Links between behaviour and resistances to acute hyperthermia and hypoxia using rainbow trout isogenic lines

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Abstract
Acute hyperthermia and hypoxia resistances are traits of growing interest in aquaculture in the context of adaptation to climate change. The feasibility of genetic improvement of these traits through selection was demonstrated in rainbow trout (*Oncorhynchus mykiss*) and other fish species. It is now important to understand what makes a genotype resistant by identifying the mechanisms underlying these resistances, which are still under debate.
In the present study, we investigated links between resistances to acute hyperthermia and hypoxia and some behaviour variables measured in moderately stressful conditions. For this purpose, we used rainbow trout isogenic lines, previously phenotyped for acute hyperthermia and hypoxia resistances. Behaviour of fish was phenotyped using an individual test as well as a group test, respectively response to a novel environment and evaluation of shoaling combined with response to a moderate thermal challenge. The use of isogenic lines allowed to relate resistance to acute hyperthermia with behaviour phenotypes.
There were significant differences in behaviour between isogenic lines. Some behaviour variables were found to be highly correlated with acute hyperthermia and hypoxia resistances. Isogenic lines with a higher activity level were found to be more sensitive to acute hyperthermia and more resistant to acute hypoxia than lines with a lower activity level. This encouraging link between behaviour and resistance phenotypes also suggest that some behaviour variables could be used as predictors for acute hyperthermia and hypoxia resistances in fish, leading to more ethical phenotyping methods than classical ones.

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

Introduction

Acute hyperthermia and hypoxia resistances are phenotypes of interest in fish for numerous fields of research such as evolutionary biology (e.g. Mandic et al., 2009; Comte and Olden, 2017), stress-coping physiology (e.g. Rytkönen et al., 2007; Saravia et al., 2021), anticipating climate change effects (e.g. Messmer et al., 2017; Potts et al., 2021) or genetics to breed robust animals (e.g. Baer and Travis, 2000; Perry et al., 2005; Debes et al., 2021).

The classical methods for phenotyping acute hyperthermia and hypoxia resistances in fish consist in increasing the temperature or decreasing the O_2 saturation of the water until fish lose equilibrium. The resistance time or the temperature/O_2 saturation at loss of equilibrium are considered as the resistance phenotypes (Beitinger et al. 2000; Roze et al. 2013).

Previously, genetic determinism for acute hyperthermia and hypoxia resistances have been demonstrated in rainbow trout (Perry et al. 2005; Lagarde et al. 2023a; Prchal et al. 2023). Especially, Lagarde et al. (2023b) showed that some genotypes were more resistant to acute hyperthermia or hypoxia using rainbow trout isogenic lines but without exploring the physiological mechanisms underlying this observed genetic variability. It is now important to understand what makes a genotype resistant or not.

The underlying physiological mechanisms of the breaking point leading to loss of equilibrium during acute hyperthermia stress are still under debate in fish and can be described at multiple inter-dependant levels (Ern et al. 2023). Main theories around these mechanisms are the failure of short-term essential organs such as the brain or the heart, the failure of mitochondria processes, the thermal inactivation of enzymes, the different temperature effects on interdependent metabolic reactions, the alteration of membrane structures and the oxygen- and the capacity-limited thermal tolerance theory (Fry 1971; Schmidt-Nielsen 1997; Pörtner 2010; Chung and Schulte 2020; Lefevre et al. 2021; Ern et al. 2023). This last theory, which validity is not making consensus (Jutfelt et al. 2018; Pörtner et al. 2018), states that loss of equilibrium happens when the organism is unable to supply enough O_2 to meet the temperature-increased O_2 demand (Pörtner 2010).

The physiological mechanisms responsible for the loss of equilibrium during acute hypoxic stress seem to be better understood in fish. As oxygen concentration decreases, rainbow trout first initiates a change in respiration, increasing ventilation rate and gills perfusion to satisfy oxygen demand (Hughes and Saunders 1970; Randall 1982). Under a certain O_2 tension threshold, called critical oxygen tension, the aerobic metabolism is not enough by itself to supply all the energy required by the whole organism due to the incapacity to extract enough O_2 (Speers-Roesch et al. 2013). Anaerobic metabolism then gradually takes over and resistance of fish depends on the capacity to produce adenosine triphosphate (ATP) with anaerobic substrate and/or the capacity to tolerate or eliminate the harmful metabolites produced by anaerobic metabolism such as lactate or H+ (Nilsson and Östlund-Nilsson 2008; Mandic et al. 2009; Bergstedt et al. 2021). In the case of rainbow trout, loss of equilibrium could be induced by a fatal increase in brain lactate (Di Angelo and Heath 1987) or the rapid decrease of energy sources such as ATP and creatine phosphate in the heart and other crucial organs (Dunn and Hochachka 1986; Mandic et al. 2009). Another potential resistance mechanism described in hypoxia tolerant species such as the Nile tilapia *Oreochromis niloticus* or the crucian carp *Carassius carassius* could be the metabolic depression, *i.e.* the ability to decrease whole organism energy demand by reducing metabolic rate (Nilsson and Renshaw 2004; Bergstedt et al. 2021). In rainbow trout, metabolic depression does not appear to be a major factor in hypoxia resistance (Dunn and Hochachka 1986) albeit some studies revealed a slight metabolic depression (Boutilier et al. 1988; Cech et al. 1990; Williams et al. 2019). Thus, acute hyperthermia and hypoxia resistance mechanisms can be related to multi-level physiological characteristics of the fish.

During previous experiments aiming at the evaluation of the resistances to acute hyperthermia and hypoxia of rainbow trout (Dupont-Nivet et al. 2014; Lagarde et al. 2023b), we observed expression of a large behavioural repertoire suggesting that behaviour may be linked to resistance phenotypes. The aim of the present paper is to test whether behaviour could be linked with acute hyperthermia and hypoxia resistances.

The concept of coping styles (or personalities) can be defined as a consistent set of behavioural and physiological traits that co-vary across environmental contexts (Koolhaas et al. 1999; Conrad et al. 2011). Individuals' coping styles could be discriminated using the proactive-reactive axis (Sih et al. 2004). Reactive individuals are defined as individuals showing, among other traits, shyness, low activity, low exploratory behaviour and low risk taking. On the other hand, proactive individuals are bold, more active and exploratory, take risk, and have an active "fight or flight" behaviour when exposed to novelty (Sih et al. 2004; Castanheira et al. 2015; Baker et al. 2018; Alfonso et al. 2020c). Potential coping styles differences were suggested in rainbow trout isogenic lines (Millot et al. 2014). Interestingly, coping styles were also 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 and Johnsson 2011), and boldness in a refuge emergence test was related with high aerobic capacity in bluegill sunfish *Lepomis macrochirus* (Binder et al. 2016). However, to our knowledge, no study has investigated the genetic relationship between behaviour and resistance to hyperthermia or hypoxia traits in fish.

Identifying mechanisms underlying resistance to acute hyperthermia and hypoxia, and whose phenotyping would be reasonably intrusive, could also lead to the development of predictors to replace the current classical phenotyping methods. Although the classical phenotyping methods of acute hyperthermia and hypoxia resistances have the advantage of directly measuring the phenotypes of interest, they have several drawbacks. Firstly, 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). However, 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 fairly quickly from acute hyperthermia or hypoxia challenges with less than 1% of mortality (Claireaux et al. 2013; Morgan et al. 2018). Secondly, acute hyperthermia and hypoxia challenges push fish to their limits with the loss of equilibrium as endpoint, generating animal suffering. Within the 3R framework (Replacement, Reduction, Refinement), active research must be conducted to reduce animal suffering in scientific experiments. Moreover, there is a growing pressure from the society on the scientific community to reduce animal suffering (Sloman et al. 2019). Therefore, what is legal today might not be tomorrow.

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

For this, we used the rainbow trout isogenic lines, previously shown to have different resistance to either hyperthermia or hypoxia (Lagarde et al. 2023b). Behaviour of fish was monitored using an individual test as well as a group test, the latter being associated with a moderate thermal stress. Measured behaviour phenotypes were related to activity (acceleration, speed, distance travelled, movement), risk taking (emergence, time staying in a risky zone) and schooling behaviour (inter-individual distance, contacts between fish). Although different individuals were phenotyped for resistance to acute hyperthermia and hypoxia and behavioural variables, it was possible to relate all these phenotypes thanks to the full genetic homogeneity existing among fish within an isogenic line.

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. Homozygous isogenic lines were originally established by two generations of gynogenesis and further maintained using neomales which are sex-reversed XX males (Quillet et al. 2007). Homozygous isogenic lines are a powerful genetic resource as fish are homozygous and genetically identical within line. Fish from a given line can therefore be considered as the replicates of a unique genotype.

Fish used in the present study were from the same rearing batch as the one reported in Lagarde et al. (2023b). Fig. 1 summarises the rearing process of the fish as well as which data were already published.

Briefly, six heterozygous isogenic lines were produced by fertilizing eggs from same-day naturally spawning females of the homozygous isogenic line B57 with the sperm of six neomales issued from the homozygous isogenic lines A02, A22, A32, B45, N38 and R23. Then, the only source of variation between heterozygous lines at this stage was expected to be the paternal genetic effect.

Fertilized 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) each hatching tray was randomly divided into three new hatching trays constituting three replicas per isogenic line (>500 eyed eggs per hatching tray, 3x6 hatching trays) as shown on Fig. 1. At 43 dpf, all replicates were transferred into 0.25 m^3 indoor tanks with no mixing between replicates (~500 larvae per tank, 3x6 tanks) supplied with the same spring water. At 145 dpf, 230 fries were kept in each of the 18 tanks by randomly removing 270 fries per tank. Removed fries were reared one more year in separate tanks and phenotyped for acute hyperthermia and hypoxia resistances for the purpose of another experiment (Lagarde et al. 2023b). At 150 dpf, the number of individuals was randomly reduced to 160 in each of the eighteen tanks which decreased 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 anaesthetized, 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 six replicates with the six lines mixed that were used to phenotype the resistance of isogenic lines to acute hyperthermia (3 replicates) and hypoxia (3 replicates) (Lagarde et al. 2023b). Among the approximately 60 fish staying in each of the 18 tanks, 6 were randomly collected for evaluating individual and group behaviour between 177 and 179 dpf according to an experimental set-up presented below. Between 182-187 dpf, resistance of the six isogenic lines to acute hyperthermia and hypoxia was measured as presented in Lagarde et al. (2023b) using the fish from the tanks with mixed lines. During all 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.

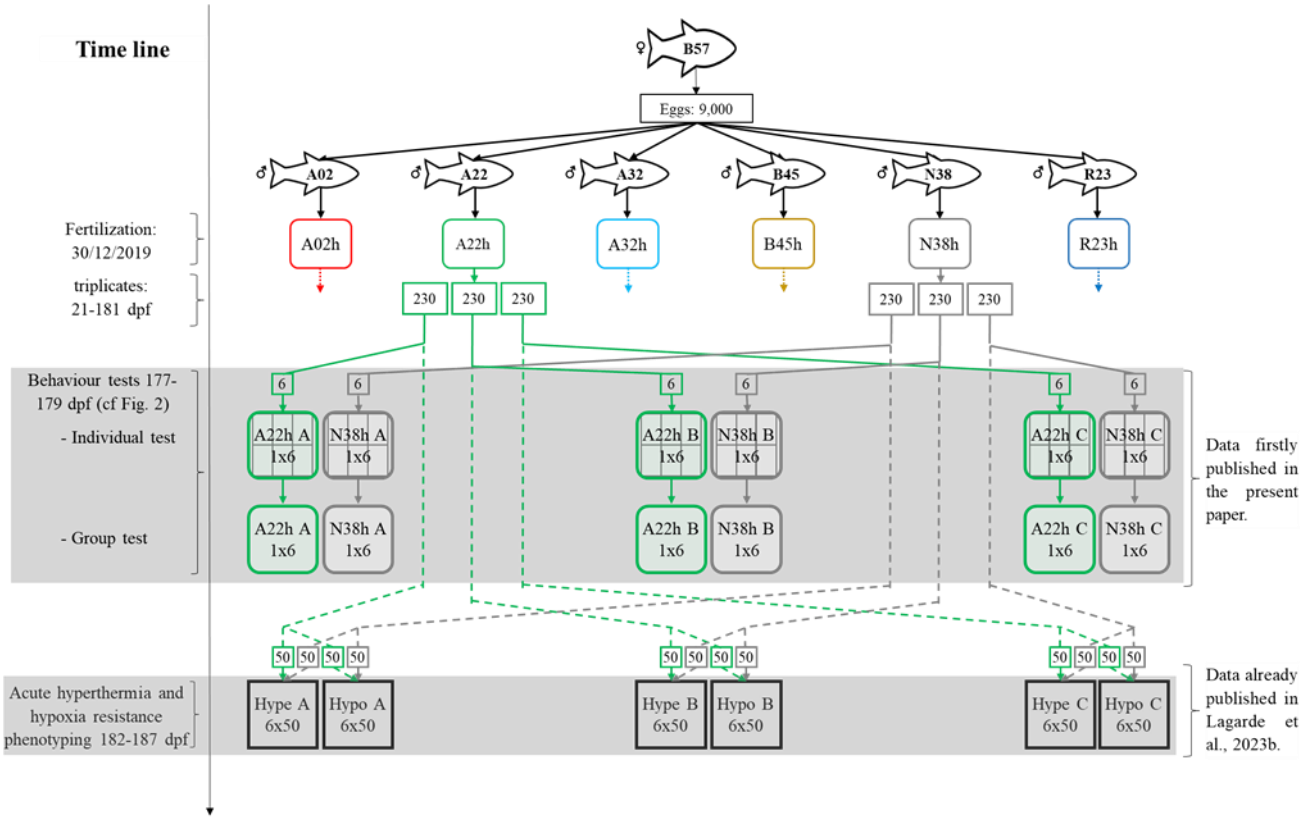

Fig. 1 Rearing history of fish. Each isogenic line is represented by a different colour. Full rearing history and experimental use was only represented for lines A22h and N38h. The four other lines were not represented so as not to overload the figure but they had a similar life history as A22h and N38h. Rectangles represent rearing tanks. Replicate rearing tanks corresponding also 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 line used in each experiment is indicated in each experiment rectangular with the first number indicating the number of isogenic line and the second the number of fish per isogenic line. Movements of fish are represented by the lines, 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. Adapted from Lagarde et al. (2023b).

1.2 General conduct of behavioural experiments

Behavioural experiments were carried out in a quiet room with no traffic for the duration of the experiments. Fig. 2 presents the diagram of the experimental devices.
Behaviour was simultaneously evaluated on six isolated individuals from the same isogenic line during 25 minutes (called individual test thereafter) and immediately after the same six individuals were grouped in a tank and exposed to a moderate heat stress during 150 minutes (called group test thereafter). Devices and duration of individual test were derived from previously published protocol used on zebrafish and seabass (Vignet et al. 2015; Alfonso et al. 2020a, b, 2021). Temperatures during the individual test and at start of the group test were set similar to the one 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, making a total of 18

sessions. Each session approximately lasted 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 to take place during morning, midday and afternoon slots.

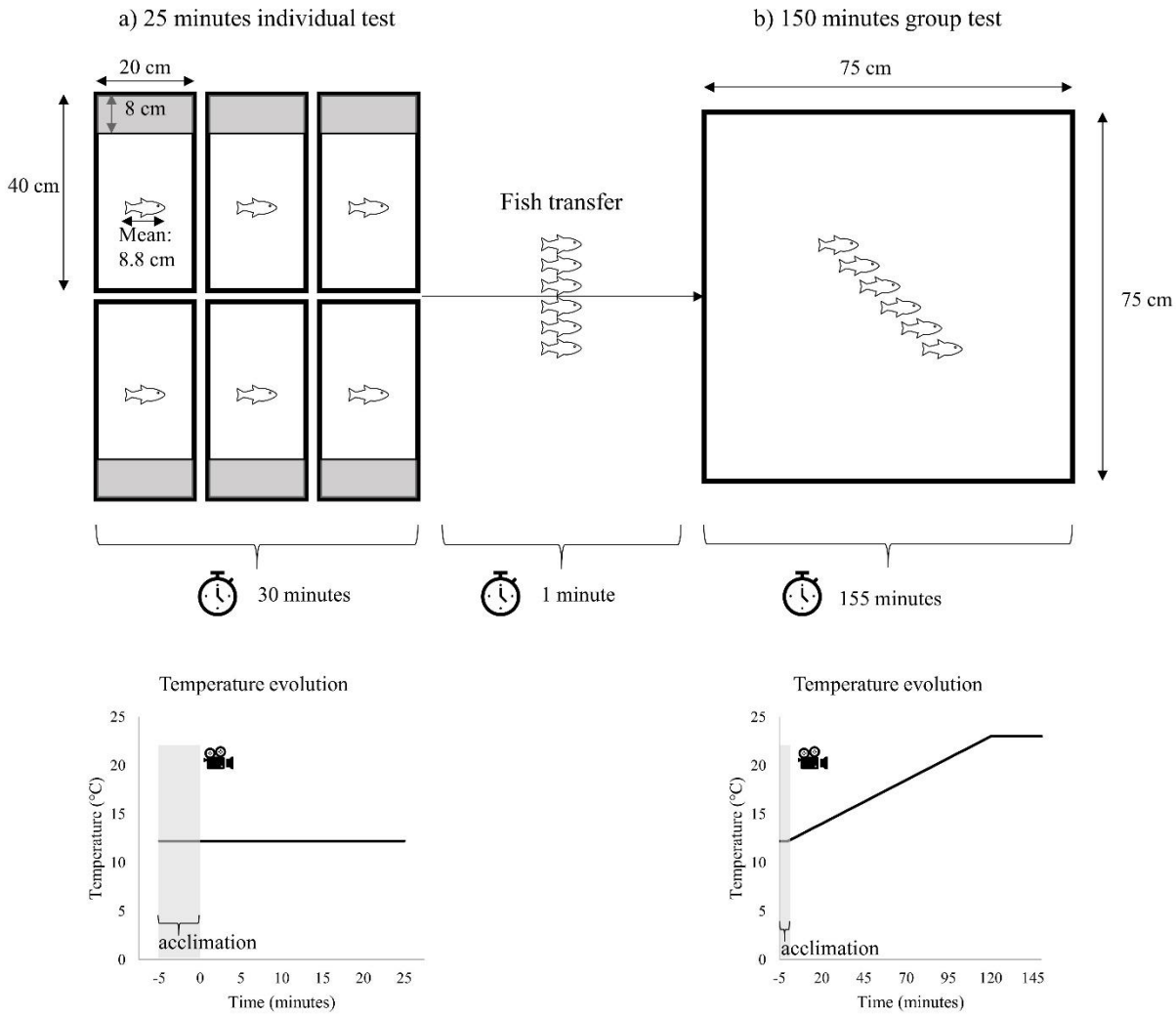

Fig. 2 Diagram of behaviour tests. a) Tanks used for monitoring individual behaviour. Grey bands represent the safe zone, which is covered with a lid opaque to visible light. The 6 fish monitored in parallel were from the same isogenic line. Temperature was kept constant during the 5 minutes of acclimation and 25 minutes of the behaviour test. b) Fish were then transferred in a unique tank for 150 minutes 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.

1.3 Conduct of individual behaviour test

The purpose of this test was to evaluate behaviour in a novel environment. Six fish from the same isogenic line were fished 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 home-made rectangular tent lit by spots placed 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 which was 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 move between the safe and risky zones. Once the door opened, fish activity was video recorded during 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 the 25 minutes, fish were immediately transferred to the group behaviour tank. At the end of each individual test, water was fully renewed in aquariums before the start of the next individual test.

1.4 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 10 cm depth water (Fig. 2.b).
Fish were gently poured out of the bucket. Once the transfer of fish from the individual aquariums to the group tank completed, fish were left 5 minutes for acclimation. Then, water temperature in the tank 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 during the next 30 minutes (Fig. 2.b). Fish activity was video recorded during 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 located on each side of the tank in order to have a homogeneous lighting. At the end of the experiment, fish were softly anaesthetized (Tricaïne MS222, 50 mg/L), quickly wiped clean, weighed and euthanized (Tricaïne MS222, 150 mg/L). Fish mean body weight was 11.6 ± 3.2 g, all lines included. Detailed isogenic lines mean body weights are given in Table S1.

1.5 Videos' behaviour variables extraction

Videos were analyzed with EthoVision XT15.0 software. Fish positions were tracked using a dynamic subtraction method with a sampling rate set at 10 frames per second. The accuracy of the tracked positions was checked by viewing the superposition of the tracked and real positions of fish in all videos and manually edited 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 group behaviour experiment, seven variables were measured related to behaviour of the group: the maximum acceleration (ACC_MAX, $cm.s^{-2}$), the average inter-fish distance (DISPERSION, cm), 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. In the group test, individual positions of fish were tracked by the software but there was frequent swapping of identity when two fish cross paths in front of the camera. This is almost unavoidable and since group variables were measured, fish identity was not useful.
To take into consideration the effect of time on behaviours, variables were extracted over several time bins, every 5 minutes for the individual test and every 30 min 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 group test.

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 traveled by the body center 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. The mobile state was averaged over 5 samples.
1 and 2	VEL_MAX	Maximum velocity	cm.s-1	The maximum velocity. Velocity is the distance moved by the body center point of the subject per unit of time. The velocity was averaged over 2 samples.
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 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.

1.6 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 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 in Millot et al. (2014). Fish body weight was only known at the level of the replica 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 was that Ethovision software was unable to 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 isogenic line through time bins on behaviour variables, we fitted linear mixed effects models with body weight as covariate, *time* (time bins) and *line* (isogenic line) as categorical fixed effects, *replica* and the interactions between *time*, *line* and *body weight* as random effects. *time* was set up as a categorical variable because a visual inspection of response variables plotted against time did not reveal any linear relationship. For EMERGENCE, we only tested *line* and *body weight* effects as this event can only happen once in the whole test. In the group test, *time* effect was confounded with the temperature increase as no control of group test, *i.e.* without temperature increase, was realized 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. Weighted terms allowing variances to differ among replica and isogenic lines and autoregressive AR1 or autoregressive moving average ARMA(1,1) covariance structures to account for temporal autocorrelation (Zuur et al. 2009) were considered to select the best model for each response variable. 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 *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 bin of *time* and *vice-versa*.
To get a synthetic view on the relationships between behaviour variables, *line* lsmeans were reduced to two principal components using PCA. Three kind 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 both individual and group tests.

1.7 Screening for correlation between behaviour and resistance variables

1.7.1 Acute hyperthermia and hypoxia resistances of isogenic lines

The methodologies and devices used to determine the mean acute hyperthermia and hypoxia resistances of each isogenic line were presented in Lagarde et al. (2023b) and will be briefly recalled 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 resistances (Fig. 1). Each replicate (named A, B and C in Fig. 1) was containing 50 fish from each of the six isogenic lines, *i.e.* a total of ~300 fish per replica.

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

As temperature increased in acute hyperthermia challenge or O_2 saturation decreased in acute hypoxia challenge, fish gradually lost equilibrium. Each time a fish was losing equilibrium, it was fished, its isogenic line was identified using PIT-tag and the time to loss of equilibrium was recorded. Fish was then anaesthetized, weighed, and euthanized.

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 difference between the temperature measured in the challenge tank and the temperature at the start of the challenge, summed each minute from the start of the challenge to the time of loss of equilibrium. CHE is the difference between a reference O_2 saturation of 90%, the O_2 saturation before the start of the challenge, and the O_2 saturation measured in the challenge tank, summed each minute from the start of the challenge to the time of loss of equilibrium. It is worth mentioning that a high CTE or CHE indicates a high resistance to acute hyperthermia or hypoxia, respectively.

The effect of isogenic line on acute hyperthermia and hypoxia resistances were analyzed by fitting a model with *line* as a fixed effect, fish *body weight* as a covariate and their interaction as explained in Lagarde et al. (2023b). *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 resistances were estimated at 15.2 g, the mean body weight, using lsmeans 2.30–0 R package (Lagarde et al. 2023b).

1.7.2 Relationship between behaviour and resistance variables

The lsmeans of the same six isogenic lines for the behavioural variables and for the resistances to acute hyperthermia and hypoxia stress were calculated using the steps mentioned in the two 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 behaviour tests (11.6 ± 3.2 g) compared to 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

Table 2 lists variables extracted from behaviour experiments and indicates significant factors if any. Fig. 3 and 4 present the raw and modelled behaviour phenotypes obtained for each isogenic line in individual and group tests, respectively, all time bins considered. Fig. S2 and S3 present the raw and modelled behaviour phenotypes obtained for each time bin for individual and group tests, respectively. For more details, modelled behaviour phenotypes data for *line* and *time* bins showed on Fig. 3-4-S2-S3 are given in Tables S2-S3-S4-S5, respectively.

2.1.1 Behaviour variables with no significant effect

Behaviour variables for which neither *time*, *line* or *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 of body weight between lines (ANOVA, N = 108, df=5; F value = 17.3; P < 2.8e-14). Details on 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 other things being equal.

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

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

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

In the individual test, *time* effect was significant for variables ACC_MAX, MOV%, RISK% and VEL_MAX without any interaction observed with *line* effects (Table 2). 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 burst of activities (high ACC_MAX and VEL_MAX) interspersed with freezing moments (low MOV%). In a second step, burst of activities 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, *time* effect was significant for some behaviour variables but always with a significant *time* x *line* interaction (Table 2). Results will be presented below.

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

In individual behaviour test, *line* effect was significant for ACC_MAX, DIST_TRAV, FRQ_CHAN, RISK% and VEL_MAX (Table 2). There were therefore significant variations in activity levels between the lines during individual test, presented on 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 (25.6 cm.s^{-2}) and higher VEL_MAX (18.1 cm.s^{-1}) than A32h (8.7 cm.s^{-1}).

In group behaviour test, *line* effect was significant and not interacting with *time* effect for ACC_MAX and DISPERSION (Table 2). Line A32h had a significantly lower ACC_MAX (716 cm.s^{-1}) than line R23h (867 cm.s^{-1}) 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 individual test, interaction between *time* and *line* effects was not significant whatever the behaviour variable (Table 2).

In group test, 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 was 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 is presented in Fig. S3. For DIST_TRAV, there were significant differences between time bins (time bin 1 higher than other time bins) for all lines but N38h. For MOV%, there were significant differences between time bins for B45h and R23h. For VEL_MAX, there were significant differences between time bins for A02h and A32h.

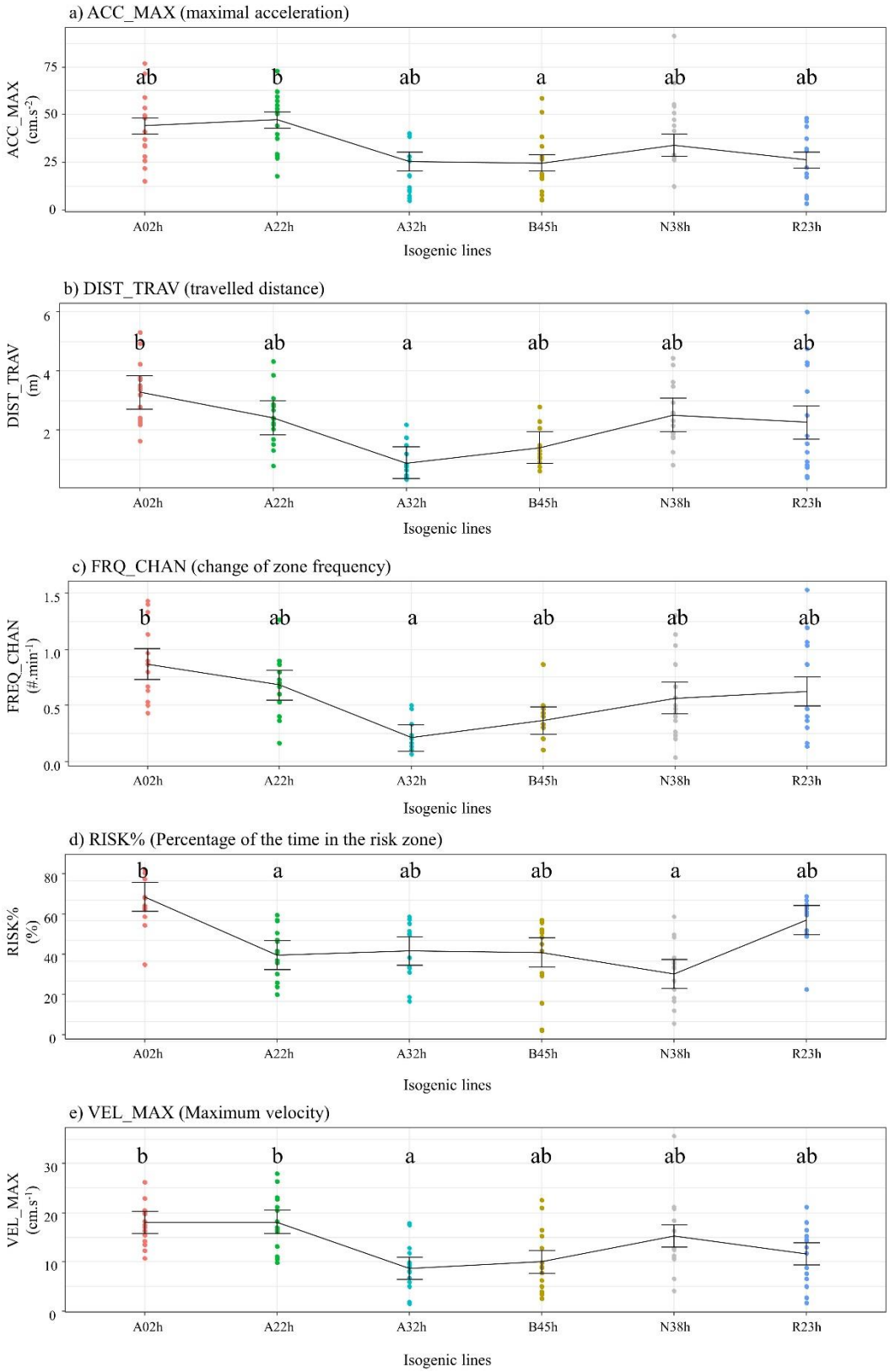

Fig. 3 Behaviour variables, from individual test, for which line effect was statistically significant: a) ACC_MAX – maximal acceleration, b) DIST_TRAV – travelled distance, c) FRQ_CHAN – change of zone frequency, d) RISK% - percentage of time in the risky zone, e) VEL_MAX – maximum velocity. Dark lines 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.

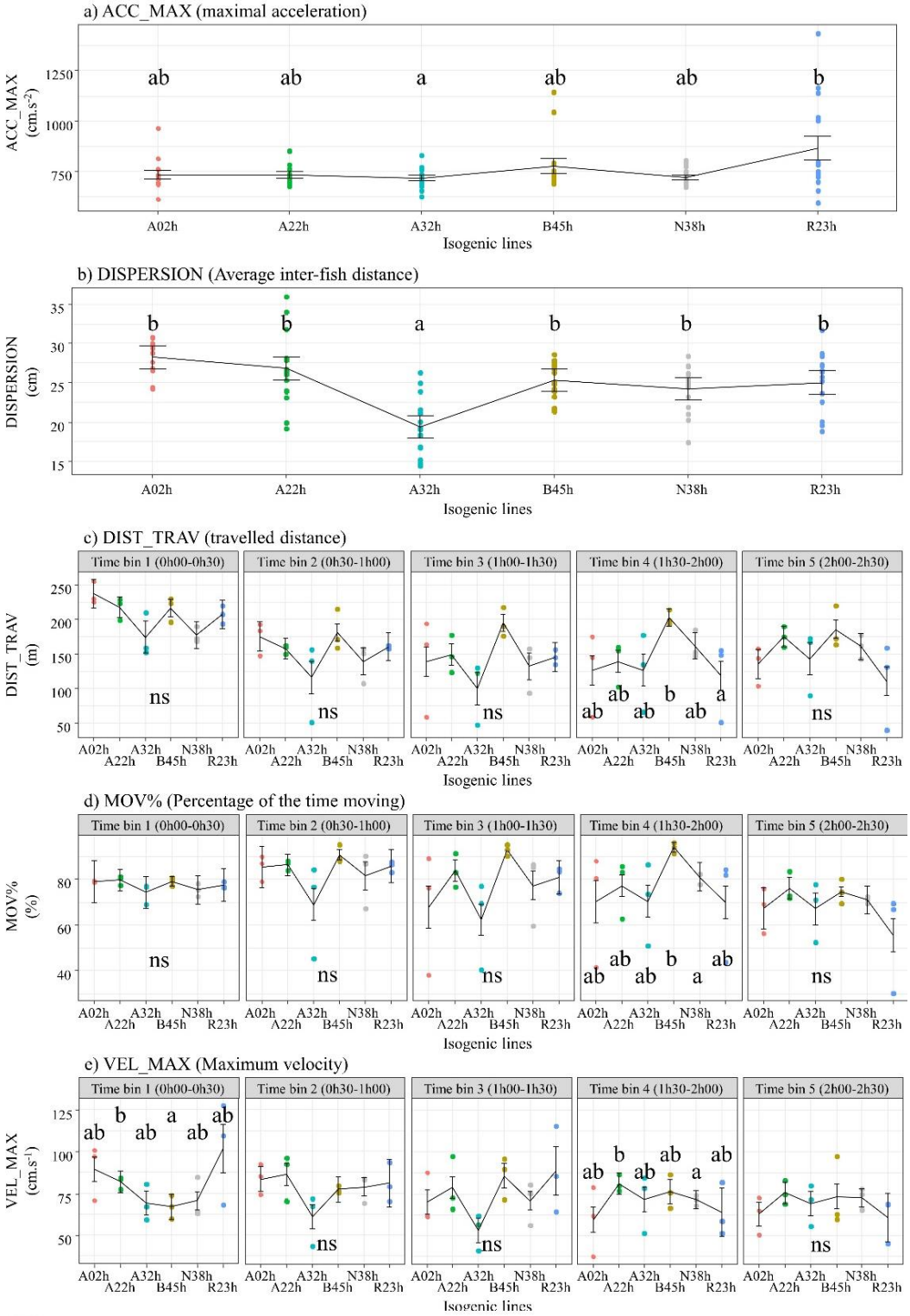

Fig. 4 Behaviour variables, from group test, for which line was statistically significant: a) ACC_MAX – maximum acceleration, b) DISPERSION – average inter-fish distance, c) DIST_TRAV – travelled distance, d) MOV% – percentage of the time moving, e) VEL_MAX – maximum velocity. Dark lines represent fits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

2.2 Relationships between behaviour variables

2.2.1 PCA

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

2.2.2 Individual behaviour test

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 4 variables, each referring to the level of activity of the fish 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.

2.2.3 Group behaviour test

In group test, interaction between *line* and *time* was significant for the variables DIST_TRAV, MOV% and VEL_MAX (Table 2). For these variables, lsmeans were therefore calculated at each time bin 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 bins merged (Fig. 5.b).

This PCA highlights a clear separation between the DIST_TRAV, MOV% and VEL_MAX variables of the first two time bins and the last two. Indeed, within time bins 1 and 2, DIST_TRAV, MOV% and VEL_MAX were moderately to highly correlated together with a r ranging between 0.30 to 0.94 (Table S7). Within time bins 4 and 5, DIST_TRAV, MOV% and VEL_MAX were strongly correlated together 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 most of the time negative, close to zero or moderate (Table S7). Interestingly, DIST_TRAV, MOV% and VEL_MAX of time bin 3 was intermediary between 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. DISPERSION variable was moderately to strongly correlated with DIST_TRAV, MOV% and VEL_MAX at time bins 1, 2 and 3 and uncorrelated at time bins 4 and 5 (Table S7). DISPERSION was also poorly correlated with ACC_MAX (Table S7).

2.2.4 Individual and group behaviour tests

Relationships between isogenic line lsmeans of behaviour variables measured in individual and group tests are presented on the PCA in Fig. 5.c and correlation coefficients are given in Table S8. Roughly, the PCA of individual and group test variables shows that the correlations between the variables of the individual and group tests gradually turned from positive to negative with

time bins in group test as time and temperature increased (Fig. 5.c). Thus, correlations between activity variables in individual test (ACC_MAX, DIST_TRAV, FRQ_CHAN and VEL_MAX) and DIST_TRAV, MOV% and VEL_MAX in group test were moderately to highly correlated when considering time bins 1 and 2 of group test, not correlated when considering time bin 3 of group test and negatively correlated when considering time bins 4 and 5 of group test (Fig. 5.c and Table S8). RISK% of individual test was negatively correlated with activity variables in group test 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.

Isogenic line mean effect on acute hyperthermia resistance was strongly negatively correlated ($r < -0.71$) with 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 5 time bins) were also slightly to moderately negative with a r between -0.11 and -0.69 (Table 3). ACC_MAX in group test was uncorrelated with acute hyperthermia resistance.

Isogenic line mean effect on acute hypoxia resistance was highly positively correlated with isogenic line mean effect on DIST_TRAV and FRQ_CHAN in the individual test ($r > 0.88$; Fig. 6.c and Table 3). Still 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: $-0.42 < r < -0.53$).

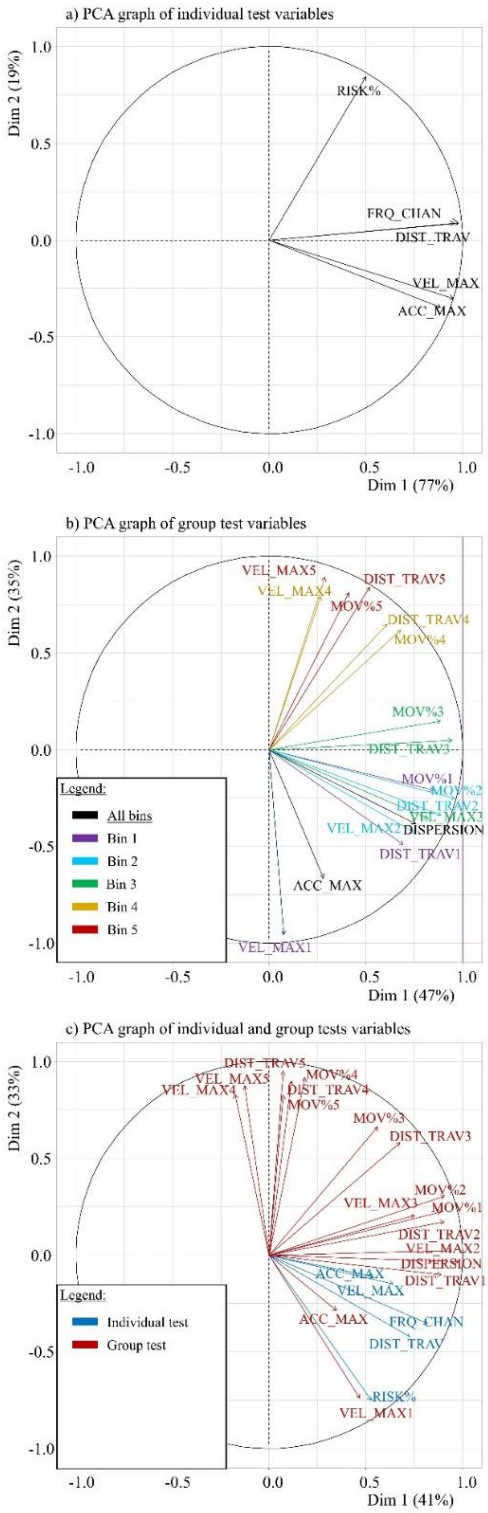

Fig. 5 PCA of isogenic lines lsmeans in behaviour tests. A) Individual test. B) Group test. ”All bins” means that there was no significant interaction between *line* and *time* bin effect for the given variable; it was therefore possible to estimate *line* lsmeans on the whole test. “Bin 1”, “Bin 2”, “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.

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

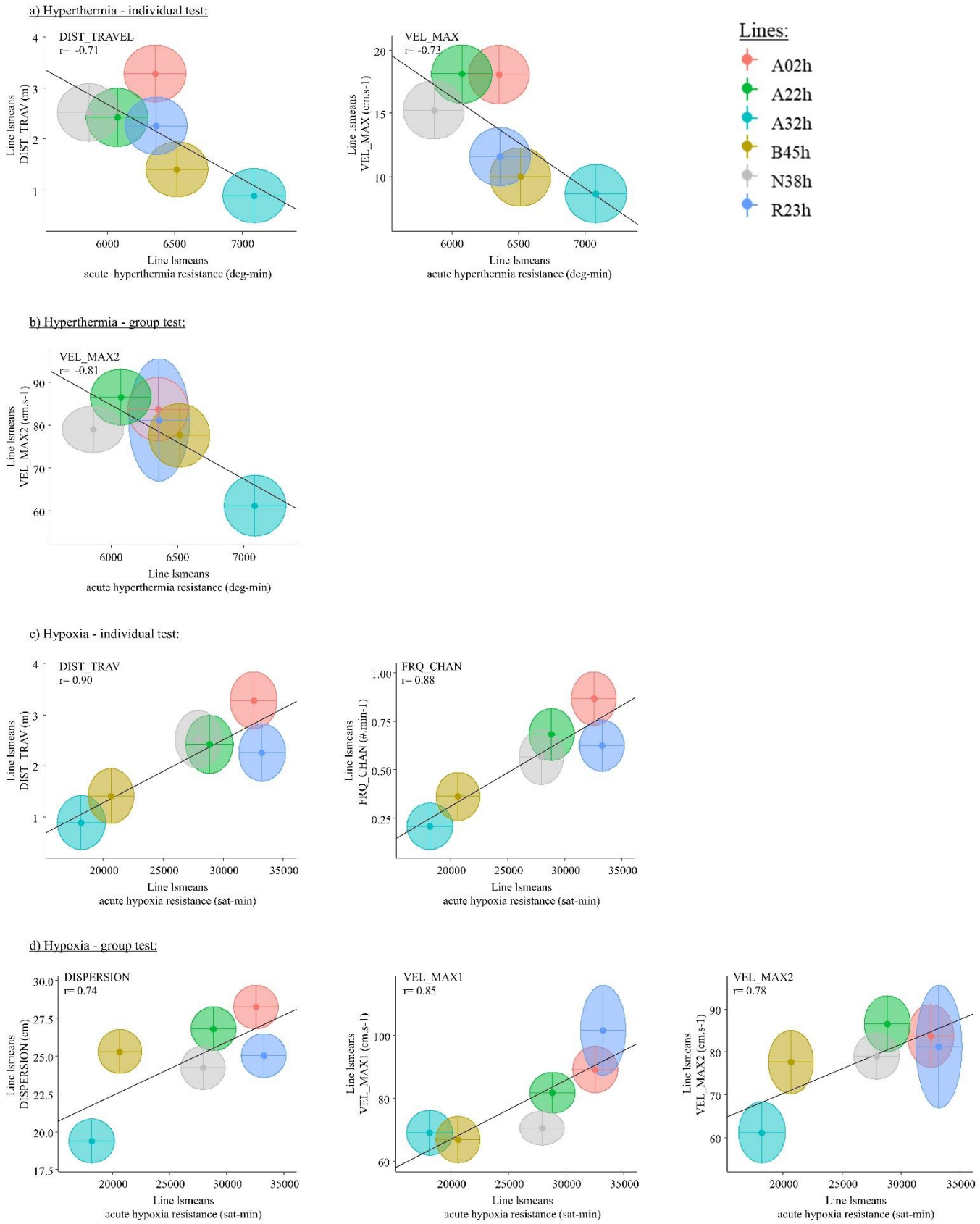

Fig. 6 Correlations between mean isogenic line effects on behaviour traits and mean isogenic line effects on acute hyperthermia or hypoxia resistances when correlations are higher than |0.7|.

3. Discussion

Behaviour was shown to be linked with numerous phenotypes such as oxygen consumption, growth, diet choice or migration in fish (Martins et al., 2011; Mittelbach et al., 2014). In this work we searched to establish if such links could also be established between behaviour variables and resistance to acute hyperthermia or hypoxia. This work is also a first step toward improvement of welfare during phenotyping process for resistance to acute hyperthermia and hypoxia using less invasive refined proxies compared to classical methods potentially more deleterious. For this, we monitored behaviour of six isogenic lines and studied links with resistances to acute hyperthermia and hypoxia phenotypes previously determined (Lagarde et al. 2023b).

3.1 Behaviours' evolution with time and isogenic lines

Regarding the evolution of behaviour variables during the course of the test (significant effect of time), the "burst and freeze" behaviour observed in the beginning of the individual test (first time bin), characterized by low moving duration and high maximum acceleration and velocity (Fig. S2.a&b&d) are representative of erratic movements linked to high level of stress (Brown et al. 2005; Staven et al. 2019). This is consistent with the stress induced by the opening of the door and the novel environment proposed. Then in following time bins, as stress is relieved, behaviour gradually turned into exploratory behaviour, characterized by an increase in moving duration and time spent in the risky zone (Fig. S2.b&c) (Ryer 2004; Malavasi et al. 2008; Millot et al. 2009).

Significant behavioural differences between isogenic lines were found in both individual and group tests, which is consistent with the existence of genetic determinism in fish behaviour as reviewed in Bell (2008) and more particularly with a previous study also using isogenic lines (Millot et al., 2014). In Millot et al. (2014), four lines were common to the present study (A02h, A22h, B45h and N38h). Albeit not significative, A02h was found to swim more than B45h in both studies (A22h and N38h were not studied for this variable in Millot et al., 2014). However, the total time spent in the risky zone relative ranking was found different between the two studies. Interpreting these similarity and difference is not possible since protocols and devices were completely different between the present study and Millot et al.'s one, in which novel object and emergence tests were used.

The interaction between *time* and *line* was significant for several behaviour variables in the group test and never in the individual test (Table 2). This may be due to the longer duration of the group test, letting time to behaviour of isogenic lines to evolve. A second hypothesis could be that the temperature increase led to a different stress-response of the lines as several studies showed different behaviour reaction to a stress depending on fish personalities in rainbow trout (Frost et al. 2013; White et al. 2017).

3.2 Behaviours' correlations within isogenic lines and tests

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

Regarding correlations between behaviour variables measured in both individual and group tests (ACC_MAX, DIST_TRAV and VEL_MAX), at the level of the isogenic line, these variables were moderately to highly positively correlated together between individual test and the two first time bins of group test (Fig. 5.c, Table S8). Consistent relationships between behaviour variables in two different contexts allows to define coping styles and to ascribe them to some lines. Thus, in our individual and group tests in which environments were novel to fish, reactivity can be related to isogenic lines showing a low level of activity such as A32h and proactivity to lines with high level of activity such as A02h. However, it is important to note that a firm personality assignment would have required more behavioural testing such as done in Cerqueira et al. (2016). As time and temperature increased, these correlations between individual and group behaviour variables loosened (Fig. 5.c, Table S8), although we cannot determine which of the confounded time and temperature is 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 or 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

In individual behaviour test, acute hyperthermia resistance was found to be rather associated with low level of activity and thus to reactivity as highly negative correlations were found with DIST_TRAV and VEL_MAX and moderately negative correlations with ACC_MAX and FRQ_CHAN. At the opposite, acute hypoxia resistance was found highly correlated with high level of activity and thus to proactivity as highly positive correlations were found with DIST_TRAV, FRQ_CHAN and moderately positive correlations with ACC_MAX, RISK% and VEL_MAX.

In the two first bins of group test, we observed a similar pattern, although less obvious: 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%).

The link between behaviour and acute hyperthermia resistance in fish remains poorly studied. Consistently with our results, Campos et al. (2018) reported that fish species with the lowest routine activity level, indirectly measured with a proxy, had the highest resistance to acute hyperthermia. Campos et al. (2018) hypothesized that fish with the lowest activity have lower resting energetic demands and thus can resist longer to acute hyperthermia as predicted by the oxygen- and the capacity-limited thermal tolerance theory. However, the validity of this theory as the primary resistance mechanism to acute hyperthermia stress is still highly debated (Jutfelt et al. 2018; Pörtner et al. 2018). In contrast with our results, Cerqueira et al. (2016) found that proactive individuals had a higher thermal preference in Nile tilapia, which was shown to be closely linked with hyperthermia resistance in fish at the level of the species (Richardson et al. 1994; Tsuchida 1995). However, no study evaluated the relationship between thermal preference and acute hyperthermia resistance at the individual level to our knowledge. The two phenotypes might therefore be different at this level, explaining the opposing result with Cerqueira et al. (2016).

In rainbows trout exposed to sub-lethal levels of acute hypoxia, divergent behaviours were identified with reactive fish staying quiet and saving energy while proactive fish were actively trying to avoid hypoxia and showed peak of 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 that recovered the hypoxia stress after return to normoxia were the reactive ones (Van Raaij et al. 1996), while we showed in the present study that isogenic lines resistant to

acute hypoxia were rather proactive. These results from the present study and Van Raaij et al. (1996) do not conflict as the phenotypes used are different. However, this raises questions about the relevance of the acute hypoxia resistance phenotype. A hypothesis would be that fish with high acute hypoxia resistance invest more energy to fight acute hypoxia compared to fish with low acute hypoxia resistance, leading to detrimental consequences on the survival once the hypoxia condition disappeared. Indeed, adaptative responses to a stress factor can have negative consequences on the organisms (Schreck and Tort 2016). Ironically, fish with high acute hypoxia resistance could be more sensitive to hypoxia in general. The link between resistance to acute hypoxia, tolerance to hypoxia and long-term survival merits further investigations, as it begins to be done for hyperthermia (Dupont-Nivet et al. 2014; Zanuzzo et al. 2019; Strowbridge et al. 2021; Bartlett et al. 2022; Ignatz et al. 2023).

3.4 Potential of behaviour as a proxy of acute hyperthermia and hypoxia resistances

In the present study, some of the behaviour variables lsmeans were highly correlated with the acute hyperthermia or hypoxia resistances lsmeans 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 demonstrate an interesting potential for using behavioural variables as novel non-stressful proxies for acute hyperthermia and hypoxia resistances in fish, improving experimental fish welfare.
However, as calculated correlations with only 6 genotypes can give misleading results, these results still need to be validated on a larger number of genotypes and different populations. In case of confirmation, it would also be necessary to optimize the way to measure behaviour as data acquisition and analysis are time consuming compared to the classical phenotyping method. For instance, in Lagarde et al. (2023b), it took around 700 minutes for phenotyping the resistance to acute hyperthermia or hypoxia stress of 300 fish, equivalent to 0.4 fish phenotyped/minute while ; in the present study, for phenotyping 6 fish, it took between 25 minutes (individual test) to 150 minutes (group test), 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 the extraction and analysis of behaviour variables from recorded videos. However, the behavioural phenotyping protocols presented here were not thought for optimization and significant time savings could be made by determining the minimum behaviour phenotyping time period required, increasing the number of fish either in individual or group tests, automation of data extraction, etc. The accuracy of behaviour variables in predicting phenotypes of resistance to acute hyperthermia and hypoxia must also be determined at the individual level to determine their value as predictors.

3.5 Implications for selective breeding of acute hyperthermia and hypoxia resistances

The correlations found between some behaviour variables and acute hyperthermia or hypoxia resistances at the level of the isogenic line possibly 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 indirect selection on certain behavioural variables. This is not surprising as, 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 impact positively or negatively fish adaptation to farming systems (e.g. Doyle and Talbot 1986). Special consideration must therefore be paid to the effects of selecting for these resistance phenotypes on the behaviour variables, if they were to be selected in a breeding program. More generally, physiological mechanisms underlying acute hyperthermia and hypoxia resistances are still not known with certitude (Nilsson and Östlund-Nilsson 2008; Ern et al. 2023) and unintended trade-offs with important breeding traits could therefore arise.
Last but not least, we showed in Lagarde et al. (2023b) that there was no strong relationship between acute hyperthermia and acute hypoxia resistances. Some lines were resistant to both acute hyperthermia and hypoxia, some lines resistant to one and sensitive to the other.
Here, we found that behaviour variables highly positively correlated with acute hyperthermia resistance were highly negatively correlated with acute hypoxia resistance and *vice-versa*. In other words, according to results from this paper, if we were to use behaviour predictors to select fish resistant to acute hyperthermia, we would select fish sensitive to acute hypoxia and *vice-versa*. Care should be taken for selective breeding to clearly define priorities regarding the trait to be selected or to identify trade off allowing selection of a sufficient resistance for both challenges. However, these opposite correlations between acute hypoxia/hyperthermia and behaviour variables are mainly due to two lines, A32h and B45h, which are highly resistant to acute hyperthermia and highly sensitive to acute hypoxia (Lagarde et al. 2023b). This once again points to the need to validate the correlations highlighted in the present study between behaviour and resistance phenotypes on more genotypes.

Conclusion

In the present study, we identified several behaviour variables strongly correlated with acute hyperthermia and hypoxia resistances at the line level using six rainbow trout isogenic lines. Globally, in individual test and in the beginning of group test, active isogenic lines were found sensitive to acute hyperthermia and resistant to 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 used as a non-invasive method for phenotyping acute hyperthermia or hypoxia resistances.
Considering isogenic lines as a case study, this must be tested using other populations to evaluate how this method could be used in selection processes. It would also be necessary to verify these correlations at the individual 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) authorized 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 authorized by the French ministry of higher education and research (APAFIS#24784_2020032510486957).

Acknowledgements

This study was supported by the European Maritime and Fisheries Fund and FranceAgrimer (Hypotemp project, n° P FEA470019FA1000016).

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