological mechanisms of acute upper thermal tolerance in fish. Physiology 38, 141–158. https://doi.org/10.1152/physiol.00027.2022

FAO, 2023. FishstatJ - Software for Fishery and Aquaculture Statistical Time Series. Food and Agriculture Organization, Roma

Fry, F.E.J., 1971. The effect of environmental factors on the physiology of fish, in: Hoar, W.S., Randall, D.J., Brett, J.R. (Eds.), Fish Physiology. Academic Press, New-York, pp. 1–98.

Gesto, M., López-Patiño, M.A., Hernández, J., Soengas, J.L., Míguez, J.M., 2015. Gradation of the stress response in rainbow trout exposed to stressors of different severity: The role of brain serotonergic and dopaminergic systems. J. Neuroendocrinol. 27, 131–141. https://doi.org/10.1111/jne.12248

Ingemansson, T., Olsson, N.U., Kaufmann, P., 1993. Lipid composition of light and dark muscle of rainbow trout (*Oncorhynchus mykiss*) after thermal acclimation: A multivariate approach. Aquaculture 113, 153–165. https://doi.org/10.1016/0044-8486(93)90348-3

Jentoft, S., Aastveit, A.H., Torjesen, P.A., Andersen, Ø., 2005. Effects of stress on growth, cortisol and glucose levels in non-domesticated Eurasian perch (*Perca fluviatilis*) and domesticated rainbow trout (*Oncorhynchus mykiss*). Comp. Biochem. Physiol. - A Mol. Integr. Physiol. 141, 353–358. https://doi.org/10.1016/j.cbpb.2005.06.006

Jin, J., Panserat, S., Kamalam, B.S., Aguirre, P., Véron, V., Médale, F., 2014. Insulin regulates lipid and glucose metabolism similarly in two lines of rainbow trout divergently selected for muscle fat content. Gen. Comp. Endocrinol. 204, 49–59. https://doi.org/10.1016/j.ygcen.2014.04.027

Jobling, M., Bendiksen, E.Å., 2003. Dietary lipids and temperature interact to influence tissue fatty acid compositions of Atlantic salmon, *Salmo salar* L., parr. Aquac. Res. 34, 1423–1441. https://doi.org/10.1111/j.1365-2109.2003.00970.x

Kamalam, B.S., Medale, F., Kaushik, S., Polakof, S., Skiba-Cassy, S., Panserat, S., 2012. Regulation of metabolism by dietary carbohydrates in two lines of rainbow trout divergently selected for muscle fat content. J. Exp. Biol. 215, 2567–2578. https://doi.org/10.1242/jeb.070581

Kolditz, C.-I., Borthaire, M., Richard, N., Corraze, G., Panserat, S., Vachot, C., Lefèvre, F., Médale, F., 2008a. Liver and muscle metabolic changes induced by dietary energy content and genetic selection in rainbow trout (*Oncorhynchus mykiss*). Am. J. Physiol. Integr. Comp. Physiol. 294, 1154–1164. https://doi.org/10.1152/ajpregu.00766.2007

Kolditz, C.-I., Paboeuf, G., Borthaire, M., Esquerré, D., SanCristobal, M., Lefèvre, F., Médale, F., 2008b. Changes induced by dietary energy intake and divergent selection for muscle fat content in rainbow trout (*Oncorhynchus mykiss*), assessed by transcriptome and proteome analysis of the liver. BMC Genomics 9, 506. https://doi.org/10.1186/1471-2164-9-506

Kolditz, C.I., Plagnes-Juan, E., Quillet, E., Lefèvre, F., Médale, F., 2010. Changes in white muscle transcriptome induced by dietary energy levels in two lines of rainbow trout (*Oncorhynchus mykiss*) selected for muscle fat content. Br. J. Nutr. 103, 629–642. https://doi.org/10.1017/S0007114509992340

Komrakova, M., Holtz, W., 2009. Factors responsible for successful chilled storage of unfertilized rainbow trout (*Oncorhynchus mykiss*) eggs. Aquaculture 286, 156–163. https://doi.org/10.1016/j.aquaculture.2008.09.019

Lagarde, H., Lallias, D., Patrice, P., Dehaullon, A., Prchal, M., François, Y., D'Ambrosio, J., Segret, E., Acin-Perez, A., Cachelou, F., Haffray, P., Dupont-Nivet, M., Phocas, F., 2023a. Genetic architecture of acute hyperthermia resistance in juvenile rainbow trout (*Oncorhynchus mykiss*) and genetic correlations with production traits. Genet. Sel. Evol. 55, 1–21. https://doi.org/10.1186/s12711-023-00811-4

Lagarde, H., Phocas, F., Pouil, S., Goardon, L., Bideau, M., Guyvarc'h, F., Labbé, L., Dechamp, N., Prchal, M., Dupont-Nivet, M., Lallias, D., 2023b. Are resistances to acute hyperthermia or hypoxia stress similar and consistent between early and late ages in rainbow trout using isogenic lines? Aquaculture 562, 738800. https://doi.org/10.1016/j.aquaculture.2022.738800

Lefevre, S., Wang, T., McKenzie, D.J., 2021. The role of mechanistic physiology in investigating impacts of global warming on fishes. J. Exp. Biol. 224, 238840. https://doi.org/10.1242/jeb.238840

Leveroni Calvi, S., Maisse, G., 1998. Cryopreservation of rainbow trout (*Oncorhynchus mykiss*) blastomeres: Influence of embryo stage on postthaw survival rate. Cryobiology 36, 255–262. https://doi.org/10.1006/cryo.1998.2084

Maddock, B., 1974. A technique to prolong the incubation period of brown trout ova. Progress. Fish-Culturist 36, 219–222. https://doi.org/10.1577/1548-8659(1974)36[219:ATTPTI]2.0.CO;2

Magnoni, L.J., Novais, S.C., Eding, E., Leguen, I., Lemos, M. F., Ozório, R.O., Geurden, I., Prunet, P., Schrama, J.W., 2019. Acute stress and an electrolyte-imbalanced diet, but not chronic hypoxia, increase oxidative stress and hamper innate immune status in a rainbow trout (*Oncorhynchus mykiss*) isogenic line. Front. Physiol. 10, 453. https://doi.org/10.3389/fphys.2019.00453

Mateus, A.P., Costa, R.A., Cardoso, J.C.R., Andree, K.B., Estévez, A., Gisbert, E., Power, D.M., 2017. Thermal imprinting modifies adult stress and innate immune responsiveness in the teleost sea bream. J. Endocrinol. 233, 381–394. https://doi.org/10.1530/JOE-16-0610

McKenzie, D.J., Zhang, Y., Eliason, E.J., Schulte, P.M., Claireaux, G., Blasco, F.R., Nati, J.J.H., Farrell, A.P., 2021. Intraspecific variation in tolerance of warming in fishes. J. Fish Biol. 98, 1536–1555. https://doi.org/10.1111/jfb.14620

Mommsen, T.P., Vijayan, M.M., Moon, T.W., 1999. Cortisol in teleosts: Dynamics, mechanisms of action, and metabolic regulation. Rev. Fish Biol. Fish. 9, 211–268. https://doi.org/10.1023/A:1008924418720

Nakagawa, S., Schielzeth, H., 2013. A general and simple method for obtaining R2 from generalized linear mixed-effects models. Methods Ecol. Evol. 4, 133–142. https://doi.org/10.1111/j.2041-210x.2012.00261.x.

Niksirat, H., Sarvi, K., Amiri, B. M., & Hatef, A., 2007. Effects of storage duration and storage media on initial and post-eyeing mortality of stored ova of rainbow trout *Oncorhynchus mykiss*. Aquaculture, 262(2-4), 528–531. https://doi.org/10.1016/j.aquaculture.2006.10.031

Perry, G.M.L., Martyniuk, C.M., Ferguson, M.M., Danzmann, R.G., 2005. Genetic parameters for upper thermal tolerance and growth-related traits in rainbow trout (*Oncorhynchus mykiss*). Aquaculture 250, 120–128. https://doi.org/10.1016/j.aquaculture.2005.04.042

Pickering, A.D., 1981. Stress and Fish. Academic Press, New-York

Pickering, A.D., Pottinger, T.G., Sumpter, J.P., Carragher, J.F., Le Bail, P.Y., 1991. Effects of acute and chronic stress on the levels of circulating growth hormone in the rainbow trout, *Oncorhynchus mykiss*. Gen. Comp. Endocrinol. 83, 86–93. https://doi.org/10.1016/0016-6480(91)90108-I

Piper, R.G., McElwain, I.B., Orme, L.E., McCraren, J.P., Fowler, L.G., Leonard., J.R., 1982. Fish Hatchery Management, U.S. Department of the Interior, U.S. Fish and Wildlife Service, Washington

Pottinger, T.G., Carrick, T.R., 1999. Modification of the plasma cortisol response to stress in rainbow trout by selective breeding. Gen. Comp. Endocrinol. 116, 122–132. https://doi.org/10.1006/gcen.1999.7355

Quillet, E., Le Guillou, S., Aubin, J., Fauconneau, B., 2005. Two-way selection for muscle lipid content in pan-size rainbow trout (*Oncorhynchus mykiss*). Aquaculture 245, 49–61. https://doi.org/10.1016/j.aquaculture.2004.12.014

Quillet, E., Le Guillou, S., Aubin, J., Labbé, L., Fauconneau, B., Médale, F., 2007. Response of a lean muscle and a fat muscle rainbow trout (*Oncorhynchus mykiss*) line on growth, nutrient utilization, body composition and carcass traits when fed two different diets. Aquaculture 269, 220–231. https://doi.org/10.1016/j.aquaculture.2007.02.047

R Development Core Team, 2022. R: A Language and Environment for Statistical Computing. The R Project for Statistical Computing, Vienna

Richardson, G.F., Gardiner, Y.T., McNiven, M.A., 2002. Preservation of rainbow trout (*Oncorhynchus mykiss*) eyed eggs using a perfluorochemical as an oxygen carrier. Theriogenology 58, 1283–1290. https://doi.org/10.1016/s0093-691x(02)00955-x

Sadoul, B., Leguen, I., Colson, V., Friggens, N.C., Prunet, P., 2015. A multivariate analysis using physiology and behavior to characterize robustness in two isogenic lines of rainbow trout exposed to a confinement stress. Physiol. Behav. 140, 139–147. https://doi.org/10.1016/j.physbeh.2014.12.006

Schreck, C.B., Tort, L., 2016. The concept of stress in fish, in: Schreck, C.B., Tort, L., Farrell, A.P., Brauner, C.J. (Eds.), Biology of Stress in Fish. Elsevier, Amsterdam, pp. 1–34.

Seibel, H., Baßmann, B., Rebl, A., 2021. Blood will tell: What hematological analyses can reveal about fish welfare. Front. Vet. Sci. 8, 616955. https://doi.org/10.3389/fvets.2021.616955

Scott, G.R., Johnston, I.A., 2012. Temperature during embryonic development has persistent effects on thermal acclimation capacity in zebrafish. Proc. Natl. Acad. Sci. U. S. A. 109, 14247–14252. https://doi.org/10.1073/pnas.1205012109

Skiba-Cassy, S., Lansard, M., Panserat, S., Médale, F., 2009. Rainbow trout genetically selected for greater muscle fat content display increased activation of liver TOR signaling and lipogenic gene expression. Am. J. Physiol. - Regul. Integr. Comp. Physiol. 297, 1421–1429. https://doi.org/10.1152/ajpregu.00312.2009

Stonecypher, R.W., Hubert, H.A., Gern, W.A., 1994. Effect of reduced incubation temperatures on survival of trout embryos. Progress. Fish-Culturist 56, 180–184. https://doi.org/10.1577/1548-8640(1994)056<0180:EORITO>2.3.CO;2

Trenzado, C.E., Carrick, T.R., Pottinger, T.G., 2003. Divergence of endocrine and metabolic responses to stress in two rainbow trout lines selected for differing cortisol responsiveness to stress. Gen. Comp. Endocrinol. 133(3), 332–340. https://doi.org/10.1016/S0016-6480(03)00191-6

Ubilla, A., Valdebenito, I., Árias, M.E., Risopatrón, J., 2016. Viability and DNA fragmentation of rainbow trout embryos (*Oncorhynchus mykiss*) obtained from eggs stored at 4 °C. Theriogenology 85, 1499–1506. http://doi.org/10.1016/j.theriogenology.2016.01.012

Wedemeyer, G.A., Barton, B.A., McLeay, D.J., 1990. Stress and acclimation, in: Schreck, C.B., Moyle, P.B. (Eds.), Methods for Fish Biology. Bethesda, Maryland, pp. 451–489.

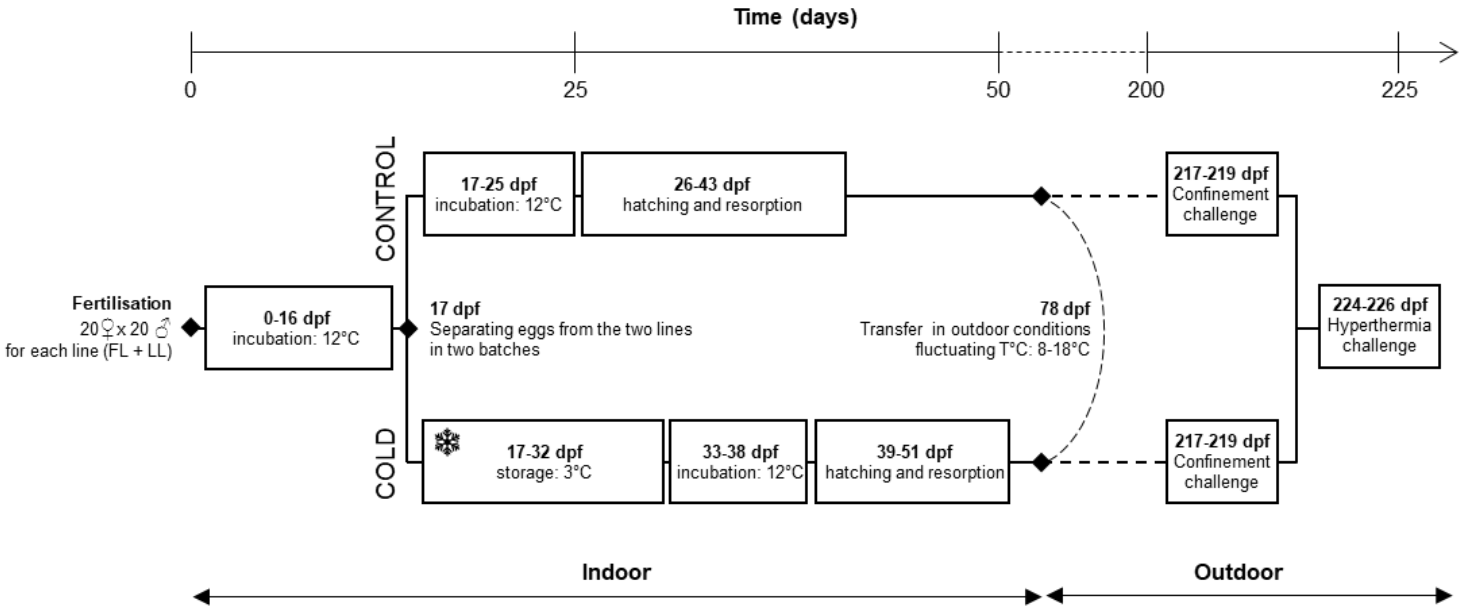
Whitehead, C., Bromage, N.R., 1978. The effects of alterations in photoperiod on ovarian development and spawning time in the rainbow trout (*Salmo gairdneri*). Ann. Biol. Anim. Biochim. Biophys. 18, 1035–1043. https://doi.org/10.1051/rnd:19780543

Captions to figures

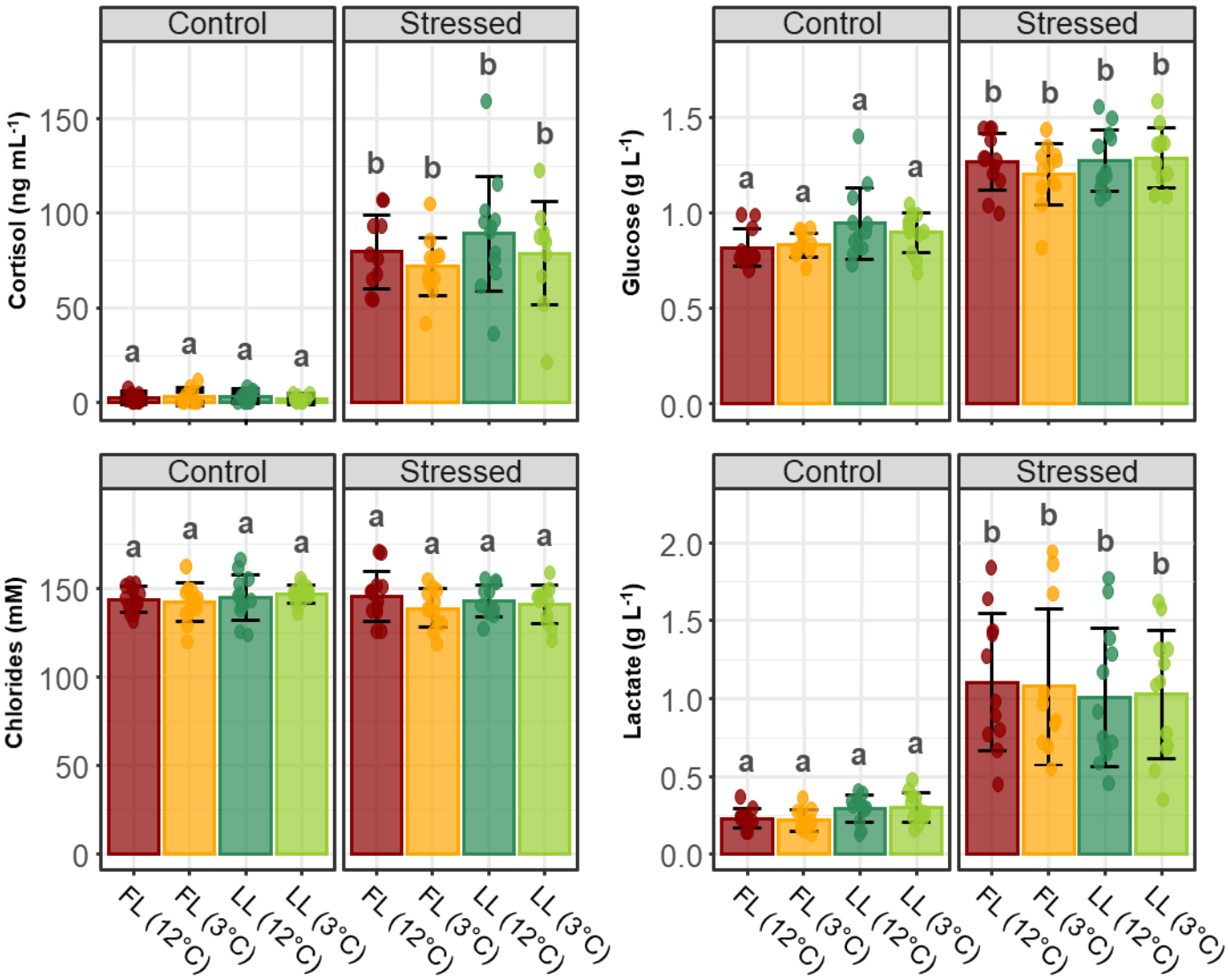
Figure 1. Schematic view of the experimental protocol (FL: fat line, LL: lean line, CONTROL: eyed eggs kept at 12°C, COLD: eyed eggs stored at 3°C for 15 days, dpf: days post fertilisation).

Figure 2. Cortisol, glucose, chloride ions, and lactate concentrations in plasma collected in juvenile fish before (Control, n = 12) and 1h after the confinement challenge (Stressed, n = 12) from the two lines (FL: fat line and LL: lean line) kept at two incubation temperatures at the eyed egg stage (3 or 12°C). Different letters denote significant differences ($P < 0.05$).

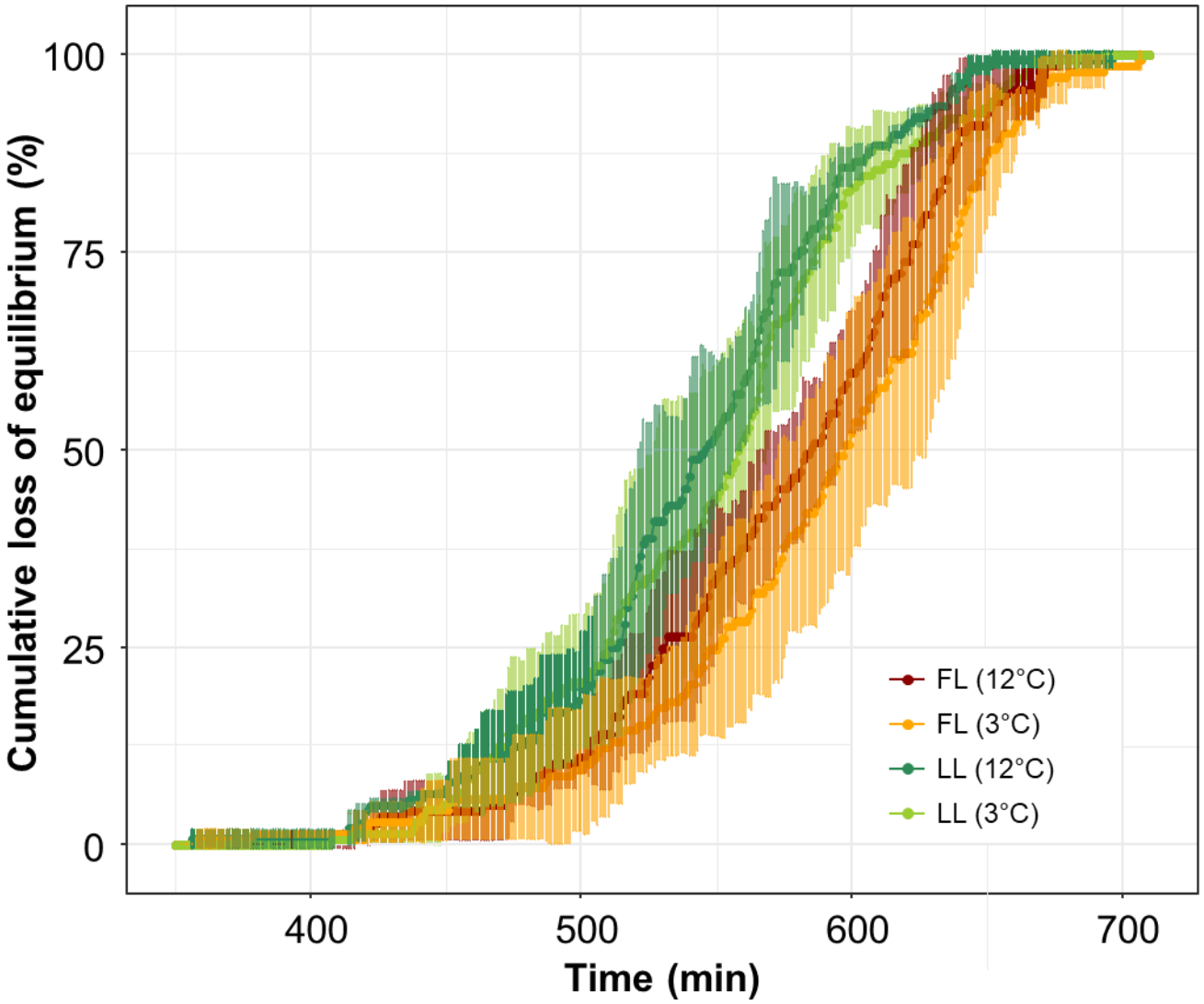
Figure 3. Kinetics of cumulative loss of equilibrium in juvenile fish from the two lines (FL: fat line and LL: lean line) kept at two incubation temperatures at the eyed egg stage (3 or 12°C). Values are means ± SD from the three replicates.


Time (days)
0
25
50
200
225
CONTROL
17-25 dpf
incubation: 12°C
26-43 dpf
hatching and resorption
217-219 dpf
Confinement
challenge
Fertilisation
20♀x 20 ♂
for each line (FL + LL)
0-16 dpf
incubation: 12°C
17 dpf
Separating eggs from the two lines
in two batches
78 dpf
Transfer in outdoor conditions
fluctuating T°C: 8-18°C
224-226 dpf
Hyperthermia
challenge
COLD
17-32 dpf
storage: 3°C
33-38 dpf
incubation: 12°C
39-51 dpf
hatching and resorption
217-219 dpf
Confinement
challenge
Indoor
Outdoor

546 Figure 1


Control
Stressed
Cortisol (ng mL-1)
Glucose (g L-1)
Chlorides (mM)
Lactate (g L-1)
FL (12°C)
FL (3°C)
LL (12°C)
LL (3°C)

547 Figure 2


Cumulative loss of equilibrium (%)
100
75
50
25
0
400
500
600
700
Time (min)
FL (12°C)
FL (3°C)
LL (12°C)
LL (3°C)

548 Figure 3

549 Table 1. Zootechnical performances of the fish from the different experimental conditions
550 (lines and incubation temperatures). Data are expressed as means ± SD. Letters denote
551 significant differences.

Parameter	n per condition	Fat line (FL)		Lean Line (LL)	
		12°C	3°C	12°C	3°C
Indoor rearing					
Deformity rate (%)	3	5.3 ± 2.1 ^{A}	4.5 ± 0.8 ^{A}	5.5 ± 1.1 ^{A}	9.6 ± 2.6 ^{A}
Survival rate (%)	3	89.9 ± 3.2 ^{A}	87.8 ± 5.8 ^{A}	91.4 ± 4.6 ^{A}	86.9 ± 2.7 ^{A}
Outdoor rearing					
Survival rate (%)	3	94.4 ± 1.2 ^{A}	89.8 ± 3.6 ^{AB}	92.5 ± 2.3 ^{AB}	80.8 ± 3.3 ^{B}
Weight at 190 dpf (g)	353-459	35.3 ± 11.1 ^{A}	29.2 ± 9.8 ^{B}	40.4 ± 11.5 ^{C}	35.2 ± 12.0 ^{A}

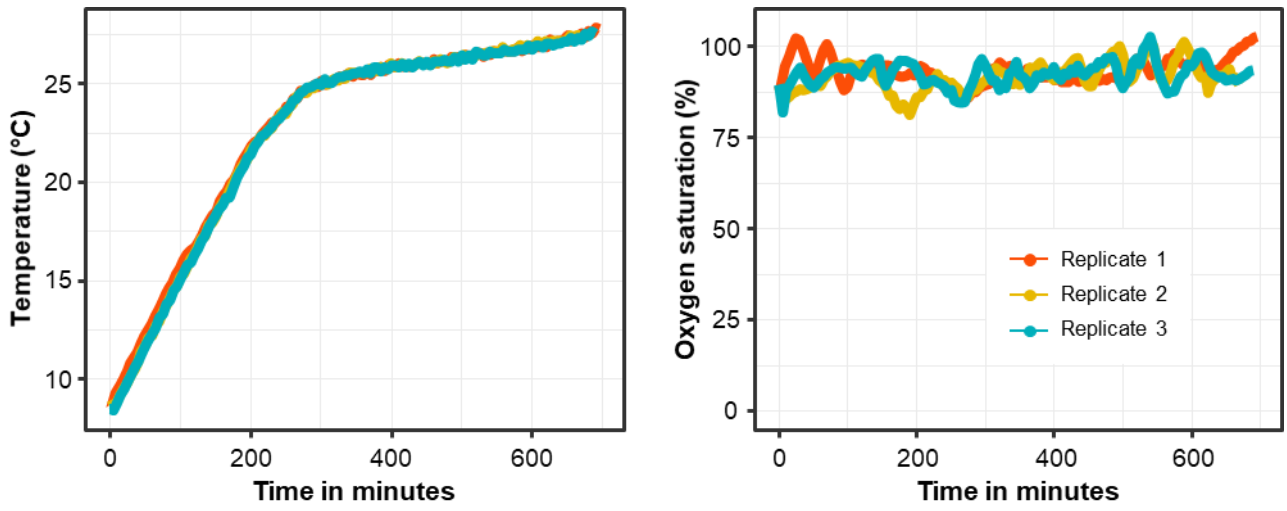
552

Table 2. Weights of the fish from the different experimental conditions (lines and incubation temperatures) sampled during the confinement and hyperthermia challenges. Data are expressed as means ± SD. Letters denote significant differences.

Challenge	Line	Incubation temperature	n	Weight (g)
Confinement	FL	12°C	24	57.3 ± 9.6^{A}
	FL	3°C	24	57.2 ± 13.7^{A}
	LL	12°C	24	62.1 ± 9.6^{A}
	LL	3°C	24	54.5 ± 8.4^{A}
Hyperthermia	FL	12°C	135	57.8 ± 13.4^{A}
	FL	3°C	139	49.0 ± 12.0^{B}
	LL	12°C	139	66.0 ± 16.9^{C}
	LL	3°C	137	59.8 ± 15.0^{A}

FL: fat line, LL: lean line

564 **Supplementary file**



565 Figure S1. Temperature and O_2 saturation over the hyperthermia challenges for the three

566 replicates.