Links between behaviour and acute hyperthermia and hypoxia resistance in rainbow trout using isogenic lines

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Abstract

In the context of adaptation to climate change, acute hyperthermia and hypoxia resistance are traits of growing interest in aquaculture. Although the feasibility of genetic improvement of these resistance traits through selection has been demonstrated in rainbow trout (*Oncorhynchus mykiss*), little is known about the mechanisms underlying such traits. This poses a problem, as genetic selection of poorly understood traits can be inefficient and even harmful. To better understand these mechanisms, the present study investigated the link between several behaviour variables, and the resistance to acute hyperthermia and hypoxia.

Six rainbow trout isogenic lines were phenotyped for behaviour variables and for acute hyperthermia and hypoxia resistance, using systematically different individuals. The behaviour variables of the fish were phenotyped using an individual test in a new environment and a group test during a moderate thermal challenge. Experimental design was robust, with 150 fish phenotyped per isogenic line for each resistance trait and 18 fish per isogenic line for behavioural traits, distributed in triplicates. Acute hyperthermia and hypoxia resistance phenotypes were related to behaviour phenotypes at the level of isogenic lines.

Significant differences in behaviour between isogenic lines were found, with certain behaviour variables being highly correlated with acute hyperthermia and hypoxia resistance. Isogenic lines with a higher activity level appeared to be more sensitive to acute hyperthermia and more resistant to acute hypoxia than lines with a lower activity level. This previously unknown link between behaviour and resistance phenotypes also suggests that some behaviour variables could be used as predictors for acute hyperthermia and hypoxia resistance in fish. This result could also lead to more ethical acute hyperthermia and hypoxia resistance

phenotyping protocols, as the current protocols in use are classified as severe by French ethics committees.

Keywords: selective breeding, behaviour, indirect phenotyping, temperature, oxygen, climate change, isogenic lines

Introduction

Resistance to acute hyperthermia and hypoxia are phenotypes of interest in fish for numerous fields of research, such as evolutionary biology (Mandic, Todgham & Richards, 2009; Comte & Olden, 2017), stress-coping physiology (Rytkönen et al., 2007; Saravia et al., 2021), forecasts for climate change effects (Messmer et al., 2017; Potts, Mandrak & Chapman, 2021), and genetics for breeding robust animals (Baer & Travis, 2000; Perry et al., 2005; Debes et al., 2021; Lagarde et al., 2023a; Prchal et al., 2023). The methods in use for measuring these resistance phenotypes consist of increasing the temperature of the water for hyperthermia phenotyping or decreasing the O2 saturation of the water for hypoxia phenotyping until the fish lose equilibrium. The resistance phenotypes include the resistance time and the temperature/O2 saturation at the equilibrium loss (Beitinger, Bennett & Mccauley, 2000; Roze et al., 2013).

Previously, genetic determinism for acute hyperthermia and hypoxia resistance have been demonstrated in rainbow trout (Perry et al., 2005; Lagarde et al., 2023a,b; Prchal et al., 2023). In particular, Lagarde et al. (2023b) showed that some genotypes were more resistant to acute hyperthermia or hypoxia using rainbow trout isogenic lines. However, little is known about the physiological mechanisms underlying the breaking point leading to loss of equilibrium during acute hyperthermia or hypoxia stress and hence about the origin of this observed genetic variability. For acute hyperthermia resistance, the main theories about these mechanisms are the failure of short-term essential organs such as the brain, the failure of mitochondria processes, the thermal inactivation of enzymes, the desynchronization of

interdependent metabolic processes because of different response to temperature, the alteration of membrane structures, and a lack of oxygen (Fry, 1971; Schmidt-Nielsen, 1997; Pörtner, 2010; Chung & Schulte, 2020; Lefevre, Wang & McKenzie, 2021; Ern, Andreassen & Jutfelt, 2023). For acute hypoxia resistance, loss of equilibrium may be caused by an inability to produce adenosine triphosphate (ATP) fast enough with anaerobic substrate to compensate the failure of the aerobic metabolism, or by damage induced by the harmful metabolites produced by anaerobic metabolism, such as lactate or H+ (Nilsson & Östlund-Nilsson, 2008; Mandic, Todgham & Richards, 2009; Bergstedt, Pfalzgraff & Skov, 2021). There is no consensus within the scientific community on the reality of these mechanisms and their relative importance, whether for resistance to acute hyperthermia or resistance to acute hypoxia (Nilsson & Östlund-Nilsson, 2008; Ern, Andreassen & Jutfelt, 2023). It seems therefore premature to breed for acute hyperthermia or hypoxia resistance, as selecting for poorly understood traits could be ineffective or harmful in the long term. Thus, it is now essential to understand why genotypes are resistant or not. Previous studies, aiming at the evaluation of the resistance to acute hyperthermia and hypoxia of rainbow trout (Dupont-Nivet et al., 2014; Lagarde et al., 2023b), have observed expression of an extensive behavioural repertoire, suggesting that behaviour may be linked to resistance phenotypes. The present paper aims to test whether behaviour may be linked with acute hyperthermia and hypoxia resistance.

In addition, a strong interest in identifying mechanisms underlying resistance to acute hyperthermia and hypoxia and whose phenotyping would be reasonably intrusive, such as behavioural ones, would be to use them as predictors to replace the current classical phenotyping methods. Indeed, although the classical phenotyping methods of acute hyperthermia and hypoxia resistance have the advantage of directly measuring the phenotypes of interest, they have several drawbacks. Firstly, acute hyperthermia and hypoxia challenges

push fish to their limits, with the loss of equilibrium as an endpoint generating animal suffering. Within the 3R framework (Replacement, Reduction, Refinement), active research must be conducted to reduce animal suffering in scientific experiments, and society has a growing pressure on the scientific community to reduce animal suffering (Sloman et al., 2019). Secondly, they affect the physical integrity of the fish. For example, mortality can be as high as 25%-55% the week following an acute hyperthermia challenge in some strains of rainbow trout (Strowbridge et al., 2021). Yet this result must be tempered as, in other strains of rainbow trout, mortality was less than 5% (Strowbridge et al., 2021) and in zebrafish and European sea bass, fish can recover reasonably quickly from acute hyperthermia or hypoxia challenges with less than 1% of mortality (Claireaux et al., 2013; Morgan, Finnøen & Jutfelt, 2018).

In the present article, we investigate whether resistance to acute hyperthermia and hypoxia may be linked to behaviour and discuss the possibility of using behaviour variables as a novel way of phenotyping these resistances.

Our methodology used the rainbow trout isogenic lines, which have been previously shown to have different resistance to either hyperthermia or hypoxia (Lagarde et al., 2023b). The behaviour of fish was monitored using an individual test and a group test, the latter being associated with moderate thermal stress. Measured behaviour phenotypes were related to activity (acceleration, speed, distance travelled, movement), risk-taking (emergence, time spent in a risky zone), and schooling behaviour (inter-individual distance, contacts between fish). The resistance and behaviour phenotypes, measured on different individuals, were related at the level of lines, thanks to the complete genetic homogeneity among fish within an isogenic line. The advantage of measuring phenotypes on different individuals is to avoid a previous experience to interfere with a later one.

1 Material and methods

1.1 Experimental fish

Experimental fish were produced at the INRAE PEIMA experimental fish farm (doi: 10.15454/1.5572329612068406E12, Sizun, France) with broodstock issued from the INRAE homozygous isogenic rainbow trout lines (Quillet et al., 2007). Homozygous isogenic lines are a powerful genetic resource, as fish are homozygous and genetically identical within a line. Therefore, fish from a given line can be considered as replicates of a unique genotype.

The fish used in the present study are from the same rearing batch as those described in Lagarde et al. (2023b). Figure 1 summarises the rearing process of the fish and the experiments conducted. Briefly, six heterozygous isogenic lines were produced by fertilising eggs from same-day naturally spawning females of the homozygous isogenic line B57 with the sperm of six neomales (sex-reversed XX males) issued from the homozygous isogenic lines A02, A22, A32, B45, N38 and R23. At this stage, the only source of variation between heterozygous lines was expected to be the paternal genetic effect. Fertilised eggs were incubated in one hatching tray per line, supplied with spring water of little thermal amplitude over the year (11.5°C - 12.4°C). At 21 dpf (days post-fertilisation), hatching trays were each randomly divided into three hatching trays with no mixing between isogenic lines (>500 eyed eggs per hatching tray, 3x6 hatching trays). At 43 dpf, each hatching tray was transferred into a 0.25 m^3 indoor tank with no mixing between hatching trays (~500 larvae per tank, 3x6 tanks) supplied with the same spring water. At 145 dpf, the number of fries was reduced to 230 in each of the 18 tanks by randomly removing exceeding fries. At 150 dpf, the number of individuals was randomly reduced to 160 in each of the eighteen tanks, decreasing the density from 7.0 to 4.5 kg/m^3. The same day, among the approximately 160 kept individuals per tank,

100 were individually anaesthetised, PIT-tagged (Biolog-id, pit-tag dimensions: 12*9*2 mm, weight: 0.1g) in the dorsal muscle just behind the head, and put back into their tanks.

At 175 dpf, PIT-tagged fish were removed from rearing tanks to form the three replicates for phenotyping the resistance of isogenic lines to acute hyperthermia and the three replicates for phenotyping the resistance of isogenic lines to acute hypoxia. The resistance to acute hyperthermia and hypoxia were phenotyped between 182 and 187 dpf as already presented in Lagarde et al. (2023b).

Among the 60 untagged fish kept in the rearing tanks, six were randomly collected to evaluate individual and group behaviour between 177 and 179 dpf according to experimental setups presented below.

During the rearing process, the maximum reached rearing density was 8.7 kg/m^3, and fish were fed to satiation with a commercial diet from Le Gouessant's company.

1.2 Conduct of acute hyperthermia and hypoxia resistance experiments

The methods and devices used to determine the mean acute hyperthermia and hypoxia resistance of isogenic lines were previously presented in Lagarde et al. (2023b) and will be briefly summarized here.

Acute hyperthermia and hypoxia challenges were performed between 182 dpf and 187 dpf. Fish mean body weight was 15.2 ± 3.5 g. Isogenic lines were phenotyped in triplicate for acute hyperthermia and hypoxia resistance (Fig. 1). Each replicate (named A, B and C in Fig. 1) contained 50 fish from each of the six isogenic lines, *i.e.*, a total of 300 fish per replicate.

For the acute hyperthermia challenge, the temperature in the challenge tank was first increased at a rate of 3.6°C/hour (12.4°C to 23.2°C in three hours). The temperature increase rate was then slowed down to 0.6°C/hour and maintained at this rate until all fish lost

equilibrium. For the acute hypoxia challenge, dissolved oxygen saturation was decreased from nearly 90% to 15% in 4.5 hours. Saturation was then stabilised around 15% for the rest of the challenge.

As temperature increased in acute hyperthermia challenge or O2 saturation decreased in acute hypoxia challenge, fish gradually lost equilibrium. Immediately after a fish lost equilibrium, it was caught, its isogenic line was identified reading the PIT tag, and the time was recorded. The fish was then anaesthetised, weighed, and euthanised.

Acute hyperthermia resistance was quantified as the cumulative thermal exposure (CTE) in degree-minutes (deg-min), and acute hypoxia resistance was quantified as the cumulative hypoxia exposure (CHE) in saturation minutes (sat-min), as described in Lagarde et al. (2023b). CTE is the sum of the differences between the temperature in the challenge tank and the temperature at the start of the challenge, calculated at each minute from the start of the challenge to the time of loss of equilibrium. CHE is the sum of the differences between a reference O2 saturation of 90%, the O2 saturation before the start of the challenge, and the O2 saturation measured in the challenge tank, calculated at each minute from the start of the challenge to the time of loss of equilibrium. A high CTE or CHE indicates a high resistance to acute hyperthermia or hypoxia, respectively.

As explained in Lagarde et al. (2023b), the effect of isogenic line on acute hyperthermia and hypoxia resistance was analysed by fitting a model with *line* as a fixed effect, fish *body weight* as a covariate, and their interaction. *line*, *body weight* and their interaction were all significant in hyperthermia and hypoxia challenges (Lagarde et al. 2023b). Because the effect of *body weight* on resistance was different between lines, the predicted lsmeans of *lines* on acute hyperthermia and hypoxia resistance were estimated at 15.2 g, the mean body weight, using lsmeans 2.30–0 R package (Lagarde et al. 2023b).

1.3 General conduct of behavioural experiments

Behavioural experiments were carried out in a quiet room with no traffic during the experiments. Figure 2 presents the diagram of the experimental devices.

Behaviour was simultaneously evaluated on six isolated individuals from the same isogenic line for 25 minutes (called individual test after that), and immediately after, the same six individuals were grouped in a tank and exposed to moderate heat stress for 150 minutes (called group test after that). Devices and duration of individual test were derived from previously published protocol used on zebrafish and seabass (Vignet et al., 2015; Alfonso et al., 2020; Alfonso, Peyrafort & Cousin, 2020; Alfonso, Gesto & Sadoul, 2021). Temperatures during the individual test and at the start of the group test were set similarly to the ones in home tanks. The same water source was used in home tanks and during the behaviour tests. Fish transfer between the two experiments was performed in buckets containing temperature-equilibrated water and lasted approximately 1 minute (Fig. 2). These behavioural assessment sessions were conducted in triplicates for each of the six lines, totalling 18 sessions. Each session lasted approximately 186 minutes: 5 minutes of acclimation, 25 minutes of individual test, 1 minute of transfer, 5 minutes of acclimation and 150 minutes of group test. Sessions were spread over three days at a rate of 6 sessions per day. Time slots of sessions were: 9h00-12h10, 10h00-13h10, 11h00-14h10, 13h00-16h10, 14h00-17h10, 15h-18h10. We arranged sessions for the three replicates of each isogenic line during morning, midday and afternoon slots so that there was no confusion between line and times slot.

1.4 Conduct of individual behaviour test

The purpose of the individual behaviour test was to evaluate behaviour in a novel environment. Six fish from the same isogenic line were caught in one of the rearing tanks according to the pre-determined session order and immediately placed randomly in six individual rectangular aquariums (20 × 40 cm, Europrix) filled with 12 L of system water corresponding to 15 cm depth of water (Fig. 2.a). The six aquariums were placed in a homemade rectangular tent lit by spots on the outside top of the tent to ensure homogeneous semi-darkness conditions. Aquariums were isolated from neighbouring aquariums by opaque white walls and backlit by an infrared device (IR floor 1 × 1 m, Noldus, The Netherlands) as infrared radiation is invisible for trout (Rader et al., 2007).

Aquariums were divided into two zones: on one side, a zone of size 20 x 8 cm covered by a plate opaque to visible light but transparent to infrared light called the safe zone, and on the other side, an uncovered zone of size 20 x 32 cm called the risky zone (Fig. 2.a). Fish were first placed in the safe zone, separated from the risky zone by a closed door. Fish were left for 5 minutes in the safe zone, and the door was then opened by a remote-controlled device, allowing fish to freely move between the safe and risky zones. Once the door opened, fish activity was video recorded for 25 minutes with a USB camera (DMK 31AU03, The Imaging Source) equipped with a 2.7–13.5-mm lens (Fujinon) and an infrared filter (The Imaging Source), using ICapture (The Imaging Source). After 25 minutes, the fish were immediately caught and placed in a bucket filled with same water. At the end of each test, water of aquariums was entirely renewed before the start of the following test.

1.5 Conduct of group behaviour test

The tank for the group test was a 75 x 75 cm tank, identical to the fish rearing tanks, filled with water at a 10 cm depth (Fig. 2.b).

Fish were gently poured out of the bucket in the group tanks. Once the fish transfer was completed, the fish were left for 5 minutes for acclimation. Then, the water temperature was linearly increased at a rate of 5.4°C/hour, corresponding to a temperature increase from 12.2°C to 23°C in 120 minutes by adding water from a heated buffer tank. Temperature was then stabilised at 23°C for 30 minutes (Fig. 2.b). Fish activity was recorded for 150 minutes with a video-surveillance camera (Full HD: 1920 × 1080 px, 105°, VIZEO—Adrien Alarme), linked to a PC with an acquisition card. The fish were lit with two spots on each tank side for homogeneous lighting. At the end of the experiment, fish were softly fished, anaesthetised (Tricaïne MS222, 50 mg/L), quickly wiped clean, weighed and euthanised (Tricaïne MS222, 150 mg/L). The fish mean body weight was 11.6 ± 3.2 g, all lines included. Detailed isogenic lines mean body weights are given in Table S1.

1.6 Videos' behaviour variables extraction

Videos were analysed with EthoVision XT15.0 software. Fish positions were tracked using a dynamic subtraction method. The accuracy of the tracked positions was checked by viewing the superposition of the tracked and actual positions of fish in all videos and manually editing if required. The Lowess smoothing method was applied to eliminate background noise.

In individual behaviour experiment, eight variables were measured: the maximum acceleration (ACC_MAX, $cm.s^{-2}$), the distance travelled (DIST_TRAV, cm), the latency to first exit from the safe zone (EMERGENCE, s), the zone change frequency (FRQ_CHAN, $\#.min^{-1}$), the absolute meander (MEANDER, $deg.cm^{-1}$), the moving duration (MOV%, % of the time), the time spent in the risky zone (RISK%, % of the time) and the maximum velocity (VEL_MAX, $cm.s^{-1}$).

In the group behaviour experiment, seven variables were measured related to the behaviour of the group: the average inter-fish distance (DISPERSION, cm), the maximum acceleration (ACC_MAX, $cm.s^{-2}$), the distance travelled (DIST_TRAV, cm), the body contact frequency (FRQ_CONTACT, $\#.min^{-1}$), the absolute meander (MEANDER, $deg.cm^{-1}$), the moving duration (MOV%, % of the time), and the maximum velocity (VEL_MAX, $cm.s^{-1}$). More details on the measured variables are given in Table 1. The software tracked individual fish positions, but in the group test, identities swapped frequently when two fish crossed paths in front of the camera. This is almost unavoidable, and since group variables were measured, fish identity was not useful.

To consider the effect of time on behaviours, behaviour variables were extracted over several time bins: every 5 minutes for the individual test and every 30 minutes for the group test. This was done to evaluate the potential effects of acclimation of the fish along the duration of the individual test and of temperature increase along the group test.

1.7 Behaviour data statistical analysis

Analyses were performed in R version 3.6.2 (R Development Core Team, 2019).

Behavioural variables were measured at the individual level in the individual and group tests (6 isogenic lines x 3 replicas x 6 individuals, 108 data points per behaviour variable and time bin). However, these individual-level data were averaged at the replica level in the statistical analysis (6 isogenic lines x 3 replicas, 18 data points per behaviour variable and time bin). As there were significant body weight differences between isogenic lines (Table S1), behavioural variables needed to be corrected by the fish body weight to disentangle the genetic and the body weight effects, as done by Millot et al. (2014). Fish body weight was only known at the replica level, as it was measured at the end of the group test and it was not possible to reassign

it to individuals of the individual and group tests a posteriori. For the group test, a supplementary reason for not being able to reassign individuals was that Ethovision software could not follow each fish individually due to frequent swapping of identity when two fish cross paths. The experimental unit was, therefore, the replica. The setup of these experiments meant that replicas were nested within isogenic lines.

To test for an effect of the isogenic line through time bins on behaviour variables, we fitted linear mixed effects models with *body weight* as a covariate, *time* (time bins) and *line* (isogenic line) as categorical fixed effects, and the interactions between *time*, *line* and *body weight*, and *replica* as a random effect. Time was set up as a categorical variable because a visual inspection of response variables plotted against time did not reveal any linear relationship. We only tested *line* and *body weight* effects for EMERGENCE, as this event can only happen once in the whole test. In the group test, the *time* effect was confounded with the temperature increase, as no control of the group test, *i.e.*, without temperature increase, was realised in the present study.

Full and reduced linear mixed models (with vs. without *time* and/or *line* and/or *body weight* and/or the different interactions between these variables) were fitted using the function lme in package nlme 3.1–153 (https://CRAN.R-project.org/package=nlme). The reduced versus full models were compared using likelihood ratio tests to determine the best model for each response variable. To select the best model for each response variable, weighted terms allowing variances to differ among replica and isogenic lines were considered. To account for temporal autocorrelation, autoregressive AR1 or autoregressive moving average ARMA(1,1) covariance structures were considered (Zuur et al., 2009). Among the compared models (with vs. without weighted terms and/or AR1/ARMA(1,1) structures), the one with the smallest Akaike's Information Criterion (AIC) was selected as the best one. Assumptions of normality,

homoscedasticity and independence of final linear mixed models were verified by visual inspection of residual-fit plots.

The predicted least-squares means (lsmeans) of *line* and *time* on each response variable were estimated using lsmeans 2.30–0 R package (https://cran.r-project.org/package=lsmeans). For behaviour variables for which the *body weight* effect was significant, lsmeans of *time* and *line* (when significant) were estimated considering a body weight of 11.6 g, corresponding to the mean body weight of fish used in the behaviour experiment. Differences among *line* and *time* lsmeans for behaviour variables were compared by Tukey's test with Type 1 error risk set at 0.05 using the same lsmeans 2.30–0 R package. For behaviour variables for which the best model included the *line* × *time* interaction, *line* lsmeans were calculated at each *time* bin and vice-versa.

To get a synthetic view of the relationships between behaviour variables, *line* lsmeans were reduced to two principal components using PCA. Three kinds of PCA were produced: a PCA with only the *line* lsmeans of individual test, a PCA with only the *line* lsmeans of group test and a PCA with the *line* lsmeans of individual and group tests.

1.8 Screening for relationships between behaviour and resistance variables

The lsmeans of the same six isogenic lines for the behavioural variables and the resistance to acute hyperthermia and hypoxia stress were calculated using the steps mentioned in the previous sections. Behaviour variables for which lsmeans of isogenic lines were highly correlated with lsmeans of isogenic lines on resistance phenotype were considered as potentially genetically linked with resistance phenotypes.

Mean body weight was slightly lower in the behaviour tests (11.6 ± 3.2 g) than in resistance challenges (15.2 ± 3.5 g) since fish had between one and two more weeks to grow in the

resistance challenges. Rankings of lines based on body weight were similar between the two experiments (Fig. S1).

2 Results

2.1 Body weight, isogenic line and time effects influence on behaviour variables

The significance of effects (*line*, *time*, *body weight* and their interactions) on the different behaviour variables is shown in Table 2. Figure 3 shows the raw and modelled behaviour phenotypes obtained for each isogenic line in the individual test, and Figure 4 shows the same in the group test. Figure S2 shows the raw and modelled behaviour phenotypes obtained for each *time* bin in the individual test, and Figure S3 shows the same in the group test. Modelled behaviour phenotypes data for *line* and *time* bins shown in Figures 3-4-S2-S3 are available in Tables S2-S3-S4-S5, respectively.

2.1.1 Behaviour variables with no significant effect

Behaviour variables for which neither *time*, *line* nor *body weight* effects were significant were EMERGENCE and MEANDER in the individual test and FRQ_CONTACT and MEANDER in the group test (Table 2). These variables were therefore not investigated further.

2.1.2 Behaviour variables with significant *body weight* effect

The overall fish body weight was 11.6 ± 3.2 g in behaviour tests. There were significant differences in body weight between lines (ANOVA, N = 108, df=5; F value = 17.3; P < 2.8e-14). Details on the mean body weight of isogenic lines are given in Table S1. The lightest line

was A32h (9.6 ± 2.6 g), and the heaviest was N38h (14.9 ± 2.8 g). *body weight* had a significant effect on ACC_MAX in individual test and had no significant effect on any of the variables measured in group test (Table 2). *body weight* effect on ACC_MAX was an increase in 2.5 $cm.s^{-2}$ per gram, *i.e.*, bigger fish had a higher acceleration, all equal.

2.1.3 Behaviour variables with significant *time* effect and no significant interaction between *time* and *line* effects

In the individual test, the *time* effect was significant for variables ACC_MAX, MOV%, RISK% and VEL_MAX without any interaction with *line* effect (Table 2). The effect of *time* on these behaviour variables is presented in Fig. S2 and Table S4. ACC_MAX and VEL_MAX were significantly higher in the first time bin and then tended to decrease. In contrast, MOV% and RISK% were significantly lower in the first time bin and then tended to increase. These evolutions reflect the change in behaviour over time. Indeed, at the beginning of the test, fish displayed bursts of high activity bouts (high ACC_MAX and VEL_MAX) interspersed with freezing moments (low MOV%). In a second step, the number of activity bursts decreased (lower ACC_MAX and VEL_MAX) and the behaviour became more exploratory with higher moving duration and time spent in the risky zone (increase in MOV% and RISK%).

In the group test, the *time* effect was significant for some behaviour variables but always with a significant *time* x *line* interaction (Table 2). Results are presented in section 2.1.5.

2.1.4 Behaviour variables with significant *line* effect and no significant interaction between *line* and *time* effects

In the individual behaviour test, the *line* effect was significant for ACC_MAX, DIST_TRAV, FRQ_CHAN, RISK%, and VEL_MAX (Table 2). Therefore, there were significant variations

in activity levels between the lines during the individual test, presented in Fig. 3 and detailed in Table S2. Line A32h had a significantly lower DIST_TRAVEL (0.9 m), FRQ_CHAN (0.2 #.min^{-1}), and VEL_MAX (8.7 cm.s^{-1}) than A02h (3.3 m; 0.9 #.min^{-1}; 18.1 cm.s^{-1} respectively). Line A02h had a significantly higher RISK% (68.5%) than lines N38h (30.3%) and A22h (39.7%). Line A22h had a significantly higher ACC_MAX (47.4 cm.s^{-2}) than B45h (24.8 cm.s^{-2}) and higher VEL_MAX (18.1 cm.s^{-1}) than A32h (8.7 cm.s^{-1}).

In the group behaviour test, the *line* effect was significant and did not interact with the *time* effect for ACC_MAX and DISPERSION (Table 2). Line A32h had a significantly lower ACC_MAX (716 cm.s^{-2}) than line R23h (867 cm.s^{-2}) and a significantly lower group DISPERSION (19 cm) than the other five lines (24-28 cm) (Fig. 4.a&b, Table S3 for details).

2.1.5 Behaviour variables with significant interaction between *time* and *line* effects

In the individual test, the interaction between *time* and *line* effects was not significant regardless of the behaviour variables (Table 2).

In the group test, the interaction between *time* and *line* was significant for DIST_TRAV, MOV% and VEL_MAX (Table 2), meaning that the effect of *time* on these behaviours was different depending on the *line* and vice-versa. Lsmeans of *line* effect for these variables were then estimated at each time bin (Table S3) and are presented in Fig. 4.c&d&e. For DIST_TRAV, there were significant differences between isogenic lines at time bin 4 (B45h higher than R23h). For MOV%, there were significant differences between isogenic lines at time bin 4, with B45h higher than N38h. For VEL_MAX, there were significant differences between isogenic lines at time bin 1 (A22h higher than B45h) and time bin 4 (A22h higher than N38h). Lsmeans of *time* effect was also estimated for each isogenic line (Table S5) and the estimates are presented in Fig. S3. Interestingly, the lines evolved differently over time during the group challenge. The distance travelled (DIST_TRAV) tended to decrease over the

course of the challenge for some lines (A02h, A22h, A32h and R23h) before increasing again to starting level for some of them (A22h and A32h). For other two activity remained constant over time at high level for B45h and lower level for N38h(Fig. S3.a). MOV% was significantly different between some time bins for B45h and R23h (Fig. S3.b). VEL_MAX was significantly different between some time bins for A02h and A32h (Fig. S3.c).

2.2 Relationships between behaviour variables

2.2.3 PCA

The Principal Component Analyses (PCAs) of isogenic *lines* lsmeans are presented in Fig. 5.a for the individual test, Fig. 5.b for the group test, and Fig. 5.c for the individual and group tests together. Only the first two components were presented, as they explained more than 74% of the variance in these three PCAs. Correlations between behaviour variables in the individual and group tests and between the individual and group tests are also presented in Tables S6-S7-S8, respectively.

2.2.4 Individual behaviour test

The PCA of individual test variables highlights two groups of individual behaviour variables (Fig. 5.a). The first group, constituted of ACC_MAX, DIST_TRAV, FRQ_CHAN and VEL_MAX, contributed to PCA axis 1 for 93% and were highly positively correlated together with a correlation coefficient (r) ranging from 0.74 to 0.98 (Table S6). These four variables, each referring to the fish's activity level and showing strong positive and consistent correlations between them, were named activity variables of individual test. The second group, constituted of RISK%, only contributed to PCA axis 2 for 82% and was nearly uncorrelated with ACC_MAX and VEL_MAX with a r ranging from 0.21 to 0.23, and

moderately correlated with DIST_TRAV and FRQ_CHAN with a r ranging from 0.51 to 0.54 (Table S6). Fish staying longer in the risky zone therefore tended to swim longer distances than others.

2.2.5 Group behaviour test

In the group test, the interaction between *line* and *time* was significant for the variables DIST_TRAV, MOV% and VEL_MAX (Table 2). Lsmeans were therefore calculated for these variables at each time bin, which are all represented by different colours on the PCA (Fig. 5.b). ACC_MAX and DISPERSION were not time bin dependant, as this effect and the interaction with *line* were not significant (Table 2); lsmeans were therefore calculated for all time bins merged together (Fig. 5.b).

This PCA highlights a clear separation between the DIST_TRAV, MOV%, and VEL_MAX variables of the first and last two time bins. Indeed, within time bins 1 and 2, DIST_TRAV, MOV% and VEL_MAX were moderately to highly positively correlated, with a r ranging between 0.30 and 0.94 (Table S7). Within time bins 4 and 5, DIST_TRAV, MOV% and VEL_MAX were strongly positively correlated, with a r ranging between 0.51 and 0.99 (Table S7). Correlations between VEL_MAX, DIST_TRAV, and MOV% of the time bins 1 and 2 and time bins 4 and 5 were mostly negative, close to zero, or moderate (Table S7). Interestingly, DIST_TRAV, MOV% and VEL_MAX of time bin 3 were intermediaries between the first and last two time bins (Fig. 5.b). Thus, correlations between VEL_MAX, DIST_TRAV and MOV% were between -0.03 and 0.91 for time bins 1, 2 and 3 and between -0.11 and 0.91 for time bins 3, 4 and 5.

ACC_MAX variable was generally not strongly correlated with the other activity variables (DIST_TRAV, MOV% and VEL_MAX), contrary to what we observed in the individual test.

The DISPERSION variable was moderately to strongly positively correlated with DIST_TRAV, MOV%, and VEL_MAX at time bins 1, 2, and 3, and uncorrelated with these variables at time bins 4 and 5 (Table S7). DISPERSION was also poorly correlated with ACC_MAX (Table S7).

2.2.6 Individual and group behaviour tests

Relationships between isogenic *line* lsmeans of behaviour variables measured in the individual and group tests are presented on the PCA in Fig. 5.c, and correlation coefficients are given in Table S8. The PCA of individual and group test variables shows that the correlations between the individual and group test variables gradually turned from positive to negative with time bins in the group test as time and temperature increased (Fig. 5.c). Thus, correlations between activity variables in the individual test (ACC_MAX, DIST_TRAV, FRQ_CHAN and VEL_MAX) and DIST_TRAV, MOV% and VEL_MAX in the group test were moderately to highly correlated when considering time bins 1 and 2 of the group test, not correlated when considering time bin 3 of the group test, and negatively correlated when considering time bins 4 and 5 of the group test (Fig. 5.c and Table S8). RISK% of individual test was negatively correlated with activity variables in the group test for time bins 4 and 5, with a r between -0.53 and -0.84.

2.3 Correlations between behaviour variables and resistance phenotypes

Correlation coefficients between isogenic *line* lsmeans on behaviour variables and isogenic *line* lsmeans on resistance phenotypes are given in Table 3. Fig. 6 presents the relationship between behaviour variables and any of the two resistance phenotypes when the |Pearson correlation| between them was above an arbitrary threshold set to 0.7.

The isogenic *line* mean effect on acute hyperthermia resistance was strongly negatively correlated ($r < -0.71$) with the isogenic *line* mean effect on DIST_TRAV and VEL_MAX in the individual test, and with VEL_MAX in time bin 2 (VEL_MAX2) in the group test (Fig. 6.a&b). Correlation between isogenic *line* mean effect on acute hyperthermia resistance and other activity variables in the individual test (ACC_MAX and FRQ_CHAN), and in the group test (DIST_TRAV, MOV%, VEL_MAX; for the five time bins) were also slightly to moderately negative, with a r between -0.11 and -0.69 (Table 3). ACC_MAX in the group test was uncorrelated with acute hyperthermia resistance.

The isogenic *line* mean effect on acute hypoxia resistance was highly positively correlated with the isogenic *line* mean effect on DIST_TRAV and FRQ_CHAN in the individual test ($r > 0.88$; Fig. 6.c and Table 3). Again, in the individual test, acute hypoxia resistance was also moderately positively correlated with other activity variables (ACC_MAX, VEL_MAX) and RISK% ($0.54 < r < 0.69$; Table 3). In the group test, acute hypoxia resistance was highly positively correlated with VEL_MAX in time bins 1 and 2 (VEL_MAX1 and VEL_MAX2) and group DISPERSION ($r > 0.74$; Fig. 6.d and Table 3). Acute hypoxia resistance was also moderately positively correlated with other activity variables (DIST_TRAV, MOV%) at the beginning of the group test (*i.e.*, in the time bins 1 and 2: $0.38 < r < 0.51$). The correlations between acute hypoxia resistance and activity variables (DIST_TRAV, MOV% and VEL_MAX) turned moderately negative at the end of the group test (i.e. in time bins 4 and 5: $-0.42 < r < -0.53$).

3 Discussion

In this work, we investigated whether links could be established between behaviour variables and resistance to acute hyperthermia or hypoxia. To this end, we monitored the behaviour of

six isogenic lines and studied links with resistance to acute hyperthermia and hypoxia phenotypes previously determined (Lagarde et al. 2023b).

3.1 Behaviours' evolution with time and isogenic lines

The *time* effect was significant for several behaviour variables during the individual and group tests. At the beginning of the individual tests (first time bin), low moving duration, high maximum acceleration and velocity was observed (Fig. S2.a&b&d), which is characteristic of a "burst and freeze" behaviour. This behaviour could be linked to a high level of stress (Brown, Gardner & Braithwaite, 2005; Staven et al., 2019) and is consistent with the stress induced by the opening of the door and the novel environment proposed. In the following time bins, as stress was relieved, behaviour gradually turned into exploratory behaviour, which was characterised by an increase in moving duration and time spent in the risky zone (Fig. S2.b&c) (Ryer, 2004; Malavasi et al., 2008; Millot, Bégout & Chatain, 2009).

Significant behavioural differences between isogenic lines were found in the individual and group tests. These differences are consistent with the genetic determinism in fish behaviour, which was reviewed in Bell (2008), as well as in an earlier study that, similarly to our own, used isogenic lines (Millot et al., 2014). In particular, Millot et al. (2014) studied four isogenic lines that are investigated here: A02h, A22h, B45h and N38h. Establishing direct links between their results and our own is not simple since protocols and devices were different; specifically, Millot et al. used novel object and emergence tests. It is interesting to note that albeit evaluated in different contexts, A02h line was more active than B45h in both studies, carried out a decade apart, supporting the hypothesis of a genetic basis for this trait.

The interaction between *time* and *line* was significant for several behaviour variables in the group test but not in the individual test (Table 2). This difference may be due to the longer

duration of the group test, allowing time for the behaviour of isogenic lines to evolve. A second hypothesis could be that the temperature increase during the group test led to a different stress response of the lines, as several studies have shown different behaviour reactions to stress depending on fish personalities in rainbow trout (Frost et al., 2013; White et al., 2017).

3.2 Correlated behaviours between tests

Behaviour variables reflecting the level of activity of fish, *i.e.* ACC_MAX, DIST_TRAV, FRQ_CHAN and VEL_MAX in the individual test, were highly correlated together at the isogenic line level (Fig. 5a, Table S6). In the group test, DIST_TRAV, MOV% and VEL_MAX were strongly correlated within each time bin (Fig. 5b, Table S7). The only exception was ACC_MAX in the group test, which was poorly correlated with the three behaviour variables mentioned above (Fig. 5b, Table S7). A strong correlation between activity variables was already found in rainbow trout for variables similar to DIST_TRAV, MOV% and VEL_MAX (Makaras et al., 2020).

The concept of coping styles (or personalities) refers to a consistent set of behavioural and physiological traits that co-vary across environmental contexts (Koolhaas et al., 1999; Conrad et al., 2011). Existence of coping style have been suggested in rainbow trout isogenic lines (Millot et al. 2014). ACC_MAX, DIST_TRAV and VEL_MAX were moderately to highly positively correlated together between the individual tests and the two first time bins of the group tests, at the level of the isogenic line (Fig. 5.c, Table S8). Consistent relationships between behaviour variables in two contexts allow us to ascribe coping styles to some lines. Thus, in the group tests we conducted, we can differentiate the lines according to their level of activity, some lines showing a low activity level, such as A32h, others with a high activity

level, such as B45h. However, it is essential to note that a robust personality assignment would have required more behavioural testing, such as in Cerqueira et al. (2016). As time and temperature increased, the correlations between individual and group behaviour variables loosened (Fig. 5.c, Table S8), although we cannot determine which of the confounded time and temperature factors was the cause. It is highly probable, however, that heat stress played a role, given that temperature is a well-known factor affecting fish behaviours such as schooling, activity, and maximum swimming speed (Hurst, 2007; Colchen et al., 2017; Kochhann et al., 2021; Pilakouta et al., 2022).

3.3 Behaviour associated with acute hyperthermia or hypoxia resistance

Interestingly, coping styles were shown to be associated with physiological traits possibly linked with acute hyperthermia and hypoxia resistance: proactivity was shown to be associated with thermal preference in Nile tilapia (Cerqueira et al., 2016), reactivity was linked to faster growth in brown trout *Salmo trutta* (Adriaenssens & Johnsson, 2011), and boldness in a refuge emergence test was related with high aerobic capacity in bluegill sunfish *Lepomis macrochirus* (Binder et al., 2016). To our knowledge, no study previous to our own has investigated the genetic relationship between behaviour and resistance to acute hyperthermia or hypoxia traits in fish.

In the individual behaviour test, acute hyperthermia resistance was found highly negatively correlated with DIST_TRAV and VEL_MAX, and moderately negatively correlated with ACC_MAX and FRQ_CHAN. In contrast, acute hypoxia resistance was found to be highly positively correlated with DIST_TRAV, FRQ_CHAN, and moderately positively correlated with ACC_MAX, RISK% and VEL_MAX. Therefore, acute hyperthermia resistance appears to be associated with a low level of activity and acute hypoxia resistance with a high level of

activity. In the two first bins of the group test, we observed a similar yet less clear pattern: acute hyperthermia resistance was still associated with low activity (high negative correlation with VEL_MAX, moderate negative correlation with DIST_TRAV and MOV%), while acute hypoxia resistance was associated with high activity (high positive correlation with VEL_MAX and moderate positive correlation with DIST_TRAV and MOV%).

Our results on acute hyperthermia resistance are consistent with the study of Campos, Val & Almeida-Val (2018), although the two studies are very different. They reported that fish species with the lowest routine activity level, indirectly measured with a proxy, had the highest resistance to acute hyperthermia. They also hypothesised that fish with the lowest activity have lower resting energetic demands and thus can resist longer acute hyperthermia.

For acute hypoxia resistance, our results are hardly comparable to those in the existing literature. In rainbow trout exposed to sub-lethal levels of acute hypoxia, divergent behaviours were identified with some fish staying quiet and not panicking while other fish actively tried to avoid hypoxia and showed peak activity (Van Raaij et al., 1996). The survival rate of fish during the post-exposition recovery period was significantly associated with behavioural strategy, as fish staying quiet during the hypoxia challenge had a higher survival rate after return to normoxia compared to fish displaying high level of activity during the hypoxia challenge (Van Raaij et al., 1996). In contrast, we showed in this study that isogenic lines resistant to acute hypoxia were rather highly active. However, the results from the present study and Van Raaij et al. (1996) do not conflict since the level of activity was measured in a hypoxia stress challenge in Van Raaij et al. (1996) which was not the case in our experiment.

3.4 Potential of behaviour as a proxy of acute hyperthermia and hypoxia resistance traits

In the present study, some behaviour variable were highly correlated with acute hyperthermia or hypoxia resistance at the level of the isogenic lines. These correlations were consistent between individual and group tests, even if they weakened with the duration and temperature increase of the group test. Together, these results suggest a potential for using behavioural variables as novel, non-stressful proxies for acute hyperthermia and hypoxia resistance in fish, improving experimental fish welfare.

However, as correlations calculated with only six genotypes can give misleading results, these results still need to be validated on more genotypes and different populations. In case of confirmation, it would also be necessary to optimise how behaviour is measured, as data acquisition and analysis of the measurements are time-consuming compared to the classical phenotyping method. For instance, in Lagarde et al. (2023b), it took around 700 minutes to phenotype the resistance to acute hyperthermia or hypoxia stress of 300 fish, equivalent to 0.4 fish phenotyped/minute, while in the present study, it took between 25 minutes (individual test) to 150 minutes (group test) to phenotype six fish, equivalent to a rate of 0.24 or 0.04 phenotyped fish/minute. In addition, while the classical resistance phenotyping method directly gives the phenotypes of resistance, the behaviour phenotyping method implies extracting and analysing behaviour variables from recorded videos. However, the purpose of the behavioural phenotyping protocols in the present study did not involve optimisation. Future studies could implement significant time savings such as determining the minimum behaviour phenotyping time period required, increasing the number of fish in individual or group tests, automating data extraction, etc. The accuracy of behaviour variables in discriminating phenotypes of resistance to acute hyperthermia and hypoxia must also be determined at the fish level to determine their value as a proxy.

3.5 Implications for selective breeding of acute hyperthermia and hypoxia resistance

The correlations found between some behaviour variables and acute hyperthermia or hypoxia resistances at the level of the isogenic line might reveal genetic correlations between some behavioural variables and resistance phenotypes. Therefore, selection for resistance to acute hyperthermia or hypoxia stress, as proposed by Perry et al. (2005), Lagarde et al. (2023a), Prchal et al. (2023), could result in the indirect selection of certain behavioural variables. This side effect is not surprising since, in practice, selected fish over several generations are known to exhibit different behaviours, such as swimming, foraging, predator avoidance, social behaviour, personality, reproduction and cognitive learning, compared to the wild type (Pasquet, 2019). Specifically, for rainbow trout, domestication was shown to reduce predator avoidance behaviours (Johnsson, 1993; Berejikian, 1995). Behaviour modifications of farmed fish through direct or indirect genetic selection can positively or negatively impact fish adaptation to farming systems (e.g. Doyle & Talbot, 1986). If they were to be selected in a breeding program, special consideration must be paid to the effects of selecting these resistance phenotypes on the behaviour variables. More generally, physiological mechanisms underlying acute hyperthermia and hypoxia resistance are still not known with certainty (Nilsson & Östlund-Nilsson, 2008; Ern, Andreassen & Jutfelt, 2023), and unintended trade-offs with important breeding traits could therefore arise.

Conclusion

In the present study, we identified several behaviour variables strongly correlated with acute hyperthermia and hypoxia resistance at the line level using six rainbow trout isogenic lines. In individual test and the beginning of group test, active isogenic lines were generally found to

be sensitive to acute hyperthermia and resistant to acute hypoxia. These relationships between behaviour and resistance to acute hyperthermia and hypoxia suggest that behaviour is a promising approach to better understand the mechanisms underlying these resistances. These results also support the idea that behaviour phenotyping could be a non-invasive method for phenotyping acute hyperthermia or hypoxia resistances.

However, these conclusions, notably the correlations between behaviour and resistance traits, must be verified in a higher number of isogenic lines and other populations of rainbow trout, as well as at the fish level.

Ethical statement

Experiments were carried out 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. Experiments were 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#24784_2020032510486957).

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Figures :

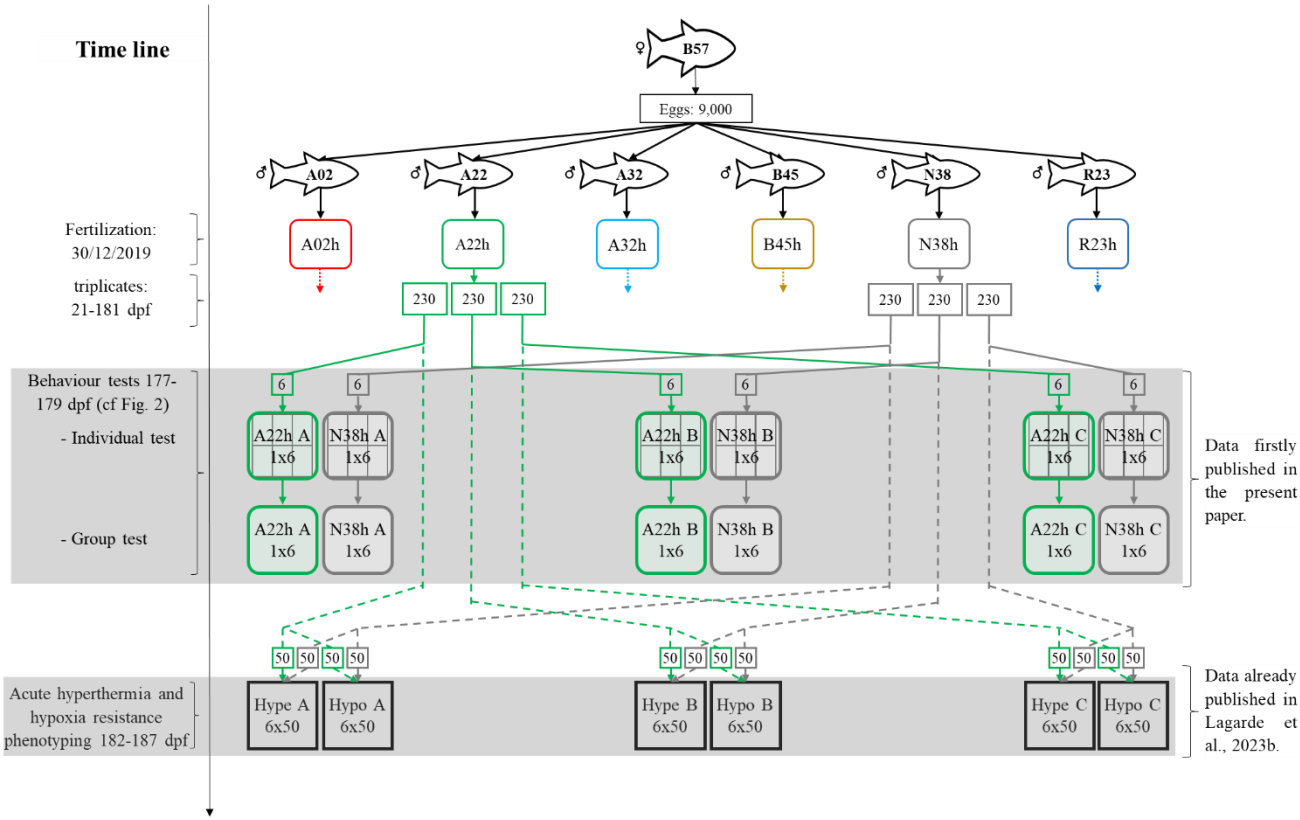

Fig. 1 Rearing history of fish. Each isogenic line is represented by a different colour. The entire rearing history and experimental use were only represented for lines A22h and N38h. The four other lines were not represented to not overload the figure, but they had a similar life history. Rounded rectangles represent rearing tanks. Replicate rearing tanks also corresponding to replicate behaviour and resistance phenotyping are named A, B and C. The theoretical number of fish is indicated inside each rectangle. The number of fish and lines used in each experiment is indicated in each experiment rectangular, with the first number indicating the number of isogenic lines and the second the number of fish per isogenic line. The lines represent fish movements, solid for fish used in behaviour tests and dotted for fish used in acute hyperthermia and hypoxia challenges. "Hype" stands for acute hyperthermia phenotyping, and "Hypo" for acute hypoxia phenotyping. Figure adapted from Lagarde et al. (2023b).

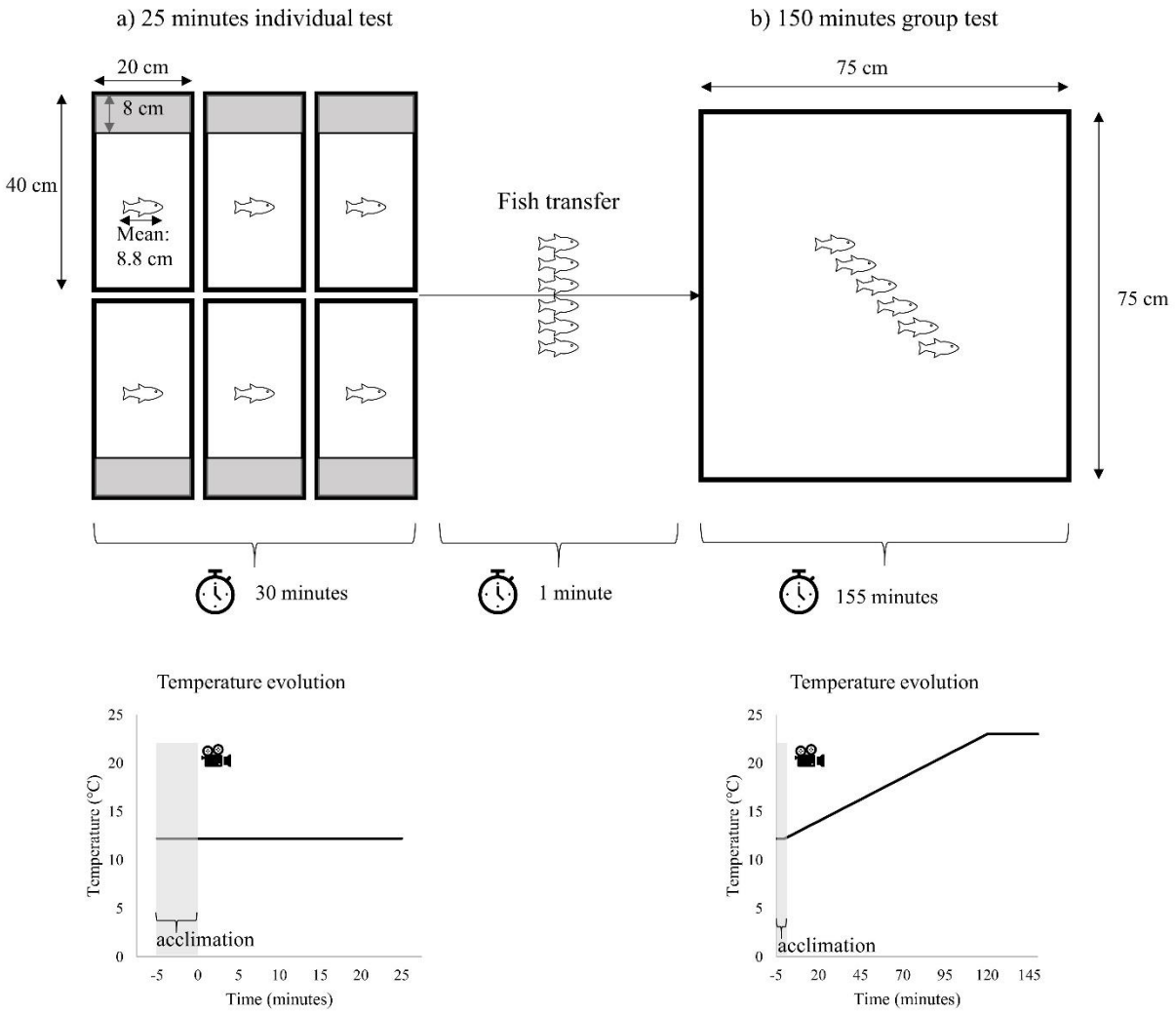

Fig. 2 Diagram of behaviour tests. a) Tanks used for monitoring individual behaviour. Grey bands represent the safe zone, covered with a lid opaque to visible light. The six fish monitored in parallel were from the same isogenic line. Temperature was kept constant during the 5 minutes of acclimation and 25 minutes of the behaviour test. b) Fish were then transferred to a unique tank for 150 minutes for a group test under moderate hyperthermia stress. Temperature was linearly increased from 12.2°C to 23.0 °C in 2 hours after five minutes acclimation.

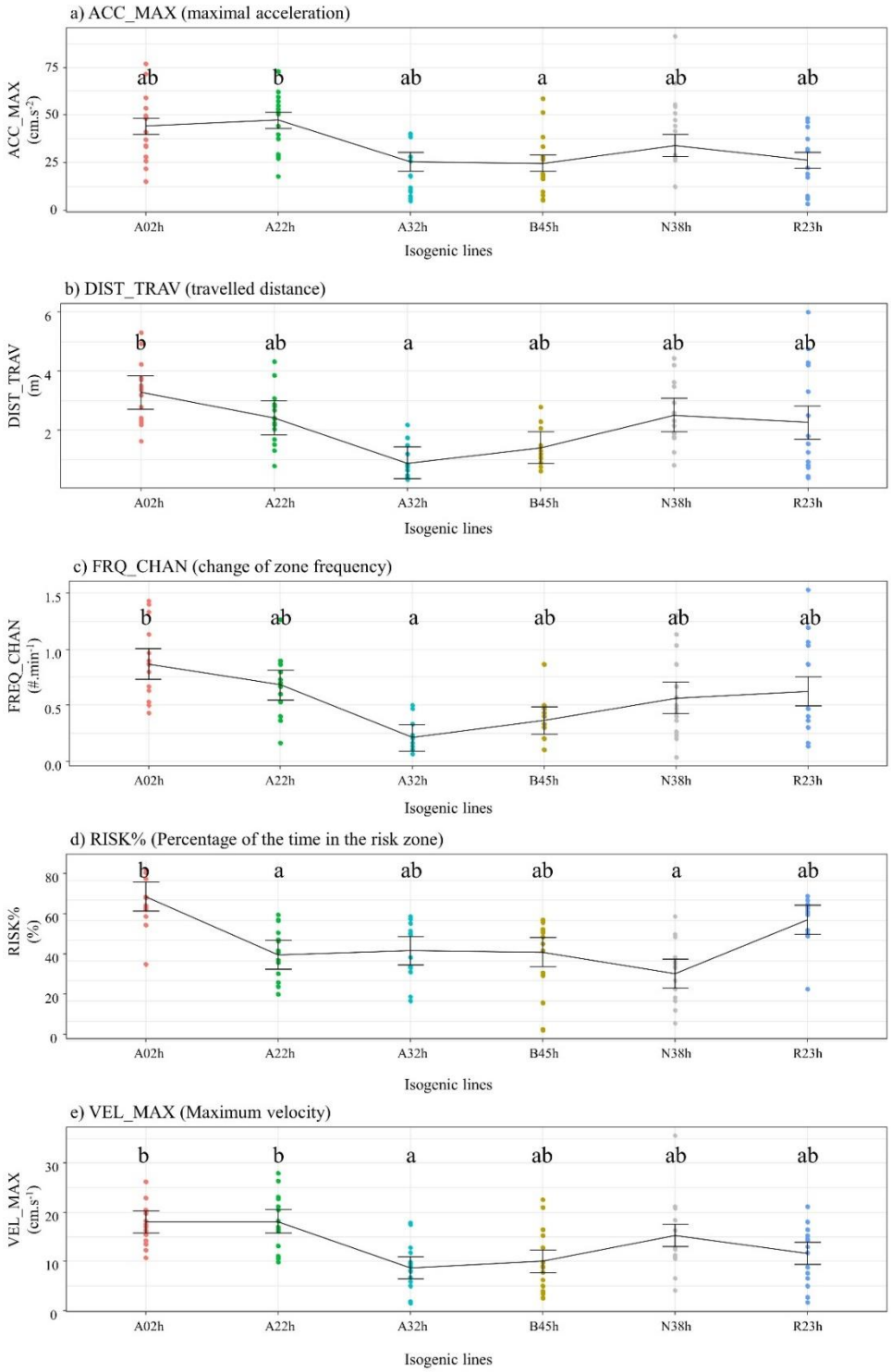

Fig. 3 Behaviour variables from individual test, for which *line* effect was statistically significant: a) ACC_MAX – maximal acceleration, b) DIST_TRAV – travelled distance, c) FRQ_CHAN – change of zone frequency, d) RISK% - percentage of time in the risky zone, e) VEL_MAX – maximum velocity. Dark lines represent fits of models. Error bars are standard errors of the model predictions. Means with same letters are not significantly different. Points represent the replicates mean behaviour variables, all time bins considered.

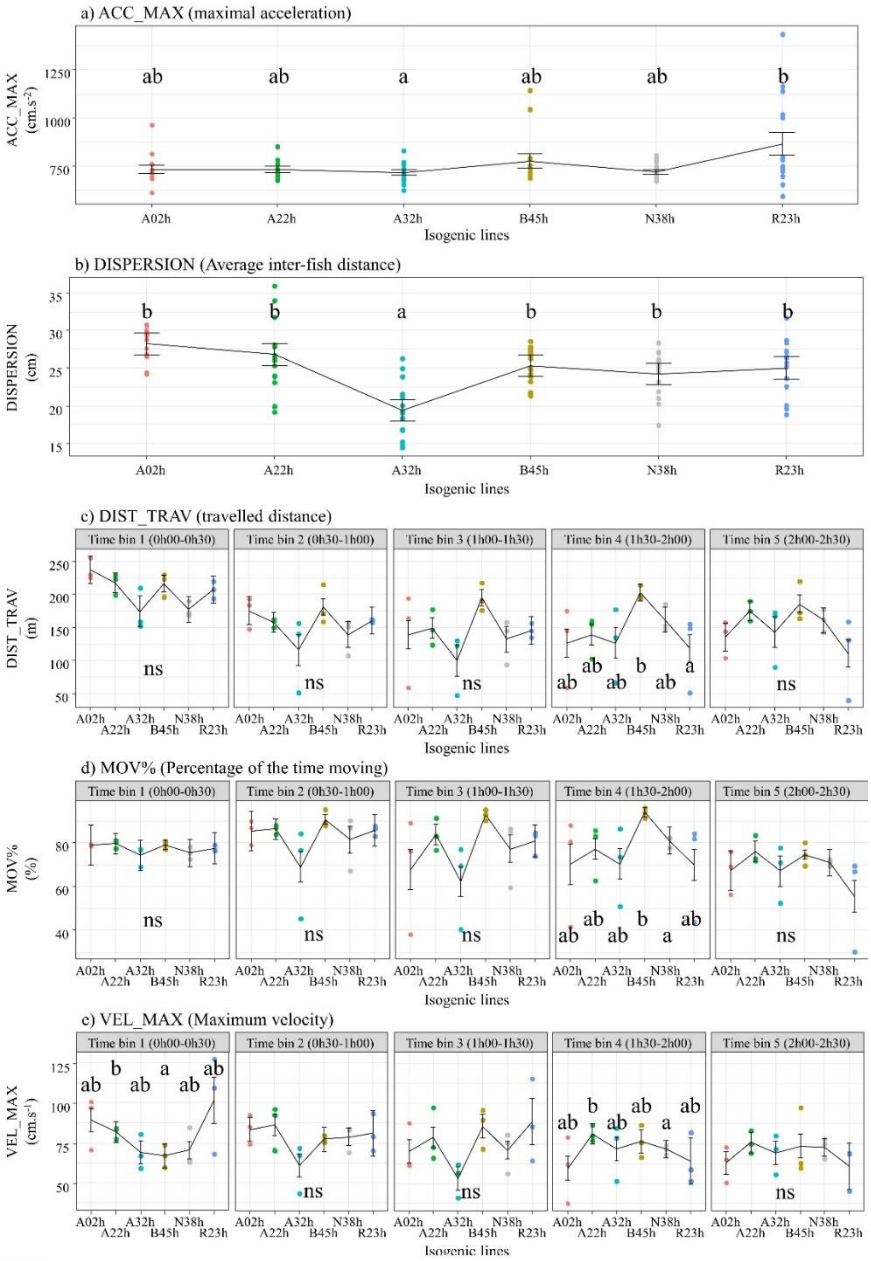

Fig. 4 Behaviour variables from group test, for which *line* was statistically significant: a) ACC_MAX – maximal acceleration, b) DISPERSION – average inter-fish distance, c) DIST_TRAV – travelled distance, d) MOV% – percentage of the time moving, e) VEL_MAX – maximum velocity. Dark lines represent fits of models. Error bars are standard errors of the model predictions. Means with identical letters are not significantly different. For ACC_MAX and DISPERSION, in the absence of interaction between *line* and *time*, points represent the replicates mean behaviour variables, all-time bins considered. For DIST_TRAV, MOV%, and VEL_MAX, behaviour variables for which the best model had a significant interaction between *line* and *time* effects, points represent the replicates' mean behaviour variables at each time bin. ns means there was no significant difference between lines.

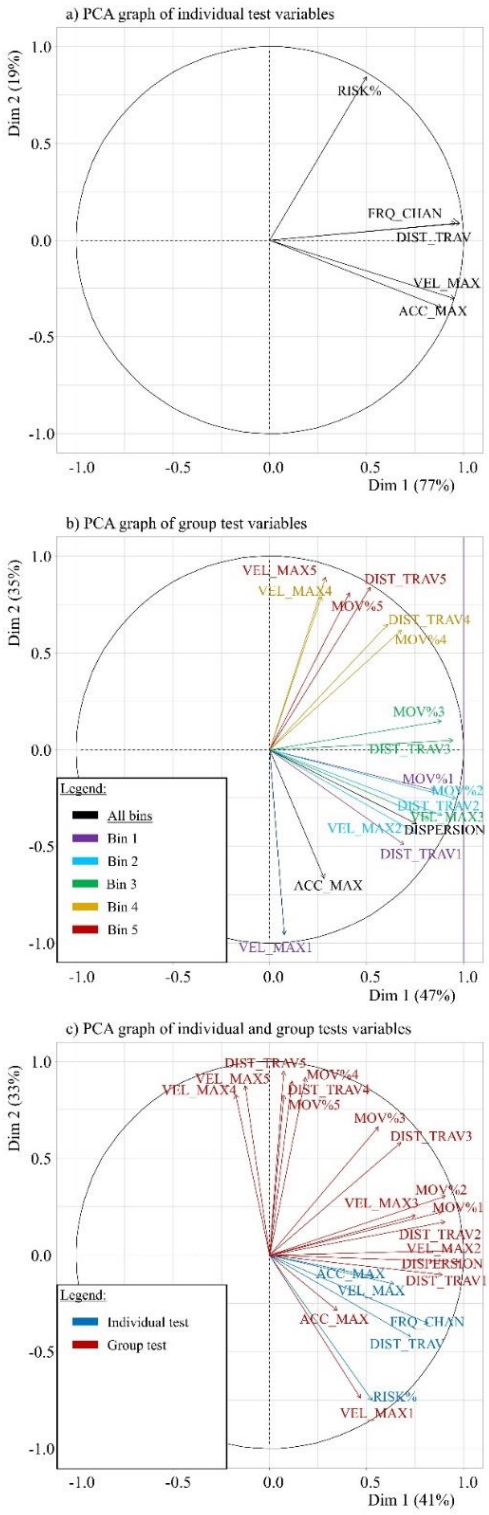

Fig. 5 PCA of isogenic lines lsmeans in behaviour tests. A) Individual test. B) Group test. "All bins" means that there was no significant interaction between *line* and *time* effects for the given variable; it was, therefore, possible to estimate *line* lsmeans on the whole test. "Bin 1", "Bin 2", "Bin 3", "Bin 4", and "Bin 5" are the lsmeans estimated at each time bin when there was a significant interaction between *line* and *time* effects. C) Individual and group tests together.

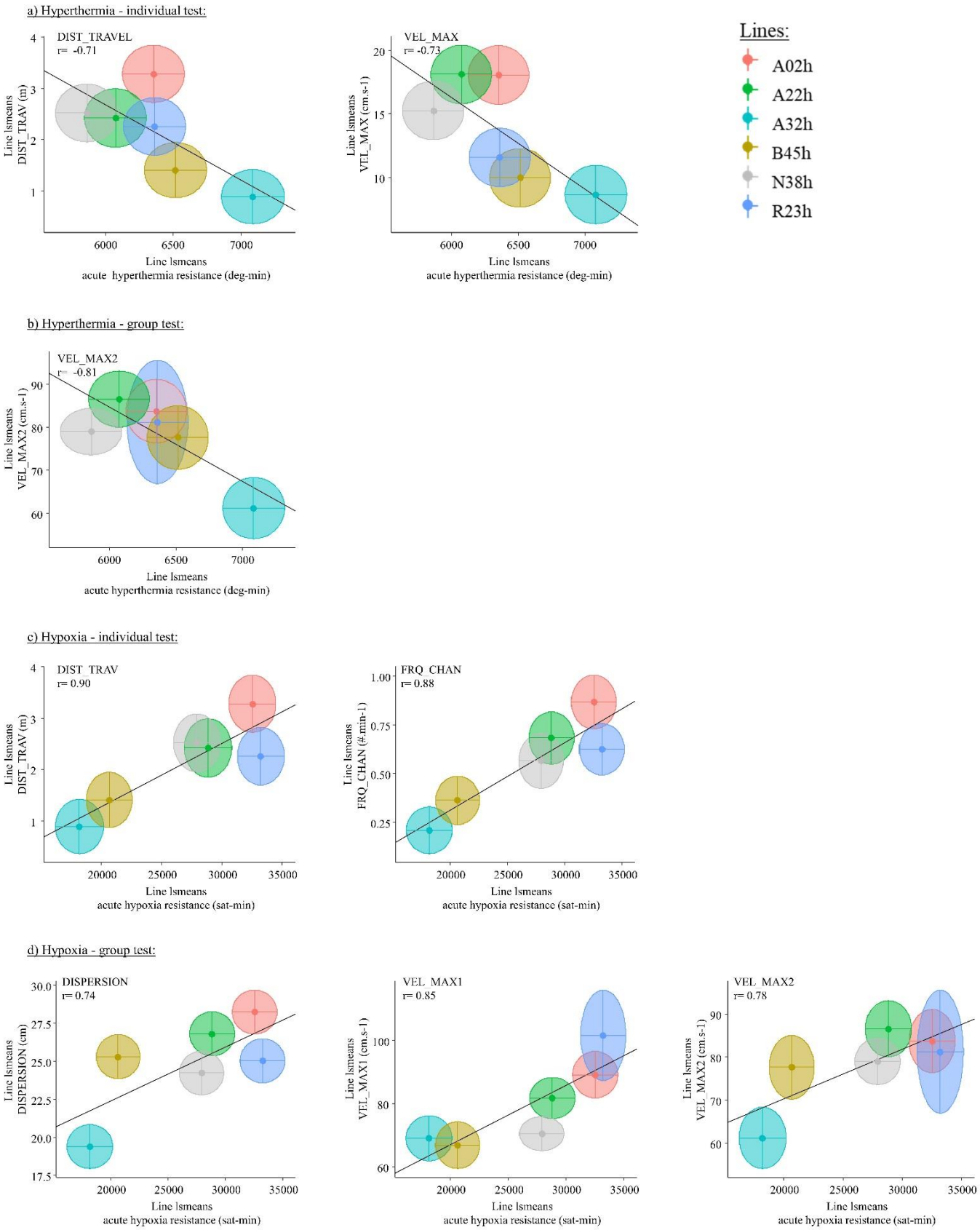

Fig. 6 Correlations between mean isogenic line effects on behaviour traits and mean isogenic line effects on acute hyperthermia or hypoxia resistances when correlations are higher than |0.7|.

Table 1 Definition of behaviour variables. Test 1 stands for individual behaviour test, and test 2 stands for group test.

Test	Abbreviation	Full name	Unit	Details
1 and 2	ACC_MAX	Maximum acceleration	$cm.s^{-2}$	The maximum acceleration. Acceleration is the difference in velocity divided by the time difference between the current sample and the previous one.
1 and 2	DIST_TRAV	Distance moved	m	The cumulated distance travelled by the body centre point of the subject from the previous sample to the current one.
1 and 2	MEANDER	Meander	$deg.cm^{-1}$	Meander is the change in direction of movement of a subject relative to the distance moved by that subject
1 and 2	MOV%	Percentage of the time moving	%	The fraction of time the subject was in moving state, *i.e.* when the subject velocity was above 1.5 cm/s.
1 and 2	VEL_MAX	Maximum velocity	$cm.s^{-1}$	The maximum velocity. Velocity is the distance moved by the body centre point of the subject per unit of time. The velocity was averaged over 2 frames.
1	EMERGENCE	Latency to exit from the safe zone	s	The time until fish first exit from the safe zone
1	FRQ_CHAN	Zone change frequency	$\#.min^{-1}$	The number of time per minute the subject cross the border between the risky and the safe zone.
1	RISK%	Percentage of the time in the risk zone	%	The fraction of time the subject remains in the risky zone.
2	DISPERSION	Average inter-fish distance	cm	The mean distance between the body centre point of a subject and the body points of all other individuals.
2	FRQ_CONTACT	Body contact frequency	$\#.min^{-1}$	The number of time per minute the subject is in the state "In contact", *i.e.* the subject body contour is in contact with the body contour of any of the other individuals.

Table 2 Best model for behaviour variables in individual and group tests

Behaviour variables	**Fixed structure best model**
Individual test	
ACC_MAX	*line, time, body weight*
DIST_TRAV	*line*
EMERGENCE	no significant effect
FRQ_CHAN	*line*
MEANDER	no significant effect
MOV%	*time*
RISK%	*line, time*
VEL_MAX	*line, time*
Group test	
ACC_MAX	*line*
DISPERSION	*line*
DIST_TRAV	*line, time, time × line*
FRQ_CONTACT	no significant effect
MEANDER	no significant effect
MOV%	*line, time, time × line*
VEL_MAX	*line, time, time × line*

Table 3 Correlations between isogenic line mean effect (lsmeans) on resistance phenotypes and isogenic line mean effect on behaviour variables. Pearson correlation coefficients above an arbitrary threshold set to |0.7| are highlighted in bold.

Test type	Behaviour variables	Time bin (if interaction)	Acute hyperthermia resistance (min)	Acute hypoxia resistance (min)
Individual test	ACC_MAX		-0.55	0.54
	DIST_TRAV		**-0.71**	**0.90**
	FRQ_CHAN		-0.69	**0.88**
	RISK%		0.19	0.56
	VEL_MAX		**-0.73**	0.69
Group test	ACC_MAX		-0.01	0.40
	DISPERSION		-0.67	**0.74**
	DIST_TRAV	bin 1	-0.20	0.51
		bin 2	-0.34	0.41
		bin 3	-0.30	0.05
		bin 4	-0.17	-0.48
		bin 5	-0.21	-0.51
	MOV%	bin 1	-0.35	0.38
		bin 2	-0.55	0.45
		bin 3	-0.44	0.05
		bin 4	-0.22	-0.42
		bin 5	-0.22	-0.44
	VEL_MAX	bin 1	-0.21	**0.85**
		bin 2	**-0.81**	**0.78**
		bin 3	-0.49	0.46
		bin 4	-0.11	-0.53
		bin 5	-0.27	-0.49