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### ► To cite this version:

Simon Pouil, Elodie Harté, Michaël Marchand, Lionel Goardon, Tina Terrenne, et al.. Cold temperature during embryonic development and its influence on responses to acute confinement and hyperthermia challenges in juvenile rainbow trout. *Aquaculture*, 2024, 593, pp.741318. 10.1016/j.aquaculture.2024.741318 . hal-04666176

**HAL Id: hal-04666176**

**<https://hal.umontpellier.fr/hal-04666176v1>**

Submitted on 20 Oct 2024

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

3

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16 **Abstract**

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

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

34

## 35 **1. Introduction**

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

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

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

60 muscle fat content, were used (Quillet et al., 2005). Given the observed differences in energy  
61 utilisation and intermediate metabolism between these lines (Kolditz et al., 2008a, 2008b,  
62 2010; Kamalam et al., 2012), we hypothesised that their responses to cold temperatures  
63 during incubation would differ.

## 64 **2. Materials and methods**

### 65 2.1. Ethics statement

66 All the experiments were conducted at the INRAE experimental facilities (PEIMA, INRAE,  
67 2021, Fish Farming systems Experimental Facility, doi: 10.15454/1.5572329612068406E12,  
68 Sizun, France) authorised for animal experimentation under the French regulation C29-277-  
69 02. The experiment was carried out according to the European guidelines; the protocols were  
70 evaluated and approved by the ethical committee CEFEA No 74 and authorised by the French  
71 Ministry of Higher Education and Research (APAFIS #31861-2021060117041206 v4).

### 72 2.2. Fish and rearing conditions

73 The study was conducted with two INRAE experimental lines of rainbow trout  
74 (*Oncorhynchus mykiss*), designated as Fat line (FL) and Lean line (LL), obtained after seven  
75 generations of divergent selection for respectively high or low muscle fat content using a non-  
76 destructive method (Distell Fish Fat Meter ®) as detailed by Quillet et al. (2007). Muscle fat  
77 content was found to be more than 3 times higher in the FL line (8.0%) than in the LL line  
78 (2.3%) in 200 g-trout after five generations of selection (Jin et al., 2014).

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

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

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

98 Fry were kept in incubators and individuals with deformities were counted and removed  
99 regularly. Before the first feeding only viable individuals were transferred into 0.3 m<sup>3</sup> indoor  
100 tanks supplied with natural spring water at 12.0°C and lit by artificial neon lights from 8:00  
101 AM to 8:00 PM, without mixing between lines and incubation temperature conditions (n = 3  
102 tanks per condition and ~270 individuals per tank). At the age of 78 dpf (average weight: 0.6-  
103 1.0 g), the fry were transferred first into covered 0.3 m<sup>3</sup> tanks under a greenhouse and  
104 supplied with lake dam water and then, at 155 dpf, into covered 1.8-m<sup>3</sup> outdoor fiberglass  
105 tanks. From 78 dpf and up to the challenges, fish were subjected to normal seasonal  
106 temperature variations throughout the concerned period (daily mean temperature: 8.0 to  
107 18.0°C) and lit by natural light. Until the beginning of the experiment, the fish were fed daily  
108 to satiation using automatic feeders with a commercial diet from Le Guessant's company.

109 The growth was followed regularly by weighing a random subsample of 50 fish from each  
110 tank while the fish individual body weight was measured at 190 dpf (n = 353-459 per  
111 condition). Mortality has been checked daily throughout the experiment. Over the rearing  
112 period, O<sub>2</sub> concentrations in the outlet water ranged from 8.5 to 9.9 mg L<sup>-1</sup> (*i.e.* 85-99 % of  
113 saturation level) while NH<sub>4</sub><sup>+</sup> and NO<sub>2</sub><sup>-</sup> concentrations ranged from 0.2 to 0.7 mg L<sup>-1</sup> and <0.01  
114 to 0.8 mg L<sup>-1</sup>, respectively (assessed on a bi-weekly basis on average).

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

### 116 2.3. Confinement challenge

117 Acute confinement challenges were performed over three days at 217-219 dpf. About one  
118 month before the challenges, fish from each line and condition were transferred into 0.3 m<sup>3</sup>  
119 tanks still supplied with lake dam water and kept separate with 50 fish in each tank (n = 3  
120 tanks per condition). Before the start of the confinement challenge, all the fish were starved  
121 for 24h and eight of them were euthanised by an overdose of anaesthetic (Tricaine MS-222,  
122 100 mg L<sup>-1</sup>), weighed using digital scales ( $\pm$  0.1 g), and immediately sampled for blood.  
123 Blood was taken from the caudal vein using heparinised syringes (heparin lithium, Sigma-  
124 Aldrich, USA). Blood samples were kept on ice until plasma was separated from whole blood  
125 by centrifugation at 2,500 g for 10 min at 4°C. Plasma samples were stored at -20°C before  
126 analysis. These fish constituted the time-zero control group before the confinement challenge.  
127 Then, 8 fish from each tank were transferred into a bucket filled with water according to the  
128 average weight per tank to reach a fish density of 200 kg m<sup>-3</sup> and kept for 4 minutes with no  
129 water renewal or oxygen supplementation. Challenged fish were transferred under flow-  
130 through water conditions into a 0.3 m<sup>3</sup> recovery tank for 1h and then euthanised as described  
131 above, weighed, and sampled for blood according to the same procedure as for fish before  
132 confinement.

133 Glucose and lactate were measured in plasma (5  $\mu$ L per measurement) using a portable digital  
134 blood glucose meter ACCU-CHEK  $\text{\textcircled{R}}$  Active (Roche Diagnostic Systems, Herts, UK) and a  
135 THE EDGE  $\text{\textcircled{R}}$  blood lactate analyser (APEXBIO, Taiwan), respectively.

136 Plasma cortisol was extracted by adding 1 mL diethyl ether to 100  $\mu$ L of plasma in a 5 mL  
137 glass tube. The sample and solvent were vortex-mixed and frozen at  $-20^{\circ}\text{C}$  for at least 1h to  
138 allow the phases to separate. The supernatant was then transferred into a 2 mL glass vial and  
139 evaporated at room temperature under a stream of nitrogen. The residue was dissolved in 100  
140  $\mu$ L of extraction buffer provided in the commercial ELISA kit (Cortisol ELISA KIT; Neogen  
141  $\text{\textcircled{R}}$  Corporation) used for plasma cortisol measurements and performed according to the  
142 manufacturer's instructions.

143 Chloride ions were measured in plasma using a commercial kit (Kit Chlorures, Biolabo  $\text{\textcircled{R}}$ ).  
144 Plasma (3  $\mu$ L), diluted at  $\frac{1}{2}$  in deionised water, reacted with 300  $\mu$ L of Hg(II) thiocyanate for  
145 5 min at room temperature, and then absorbance was read at 500 nm. For both cortisol and  
146 chloride ions, measurements have been performed in triplicates, and values were calculated  
147 from reference standard curves.

#### 148 2.4. Hyperthermia challenges

149 Acute hyperthermia challenges were performed at 224-226 dpf. At 191 dpf, fish were  
150 individually PIT-tagged (Biolog-id  $\text{\textcircled{R}}$ ) and grouped into 3 separate 0.3  $\text{m}^3$  tanks containing  
151 each 200 fish, *i.e.* 50 fish per line and incubation temperature. PIT tags (pit-tag length: 12  
152 mm, diameter: 2 mm, weight: 0.092 g) were injected horizontally into the dorsal muscle just  
153 behind the head. Animals were starved for three days before challenges according to Lagarde  
154 et al. (2023b). The evening before each challenge, fish were transferred into the challenge  
155 tank (0.3  $\text{m}^3$ ), supplied with the same lake dam water as the one used in the rearing tanks, and  
156 left alone for the night for acclimation. Temperature was gradually increased from  $8.5^{\circ}\text{C}$  to  
157  $27.5^{\circ}\text{C}$  by renewing challenge tank water with heated water from a buffer tank. Temperature



158 was first quickly increased at a rate of 0.7°C every 10 minutes until 22°C. This period was  
159 followed by a slower temperature increase at a rate of 0.1°C every 15 minutes until all fish  
160 have lost equilibrium. This slower temperature increase was intended to increase the between-  
161 fish variability of acute hyperthermia resistance phenotypes (Lagarde et al., 2023b). Water  
162 was oxygenated to keep oxygen levels near saturation (Figure S1). Temperature and O<sub>2</sub>  
163 concentration and saturation were recorded every 5 min during challenges using electronic  
164 probes (HQ30d, Hach Company, Loveland, CO, USA), while NH<sub>4</sub><sup>+</sup> concentration was  
165 checked twice, *i.e.* at peak of loss of equilibrium - determined as the sudden increase in the  
166 number of fish losing equilibrium - and at the end of the challenge, using a commercial  
167 colorimetric kit (LCK 304, Hach Company, USA). CO<sub>2</sub> concentration was measured  
168 continuously with a CO<sub>2</sub> analyzer (Oxyguard, Denmark). The maximum concentrations  
169 measured were 2.6 mg L<sup>-1</sup> and 9 mg L<sup>-1</sup> for NH<sub>4</sub><sup>+</sup> and CO<sub>2</sub>, respectively.

170 As the temperature increased, fish were gradually losing equilibrium. When a fish lost  
171 equilibrium, it was removed from the tank, its origin was identified using the PIT tag and the  
172 exact time of loss of equilibrium was recorded (Time<sub>loss</sub>) as the phenotype of interest (see  
173 Section 4 for details). Fish were then softly anesthetised (Tricaine MS-222, 50 mg L<sup>-1</sup>),  
174 weighed using digital scales ( $\pm$  0.1 g), and euthanised by an overdose of anaesthetic (Tricaine  
175 MS-222, 100 mg L<sup>-1</sup>). Challenges ended when the last fish lost its equilibrium.

## 176 2.5. Data analysis

177 For the deformity rate and survival rates, the assumptions of normality and homoscedasticity  
178 were not met. Hence, non-parametric Kruskal-Wallis tests followed by Dunn's tests with  
179 Bonferroni adjustment for p-values were used to analyse the differences between  
180 experimental conditions. Body weight differences among experimental conditions at 190 dpf,  
181 *i.e.* before the confinement and acute hyperthermia challenges, were analysed by analysis of  
182 variance (ANOVA) and Tukey's test for multiple pairwise comparisons. ANOVA

183 assumptions of normality and homoscedasticity were verified by visual inspection of residual-  
184 fit plots.

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

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

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

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

### 206 **3. Results**

207 The incubation temperature exhibited no discernible impact on the fry's deformity rate from  
208 hatching to the first feeding, with an average value of 6% across all experimental conditions  
209 ( $H_{(3)} = 7.21$ ,  $P = 0.07$ ). Similarly, the survival rate during this phase remained unaffected by  
210 the incubation temperature ( $H_{(3)} = 2.13$ ,  $P = 0.55$ ), with an average of 90% observed across all  
211 conditions during the indoor rearing phase (Table 1). In the outdoor rearing phase, survival  
212 rates slightly differed according to experimental conditions ( $H_{(3)} = 8.44$ ,  $P = 0.04$ ) with  
213 survival rates significantly lower in LL kept at 3°C ( $80.8 \pm 3.3$  %) than FL under control  
214 temperature ( $94.4 \pm 1.2$  %). Interestingly, within the same line, we did not find significant  
215 effects of incubation temperature on survival rate (Table 1). As anticipated, the average  
216 individual weight, measured at 190 dpf one month before the confinement and hyperthermia  
217 challenges, was affected by both line ( $F_{(1)} = 103.76$ ,  $P < 0.001$ ) and incubation temperature  
218 ( $F_{(1)} = 107.23$ ,  $P < 0.001$ ). Fish from the LL line surpassed those from the FL line in size  
219 ( $40.4 \pm 11.5$  g vs  $35.3 \pm 11.1$  g in normal temperature conditions), and those subjected to cold  
220 storage during incubation exhibited smaller sizes ( $35.2 \pm 12.0$  g and  $29.2 \pm 9.8$  g for LL and  
221 FL, respectively) (Table 1). Basal blood parameters (i.e. chloride ions, cortisol, glucose, and  
222 lactate) were similar between both lines (Figure 2).

223 Following the confinement challenge, there was a significant increase in plasma cortisol  
224 levels ( $F_{(1)} = 925.47$ ,  $P < 0.001$ ) with values ranging from  $2.6 \pm 2.8$  to  $80.0 \pm 24.0$  ng mL<sup>-1</sup>,  
225 before and after the challenge respectively. On the other hand, neither the genetic lines nor  
226 incubation temperature at the eyed eggs stage affected the cortisol levels in both control and  
227 challenged fish (Figure 2). The same trend was observed for glucose ( $F_{(1)} = 184.86$ ,  $P <$   
228  $0.001$ ) and lactate concentrations ( $F_{(1)} = 327.42$ ,  $P < 0.001$ ) with significant increases in  
229 concentrations after the confinement challenge for both lines but no significant difference  
230 between lines and incubation temperatures. Chloride ion concentrations were similar between

231 the experimental conditions and remained constant over the challenge ( $143 \pm 10$  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 ( $\text{Time}_{\text{loss}}: 547 \pm 59$  min) than FL ( $\text{Time}_{\text{loss}}: 581$   
237  $\pm 64$  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  $12^{\circ}\text{C}$  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

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

260 Stress responses in teleost fishes involve three phases: primary, secondary, and tertiary. The  
261 primary response includes the release of catecholamines and cortisol, triggering secondary  
262 responses such as increased plasma glucose, lactate, and heart rate, as well as decreased  
263 plasma chloride, sodium, potassium, liver glycogen, and muscle protein (Pickering, 1981;  
264 Mommsen et al., 1999; Barton, 2002). These secondary responses can lead to tertiary  
265 responses, including reduced growth rate, metabolic scope, disease resistance, reproductive  
266 capacity, and altered behaviour and survivability (Wedemeyer et al., 1990; Barton and Iwama,  
267 1991; Mommsen et al., 1999). Given the intensity and duration of the stressor, the challenges  
268 in this study can be considered as acute (Schreck and Tort, 2016). The hyperthermia challenge  
269 induced a loss of equilibrium, indicating a phenotype of resistance (Fry, 1971). In contrast,  
270 the confinement challenge focused on primary stress responses, measuring cortisol synthesis,  
271 and secondary responses through investigations on energy metabolism (glucose and lactate)  
272 and hydromineral balance (chloride ions).

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

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

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

287 In our experiment, we found that acute stress led to a comparable release of plasma cortisol in  
288 juvenile fish, regardless of whether they were exposed to cold temperature storage at the eyed  
289 eggs stage. This pattern was consistent for both FL and LL fish lines, suggesting a uniform  
290 regulation of cortisol synthesis and release in both lines. Our confinement protocol, which  
291 involves a shorter stress duration than the protocol used by Pottinger and Carrick (1999) and a  
292 1-hour recovery period, has proven effective in revealing genetic influences on cortisol  
293 response, as shown in preliminary investigations with other rainbow trout genetic lines  
294 (unpublished data). This suggests that, even after seven generations of divergent selection for  
295 fat content, the two experimental lines still exhibit a similar stress response after 1h of  
296 recovery.

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

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

311 The hyperthermia challenges conducted in this study over three days were highly repeatable,  
312 with consistent temperature rises and oxygen saturation levels across all three tests (Figure  
313 S1). Due to the consistent starting temperature (8.5°C) and the high repeatability of  
314 temperature rise profiles, we did not calculate cumulative thermal exposure (CTE) in degree-  
315 minutes, as defined by Perry et al. (2005) and used by Lagarde et al. (2023a). Currently, there  
316 is no consensus on the mechanisms behind the loss of equilibrium in acute hyperthermia  
317 conditions in fish, with differences observed between species (Lefevre et al., 2021; Desforges  
318 et al., 2023; Ern et al., 2023). Factors such as life stage, body size, phenotypic plasticity, and  
319 genetic background influence intraspecific fish hyperthermia resistance (McKenzie et al.,  
320 2021). This resistance phenotype, as measured here, has been demonstrated to be heritable ( $h^2$   
321  $\approx 0.3-0.4$ ; Perry et al., 2005; Lagarde et al., 2023a), with strong variability observed between  
322 rainbow trout isogenic lines (Lagarde et al., 2023b). These results indicate a significant  
323 genetic component in hyperthermia stress resistance, explaining the differing resistance  
324 observed between the two experimental lines in this study.

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

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

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

#### 347 **4. Conclusion**

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

#### 355 **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.

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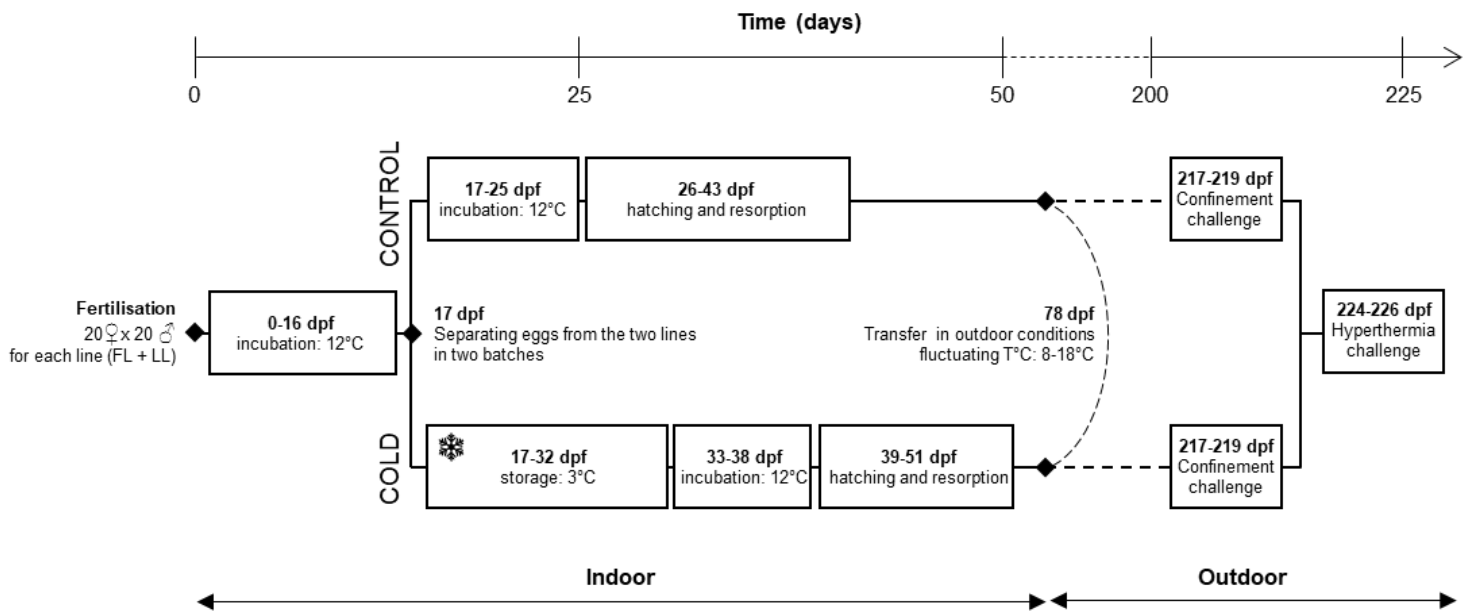
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534

535 **Captions to figures**

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

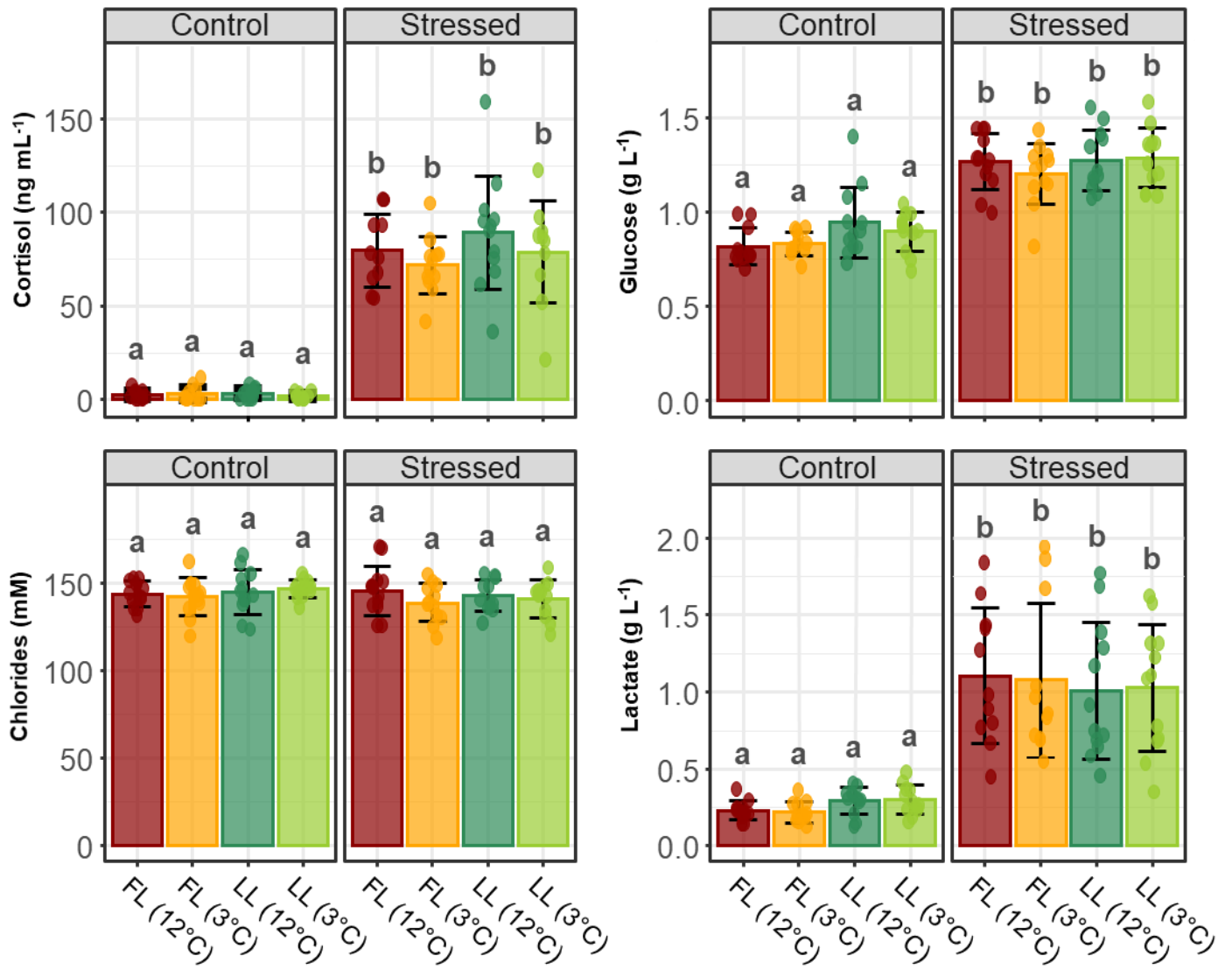
539 Figure 2. Cortisol, glucose, chloride ions, and lactate concentrations in plasma collected in  
540 juvenile fish before (Control, n = 12) and 1h after the confinement challenge (Stressed, n =  
541 12) from the two lines (FL: fat line and LL: lean line) kept at two incubation temperatures at  
542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

543 Figure 3. Kinetics of cumulative loss of equilibrium in juvenile fish from the two lines (FL:  
544 fat line and LL: lean line) kept at two incubation temperatures at the eyed egg stage (3 or  
545 12°C). Values are means  $\pm$  SD from the three replicates.

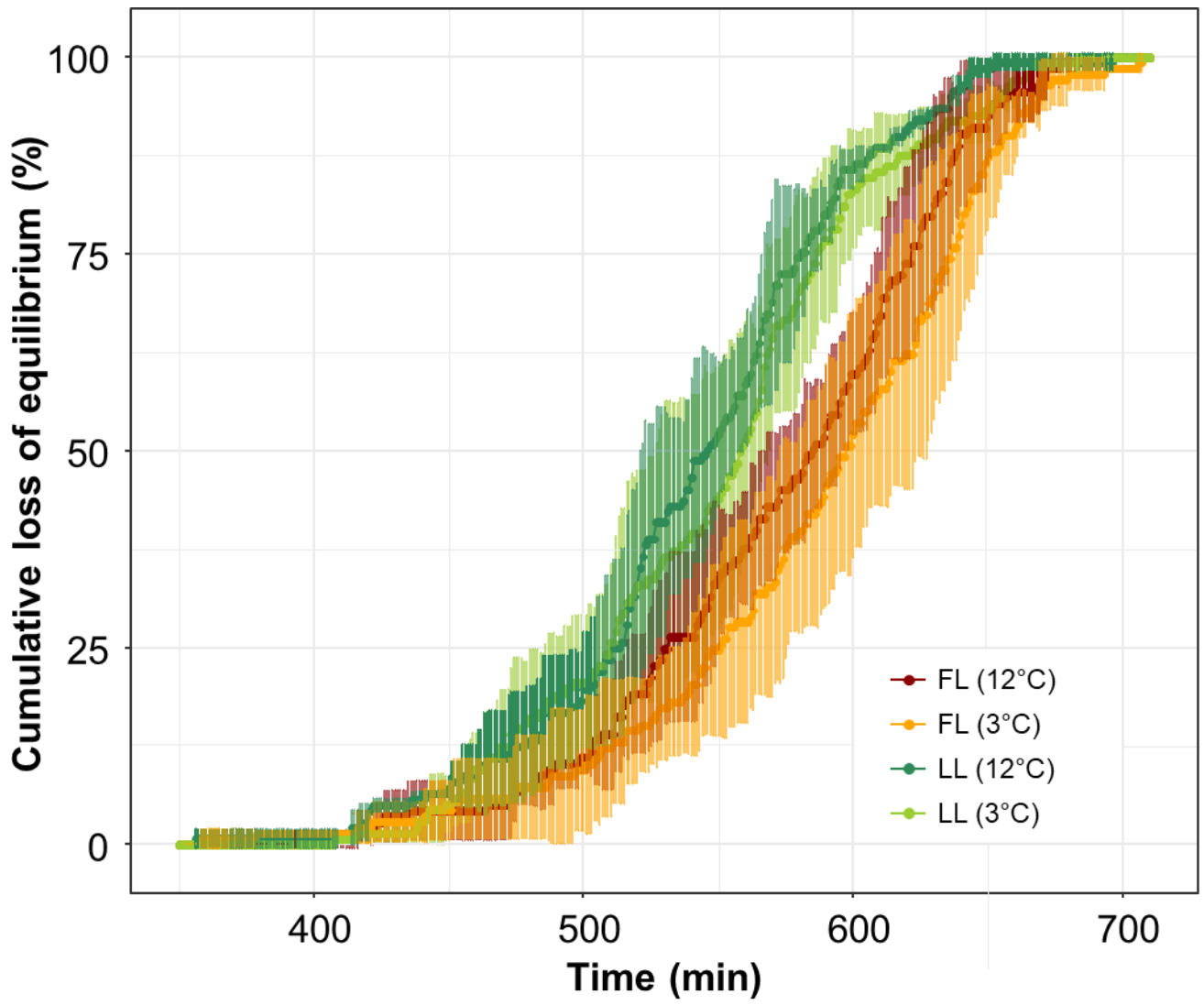


546 Figure 1





547 Figure 2



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  $\pm$  SD. Letters denote  
 551 significant differences.

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

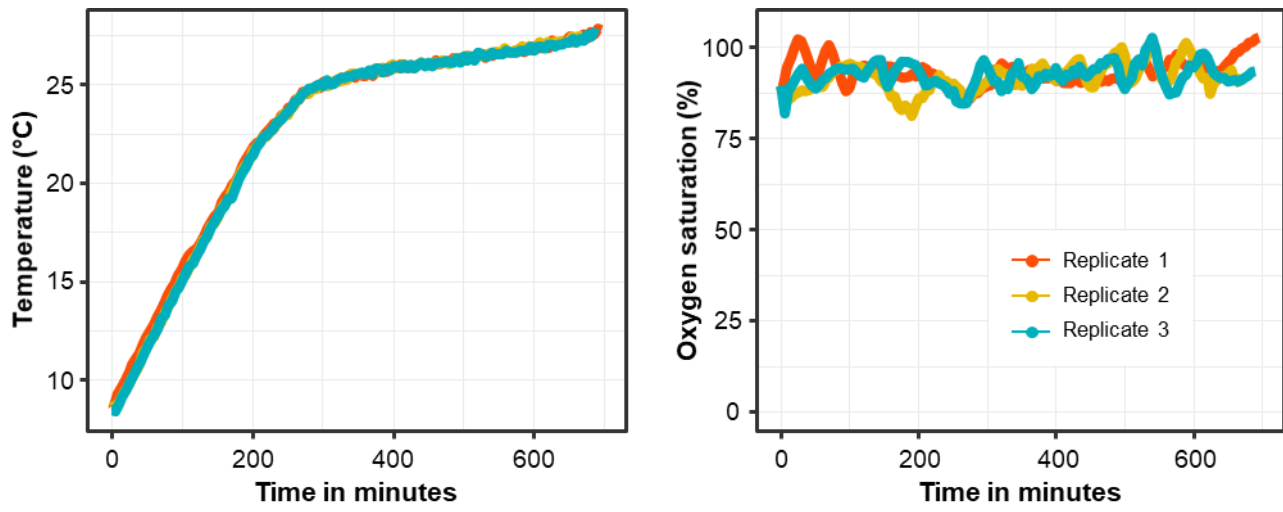
552

553 Table 2. Weights of the fish from the different experimental conditions (lines and incubation  
 554 temperatures) sampled during the confinement and hyperthermia challenges. Data are

Challenge	Line	Incubation temperature	n	Weight (g)	555	556	557	558	559	560	561
Confinement	FL	12°C	24	57.3 ± 9.6 <sup>A</sup>							
	FL	3°C	24	57.2 ± 13.7 <sup>A</sup>							
	LL	12°C	24	62.1 ± 9.6 <sup>A</sup>							
	LL	3°C	24	54.5 ± 8.4 <sup>A</sup>							
Hyperthermia	FL	12°C	135	57.8 ± 13.4 <sup>A</sup>							
	FL	3°C	139	49.0 ± 12.0 <sup>B</sup>							
	LL	12°C	139	66.0 ± 16.9 <sup>C</sup>							
	LL	3°C	137	59.8 ± 15.0 <sup>A</sup>							

562 ers denote significant differences.

563 FL: fat line, LL: lean line



565 Figure S1. Temperature and O<sub>2</sub> saturation over the hyperthermia challenges for the three  
566 replicates.