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Personality and plasticity in two isogenic lineages of the mangrove rivulus, Kryptolebias marmoratus

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Personality and plasticity in two isogenic lineages of the mangrove rivulus, *Kryptolebias marmoratus*

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Abstract

Animals are capable of displaying different reactions over time and/or contexts, in other words, personality, which is now the subject of a large number of scientific studies. An animal's personality is evaluated by means of a number of behavioral traits, such as boldness, exploration or aggression. While the variation of these traits across contexts is regularly studied, their variation over time, plasticity, is less documented. For this study, boldness was selected to evaluate the personality and plasticity of the mangrove rivulus, *Kryptolebias marmoratus*. The interest of using this fish for this type of studies, is that it produces isogenic lineages. Being one of the only two vertebrate species that self-reproduce, the parents are able to produce offspring identical to themselves. This allows researchers to disregard genes as the source of behavioral variation. Using two isogenic lineages (EPP and DC4) of the mangrove rivulus, the aim of this study is to verify if these fish present a personality and if they are changing behavior over time thus, are being plastic. But also, to compare those two lineages who differ only by their degree of genetic variation to verify if they present differences in personality and plasticity.

Key words: personality, plasticity, behavior, behavioral traits, mangrove rivulus, boldness, shelter test.

Foreword: This master's thesis is not written in a usual thesis format, it is written in an article format. It is therefore, composed of a shorter body of text, followed by a significantly bigger annex part.

Introduction

Personality, plasticity, and predictability

Animal's behavior differs over time and contexts, this is why studying their behavior is important (Lehner, 1987). An animal possesses a set of behaviors, known as personality traits. A personality trait was defined by Carter *et al.,* 2013 as "A specific aspect of a behavioral repertoire that can be quantified and that shows between-individual variation and within-individual consistency". Among those traits figure boldness, aggression, or even exploration, while these traits are regularly studied, their variation over time receives very little attention. However, it is known that an animal's behavior can vary over time, even in an identical context (Dall & Griffith, 2014). It is inconceivable to discuss the study of behavior without defining three concepts: personality, plasticity, and predictability (**figure 1**). Personality is the way an individual behaves in a given context (Biro *et al.,* 2018). This trait can be assessed using the centered intercept of the behavioral reaction norm (Kermany *et al.,* 2023). Plasticity is the way in which an individual will adapt their behavior over time and contexts, and is assessed by the slope, which represents the degree of variation in behavior (Jolles *et al.,* 2019; Kermany *et al.,* 2023). Predictability is defined as the degree of variability of an individual in a given context, which is the amplitude of the residual variation (Cornwell *et al.,* 2023; O'Dea *et al.,* 2022). Originally, the differences in behavior between individuals were explained by the fact that they had differences in the way they acquired their energy. Some behaviors are more energy consuming than others and therefore, these differences came from there (Mitchell & Biro, 2017).

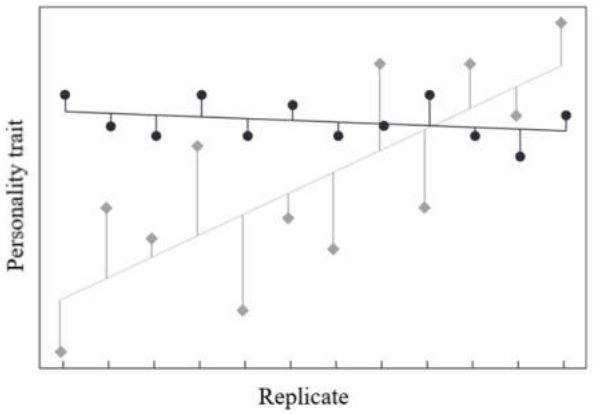

Figure 1: *This figure is a fictitious demonstration of intra-individual variability between a grey and a black subject, they have been tested many times and exhibit certain behaviors. A linear regression of this fictional data is presented for each of the two subjects. The y-intercept on the y-axis indicates the personality of the subjects, the slope of the line their plasticity and the vertical lines the residuals per time point, together they attest the*

intra-individual behavioral variability of the subject. Here, the black subject exhibits a high personality score, low plasticity, and low intra-individual behavioral variability, while the gray subject exhibits a low personality score, high plasticity, and high intra-individual behavioral variability (adapted from Jolles et al., 2019).

Plasticity is usually considered as intra-individual variation, but it is not. Plasticity is the differences between individuals in intercepts (score of the personality trait studied) and slopes regarding a specific context. Once changes in time and contexts have been considered, in addition to factors such as hunger, thirst, and light, which are in other words, the factors that are not experimentally controlled. The only remaining source of variation is intra-individual variation. Intra-individual variation can change over time and contexts, depending on the subject's learning abilities. This variation could be considered as a brain generated behavioral flexibility to enhance the non-predictability of the subject in order to increase its chances of survival. Depending on the context, some individuals are much more predictable than others (Biro & Adriaenssens, 2013). Unlike plasticity, which can be modulated according to context, the degree of predictability of a behavior appears not to be modulable according to context (Cleasby *et al.,* 2015). Some studies have revealed that behavioral predictability is important in sexual selection by females. Indeed, females tend to choose males whose behavior is predictable. For example, females of *Pelvicachromis pulcher*, a fish belonging to the *cichlidae* family, have a preference for males with consistent behavior, regardless of whether a male is very aggressive or not as long as his behavior is predictable (Scherer *et al.,* 2018).

To measure the differences in personality among individuals, five traits are mainly studied: exploration, aggressiveness, activity, boldness, and sociability. In the laboratory, Boldness is measured by "open field tests" (Dall *et al.,* 2012). The advantage of being in the laboratory for this type of test is that all variables can be controlled and remain the same during the test, and thus do not influence the animal's behavioral response. The individuals of a population display different behaviors from one another. This means that out of all the behaviors observed in this population, each individual expresses only a certain number of them, which implies that each individual presents a "behavioral type" that is unique to them and that is different from the other individuals of the population (Hertel *et al.,* 2020). In general, the boldest, most aggressive and exploring individuals are less likely to change their behavior over time and are therefore more predictable than shy and non-aggressive individuals (Guayasamin *et al.,* 2017). For example, three-spined sticklebacks, *Gasterosteus aculeatus* that are considered shy, modify their behavior and tend to be bolder when placed with conspecifics considered bold. Bold individuals, on the other hand, do not seem to modify their behavior in

the presence of shy individuals (Jolles *et al.,* 2014). In hierarchical species, the animal's status within the group influences the plasticity of behavior. For instance, when a rooster is dominant in a henhouse, it displays more developed sexual attributes, is more aggressive, is more vigilant and feeds less than non-dominant individuals (Cornwallis & Birkhead, 2008). Behavioral plasticity is potentially heritable, as plastic individuals are more likely to be able to modulate their behavior in response to changes in their environment, and thus pass these traits on to their offspring (Cornwell *et al.,* 2019).

Boldness behavior assessment

Among the most studied behavioral traits is boldness, which is the trait that will be studied in the present study. Boldness can be seen as the willingness to take a potential risk in an effort to find something that will enhance the fitness of an individual, whether it is the search for food, a mating partner, or a new habitat (MacGregor *et al.,* 2021).

Boldness can be assessed in several ways: either by simulating the presence of a predator in the animal's environment, then measuring the time it takes the animal to leave its refuge and, for example, to resume its foraging activities (Frost *et al.,* 2006). Those defined as bold, are those who come out of their hiding place the fastest. Another way of assessing boldness, is to bring the subject into a new environment containing a shelter and then measure the latency before the first emergence, or if the animal tends to move along the walls (thigmotaxis) or out in the open, without generating any prior stress. Those who get out the fastest and are out in the open, are defined as the boldest. This second way of assessing boldness can be achieved by using a shelter test (see **The shelter test**) (Carter *et al.,* 2012).

In 2019, jolles *et al.* conducted a study on the boldness of three-spine sticklebacks, *Gasterosteus aculeatus*. This study shows that the shyest individuals are generally the most plastic. But they also highlighted potential biases, due to the housing in common aquariums, because of the gregarious nature of this species. Indeed, group housing can modify the behavior of the individuals and thus cause fluctuations in the results of the experiment. Since there is a certain heritability to behavioral traits, such as boldness, this implies that personality differences are due to genetic differences between individuals (Conrad *et al*., 2011). For this reason, it seems interesting to conduct the same study on individuals with minimal genetic differences.

The mangrove rivulus

The mangrove rivulus (*Kryptolebias marmoratus*) is a fish living in the mangroves of North, Central and South America and shares a particularity with only one other vertebrate, it

reproduces by self-fertilization. The second species to reproduce by self-fertilization is also a rivulus species, *Kryptolebias hermaphroditus* (Tatarenkov *et al.,* 2010). Self-fertilization can lead to a genetic bottleneck because traits like life-history traits are very much subjected to selection pressure. In a given environment, some of these traits would be predominant in an inbred population, as they enable individuals to be perfectly adapted to their environment. But since genetic variability within the population is low, if a drastic change in environmental conditions were to occur, very few individuals would be able to survive, driving the population to extinction. This diminution in population fitness as result of inbreeding is called inbreeding depression (Wright *et al.,* 2008). This is probably one of the reasons why this mode of reproduction has not been conserved in vertebrates (Tatarenkov *et al.,* 2012). The fact that rivulus generates isogenic lineages offers an grea opportunity to examine the link between the genetics of an individual and its personality.

In this species the majority of individuals are hermaphrodites possessing an ovotestis and according to the populations there may be between 1 and 45 % of males, no female individual exists. Even if the dominant mode of reproduction is self-fertilization, it happens that cross-fertilization occurs between a male and a hermaphrodite. The frequency at which it occurs is assumed to be low but is not known (Marson *et al.,* 2019; Tatarenkov *et al.,* 2012). This cross-fertilization occurs when a hermaphrodite lays an unfertilized egg and a male releases his sperm on it. Males that are born male without ovarian tissue, are called primary males. Males can also come from hermaphrodites whose ovarian tissue is no longer functional, they are then called secondary males (Avise & Tatarenkov, 2015). Temperature seems to play a role in the appearance of secondary males, between 18 to 20°C during incubation, the proportion of male augment but its modus operandi is not yet known (Garcia *et al.,* 2016; Kelley *et al.,* 2016).

The rivulus is a solitary fish, it is rather aggressive towards the members of its own species whom it rarely meets. The parents are known to predate their own eggs and their own juveniles. Juveniles raised in small groups are more tolerant of other group members but, are still aggressive. To avoid unnecessary aggression and injury, these animals are generally kept in separate containers in laboratories (Tatarenkov *et al.,* 2010 ; Taylor, 2012). It would be tempting to assume that individuals carrying the same genotype would be reacting the same way to the same situation, but individuals carrying the same genotype sometimes show very different behaviors from each other. During their growth, these fish are much more plastic than adults. This high plasticity during juvenile phase may potentially be explained by the fact that the mangrove is a constantly changing environment and therefore, juveniles must adapt their

behavior rapidly to survive (Edenbrow & Croft, 2011). The phenotypic plasticity of the rivulus is such that when the water in which it is immersed is too polluted by compounds like H_2S, the cells of its gills shrink and the fish is capable of emerging to breathe in the open air, through the skin without losing too much water. This change of phenotype is reversible (Earley *et al.,* 2012).

Link between genetics and behavior

DNA methylation is one of the hypotheses investigated in an effort to explain the behavioral differences between individuals with identical genomes. Epigenetic modifications of the parent are unlikely to be transmitted to the offspring because of their reprogramming during embryogenesis. This time period during which the DNA methylations are rearranged is relatively long, during this period the environment in which the fish are as well as any change within it, influence the epigenetic modifications of the rivulus (Berbel-Filho *et al.,* 2020). This could imply that adults would be adapted to a broad variety of ecological niches through high phenotypic plasticity (Fellous *et al.,* 2018). Histone acetylation is also an epigenetic modification that can influence rivulus behavior. The enzymes involved in this process can impact processes such as gametogenesis or neurogenesis. These DNA modifications allow certain genes to be transcribed or not at given times during the development of the rivulus, which could potentially impact its phenotypic plasticity and therefore its behavior (Fellous *et al.,* 2019).

The rivulus is therefore an excellent candidate for studies on personality, predictability, and behavioral plasticity. The advantage of being from isogenic lineages is that genotypic replicates can be easily obtained, allowing genotypic differences to be excluded as a source of variation in behavior. During the course of this research, two lineages have been studied. The EPP lineage from the Emerson Point Preserve and the DC4 lineage from Dove Creek, both in Florida, USA (Mathiron & Silvestre, 2023).

These fishes live in an environment that can change drastically over a 24-hour period, the mangrove. In these isogenic lineages, the individuals present few different genotypes and therefore, it would be logical to think that they must present behavioral plasticity to survive in the unwelcoming environment that is the mangrove. The aim of this study is firstly, to quantify whether individuals who are practically genetically identical to each other show differences in terms of personality and plasticity. Secondly, to see whether there are any differences in personality and plasticity between the EPP and DC4 lineage, using the shelter test to assess boldness.

Materials and methods

Ethical note

The rivulus housing conditions and experimental procedures were approved by University of Namur Local Research Ethics Committee, in accordance with Belgian animal protection standards. Agreement number of the laboratory: LA1900048.

Subjects and housing conditions

Eggs from the EPP and DC4 lineages were collected from the breeders in the lab, who came from individuals collected in the field. The DC4s were collected in 2010 by Ryan L. Earley and Scott Taylor, in the Florida Keys (USA) at Dove Creek (GPS coordinates: N25◦01'45.64", W080◦29'49.24"). The descendants of these fish that were used in this experiment were F7; F8; F9; F10 and F11. The EPPs were collected by Valentine Chapelle in 2019 at Emerson Point Preserve, in Florida (USA) (GPS coordinates: N27°53'29.80", W82°62'55.01"). The descendants of these fish that were used in this experiment were F4 and F5. All these fish are kept in a thermoregulated chamber in the UNamur laboratory, URBE.

To calculate the N required to be able to highlight this "lineage" effect on boldness and plasticity, an a priori test was carried out with G*Power 3.1.9.2© software. This test requires specification of which test family and which statistical test will be performed. Here, a given variable (e.g. personality and plasticity) will be tested to measure how it varies as a function of a fixed factor: the effect of lineage (2 groups = 2 lineages). An ANOVA 1 (family: F test) will therefore be used. Data found in the study by James *et al*. (2018) were used, they compared behaviors including boldness between 4 lineages of mangrove rivulus and calculated the effect size, which turned out to be 0.2658752. An alpha threshold of 0.05 and a power of 75% were selected. The result was N=50 for both lineages, so 100 individuals all together. One EPP individual died during trials and could not be replaced, so N = 50 for DC4 and N = 49 for EPP.

To ensure 50 subjects from each lineage, eggs were collected and placed individually in the wells of twelve-well plates filled with 12ppt saline water made with "Instant Ocean™" sea salt. They were individually identified with a number and the genitor's number, as well as the generation of the larvae were also noted. They were then placed in an incubator at 27°C until hatching. After hatching, the larvae were placed in individual jars. The larva's identification number, that of its parent, and its date of birth were noted on the jar to ensure that the larvae was always identifiable. The water in the jars was also at 12 ppt and maintained at 26°C in a

temperature-controlled chamber, with a 12/12 day/night cycle. Larvae were fed once a day with 1 mL of live artemia. The larvae were kept in these jars until they were 50 days old, the day of the first shelter test. When the larvae had grown into fish they were individually placed in larger tanks. Still in 12 ppt water, enrichment for them to hide and lay their eggs was added. They were fed once a day with 3mL of live artemia.

The shelter test

At the age of 50 days, the first shelter test was carried out, resulting in 6 replicates, one week apart. The shelter test arena (**figure 2**) consisted of a ten-liter bucket of 18 cm in diameter. On the side of the bucket, a 5 cm diameter hole was cut, and a pipe corner was attached to it, using water resistant silicone. A slot had been cut in the part protruding into the bucket, to be able to pass a plastic trap door to block the fish into the pipe corner during the habituation period. The pipe corner was closed with a lid as soon as the fish was placed inside of it, to make sure it didn't jump out of it, and prevented it from seeing the experimenter, thereby avoiding causing stress that could have biased the experiment.

Figure 2: *Test arena used to assess boldness the pipe corner in grey (right side of the figure) is the acclimatization zone and the test arena in white (left side of the figure).*

The experiment itself was based on Chapelle, 2018 and consist of adding 1,5 liters of 12 ppt salted water into the arena and placing the fish in the pipe bend for 10 minutes to get used to its new environment. Once the 10 minutes were over, the recording was started (using SONY® HDR-CX625 camera) while the fish was still in the acclimatization zone to avoid causing fear to it, and the trap door was lifted so that the fish had access to the entire test area for the 30 minutes of the test. When the test was completed, the recording was stopped, and the fish was transferred back to its individual tank and placed back into the temperature-controlled chamber. In-between each test, the water was changed, and the arena was rinsed with clear water to avoid the detection of stress hormones left by the previous fish. It should be noted that

the room in which the experiments were conducted, was at the same temperature as the thermoregulated chamber to avoid any discomfort, or changes in behavior for the fishes tested. Pre-tests were run prior to the experiment to validate the experimental design.

Data analysis

The videos, which were stored on memory cards were then exported to the Ethovision™ software (Noldus Ethovision XT 13™). Each arena was manually calibrated to indicate the internal zone, the shelter entry, and the shelter (**figure 3**). The detection of each fish was also verified manually to ensure that the software detected the fish and not its reflection, or a shadow. The software analyzed each video separately and provided a table with distances and times. The table was later imported into the R Studio™ software (version R 4.3.2).

Figure 3: *Zone attribution in the arena, in orange: the external zone; in yellow: the internal zone; in red: the shelter entry; in blue: the shelter. Yellow arrows are for calibration scale.*

First, a correlation matrix of the response variables was made (**figure 4**) to display the level of correlation between them, using the rcorr function (Hmisc package) and corrplot function (PerformanceAnalitycs package). After that, a PCA was performed to determine which response variables to study using the dudi.pca function (ade4 package). Then, due to a high number of 0s in the data, representing fish that had not emerged from the shelter, it was decided to separate the data analysis in two parts, using different types of models. The first part of the analysis was to assess if one of the two lineages had a higher probability of emerging from the shelter and, once emerged, if fish from one lineage spent more time outside the shelter than the other, only considering the fish that exited the shelter. For this purpose, two linear mixed effects models (GLMER) (glmer function in lme4 package) were run. The first one to assess the emergence probability, it is the probability that the fish emerged divided by the probability that

it didn't emerge which is given by the exponential of the standard deviation for the lineage. The second, to assess the time spent outside the shelter, which is the probability that the fish of a lineage spent more time outside divided by the probability that it didn't, and was also given by the exponential of the standard deviation for the lineage.

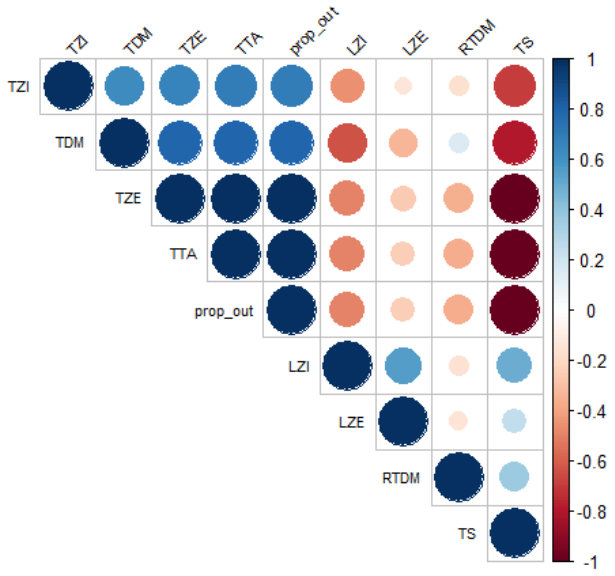

Figure 4: *Correlation matrix between the response variables, anticorrelated variables are in red (0 to -1 on the axis), correlated variables are in blue (0 to 1 on the axis).*

The second part of the analysis was to assess fish personality. This was done by using a linear mixed effects model (LMER) with the proportion of time spent outside the shelter (prop_out) as the response variable. Personality is determined by the intercept value of the model (0.10), below which fish are considered shy and above which bold (Jolles *et al.,* 2019). Other response variables, such as latency before first exit (LZE), relative total distance moved (RTDM) which was the total distance moved divided by total time spent in the arena, total time spent in the arena (TTA) and the ratio of time spent in the inner zone to total time in the arena (RTZI), were also considered analyzed using LMER, except RTZI who was assessed by a GLMER (due to the distribution of data), models to assess rivulus personality. For each response variable considered, the generation, replicate and lineage were fixed effects, and the fish ID and parent effect were random effects, the parent effect was judged non-significant by ranova (lmerTest package) . Because initially the requirements of homoscedasticity of variances and normality of distribution of residuals were not met for the TTA, RTDM and LZE variables, a Yeo-Johnson transformation (bestNormalize package) was performed to ensure that the data could be used. For the model selection, a top-down approach was applied. Starting with the most complicated model containing all fixed effects and their interactions, an anova was run

for each model and the least significant parameter was removed. Once all the models were made, an anova vas used to compare them with each other, the model with the lowest AIC was selected (see **annex 1.B** for model selection).

Conditional repeatability was measured using the rptR package, it was done for the variables mentioned above, with 1000 bootstrap iteration. Repeatability allows to quantify the reproducibility of a measured parameter, for example the consistency of a behavior over time and context for individuals in a population (Stoffel & Schielzeth, 2017). In this case it was the repeatability of boldness over temporal replicates for both lineage for each fish (using ID as random effect). If the repeatability value R is significative, it means that the fish differs more from itself over the six replicates than it does from the others indicating the presence of a personality.

Plasticity was assessed following the tutorial provided by Hertel *et al.,* 2020, it is the link between individual intercepts and slopes (replicates) that determines the plasticity of behavior. Random intercept for the fish ID and individual random slopes for the replicate, to assess the variability of the boldness behavior across replicates. Two LMERs were made (lme4, tidybayes and broom.mixed packages) for prop_out, LZE, RTZI and RTDM, with lineage and replicate as fixed effect, in the first model fish ID is the random effect and in the second, the interaction between replicate and ID as random effect (1+Replicate|ID) (see **annex 1.C** for models). Those models were then turned into a data frame containing random intercepts and slopes values for each fish. They were then plotted to display the slopes (**figure 10**), each slope corresponds to a fish. The steeper is the slope, the more plastic is considered the fish.

Results

The PCA performed on the response variables regarding the boldness test (**figure 5**), explains 74.1% of the variation in fish behavior on axes 1 (56,7%) and 2 (17.4%). On axis 1, the variables of the shy-bold continuum are represented. On this axis are represented prop_out, TTA, TZE and TZI, TS is also represented and is anticorrelated to variables just mentioned. The second axis represents RTDM during the different replicates. LZE, LZI and TDM are not well represented on those axes and will not be analyzed further, except for LZE because it's a strong boldness indicator. These results associated with the correlation matrix presented above allowed the selection of the response variables to better quantify boldness behavior.

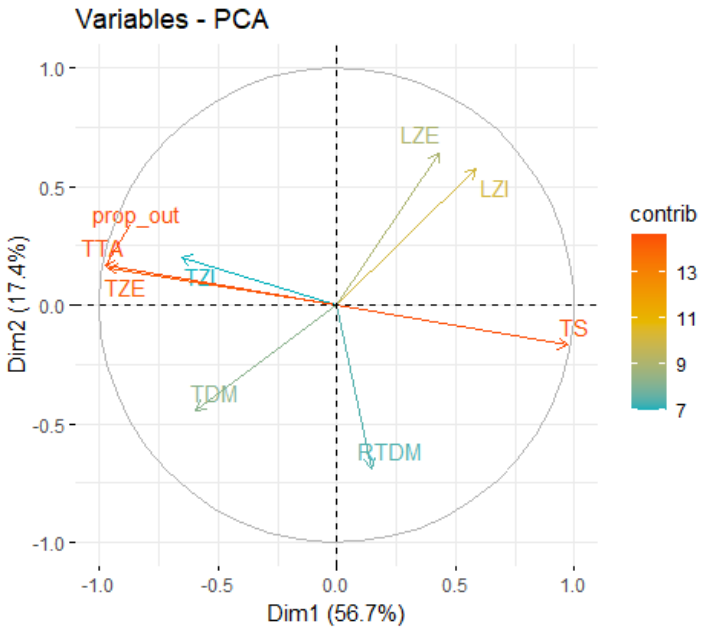

Figure 5: *PCA showing the different explanatory variables on the shy-bold axis, and their contribution.*

Due to the high number of fish not emerging from the shelter, a logistic model with the probability of fish emergence as a function of lineage was run, and the probability of emergence of EPPs is 81.40% greater than that of DC4s (p = 0,0887, non-significative). A second model, taking only emerged fish into account, shows that once emerged, EPPs spend 66.85% more time outside the shelter than DC4s (p = 2,67e-08, significative).

Over the course of the 6 replicates, the fish spent an average of 16.28% of their time outside the shelter. The proportion of time spent outside the shelter decreased between replicates 1 and 6, from 20.94% to 13.88%. However, this proportion of time varies according to the lineage considered: EPPs spend an average of 21.00% of their time outside the shelter, while DC4s spend only 11.67% of their time outside the shelter. Despite this habituation to the experimental procedure, the fish showed differences in behavior, that can be significantly attributed to a lineage effect, but also to a replicate effect. The **table 1** summarizes the model and shows p-values.

The value that determines whether a fish is bold is that of the model intercept, the results of which are shown in **Table 1** below. Below 0,10 fish are considered shy, and above 0,10 bold. DC4s are more shy, they tend to spend less time outside the shelter than EPPs.

Table 1: *Outputs of the LMER model used to monitor rivulus boldness. Model was: (prop_out~Lineage+Replicate+(1|ID)+(1|Parent)). Bold numbers indicate significant p-value.*

	Proportion of time out of shelter			
Fixed effects	*Estimates*	*CI*	*p*	*df*
Lineage [EPP]	0.10	0.05 – 0.15	**1.530e-04**	584.00
Replicate [2]	-0.02	-0.06 – 0.03	4.702e-01	584.00
Replicate [3]	-0.05	-0.10 – -0.01	**1.392e-02**	584.00
Replicate [4]	-0.07	-0.11 – -0.03	**1.805e-03**	584.00
Replicate [5]	-0.07	-0.11 – -0.02	**2.351e-03**	584.00
Replicate [6]	-0.07	-0.11 – -0.03	**1.535e-03**	584.00

The latency before first exit (LZE) was also tested for fish that emerged from the shelter and decreased between replicates 1 and 6, decreasing form 494,59 s to 349,05 s (**figure 6**). The mean LZE for EPPs was 376,43 s and 387,90 s for DC4s. Even if the fish did exit the shelter earlier, they did not increase their time out of the shelter. There is no significative effect of the replicate for this parameter (**table 2**).

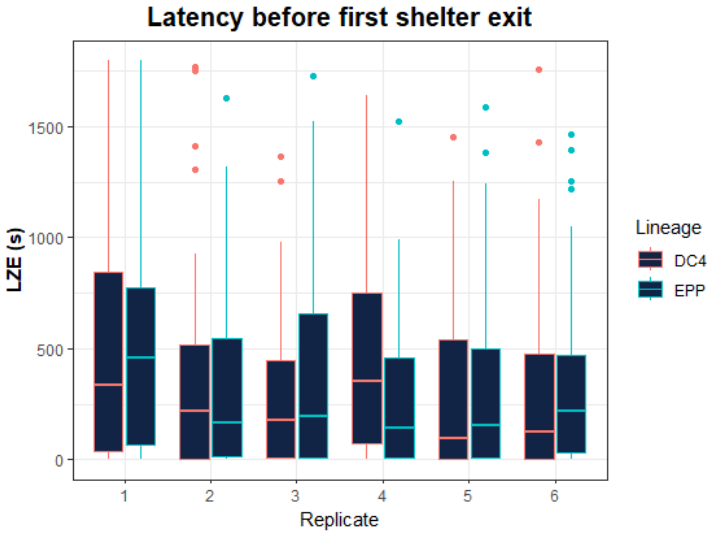

Figure 6: *Latency before first shelter exist for the two lineages according to the replicate.*

Table 2: *Outputs of the LMER model used to monitor rivulus boldness. Model was: (LZE~Replicate+(1|ID)).*

	Latency before first exit			
Fixed effects	*Estimates*	*CI*	*p*	*df*
Replicate [2]	-0.19	-0.46 – 0.08	1.615e-01	586.00
Replicate [3]	-0.06	-0.33 – 0.20	6.314e-01	586.00
Replicate [4]	-0.03	-0.29 – 0.24	8.402e-01	586.00
Replicate [5]	-0.15	-0.41 – 0.12	2.739e-01	586.00
Replicate [6]	-0.01	-0.28 – 0.25	9.254e-01	586.00

The total time spent in arena (TTA) decreased between replicates 1 and 6, from 410,04 s to 321,14 s. The fish spent less time outside as the replicates progressed. EPPs (mean TTA = 429,23 s) spent on average more time outside the shelter than DC4s (mean TTA = 259,36 s) (**figure 7**). **Table 3** shows that the lineage as well as replicates 4 and 5 were significant, but the interaction between lineage and replicate was not.

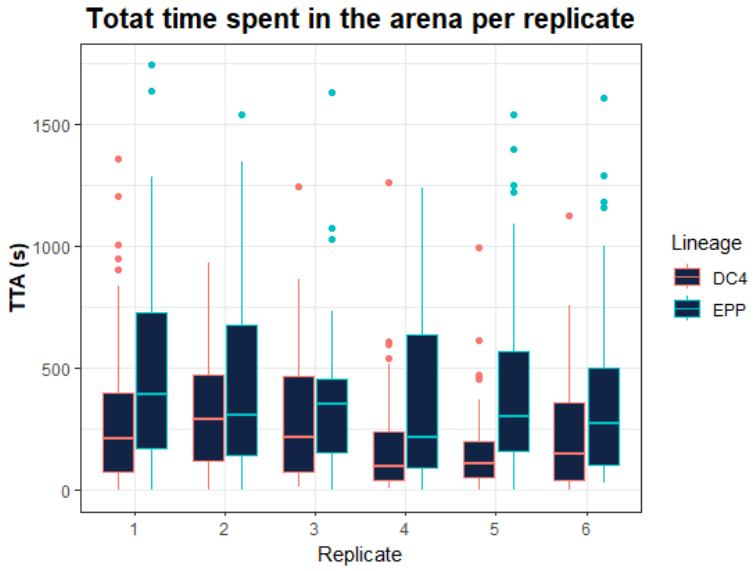

Figure 7: *Total time spent in the arena per replicate, according to lineages.*

Table 3: *Outputs of the LMER model used to monitor rivulus boldness. Model was: (TTA~Lineage+Replicate+Lineage:Replicate+(1|ID)). Bold numbers indicate significant p-value.*

		Totat time in arena		
Fixed effects	*Estimates*	*CI*	*p*	*df*
Lineage [EPP]	0.53	0.13 – 0.92	**9.405e-03**	488.00
Replicate [2]	0.14	-0.20 – 0.49	4.157e-01	488.00
Replicate [3]	0.05	-0.30 – 0.41	7.695e-01	488.00
Replicate [4]	-0.38	-0.74 – -0.02	**3.904e-02**	488.00
Replicate [5]	-0.42	-0.79 – -0.05	**2.640e-02**	488.00
Replicate [6]	-0.28	-0.64 – 0.09	1.390e-01	488.00
Lineage [EPP] × Replicate [2]	-0.26	-0.75 – 0.22	2.904e-01	488.00
Lineage [EPP] × Replicate [3]	-0.27	-0.77 – 0.23	2.863e-01	488.00
Lineage [EPP] × Replicate [4]	0.15	-0.35 – 0.65	5.471e-01	488.00
Lineage [EPP] × Replicate [5]	0.26	-0.25 – 0.76	3.165e-01	488.00
Lineage [EPP] × Replicate [6]	0.10	-0.41 – 0.61	7.030e-01	488.00

The relative total time spent in the internal zone of the arena (RTZI) decreased between replicates 1 and 6, from 0,09 s to 0,03 s. Which is consistent with the diminution of TTA, but not with the increase of RTDM. The DC4s spent slightly more time in the internal zone (mean RTZI = 0,06 s) than EPPs (mean RTZI = 0,05) (**figure 8**), this helps to quantify the thigmotaxis of fish. **Table 4** shows that the replicate had a significative effect, but not the lineage.

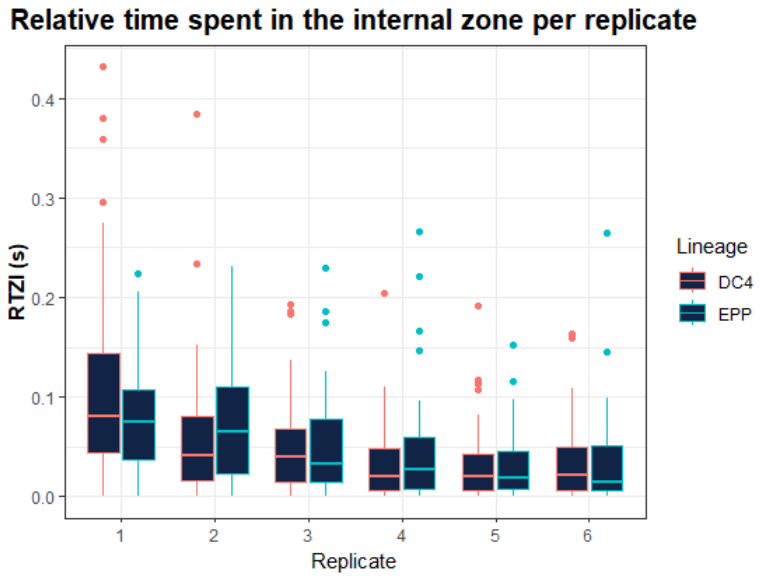

Figure 8 : Relative time spent in the internal zone of the arena per replicate, according to lineage.

Table 4 : *Outputs of the GLMER model used to monitor rivulus boldness. Model was: (RTZI~Lineage+Replicate+(1|ID)). Bold numbers indicate significant p-value.*

	Relative total time spent in the internal zone			
Fixed effects	*Estimates*	*CI*	*p*	*df*
Lineage [EPP]	0.92	0.71 – 1.20	5.370e-01	435.00
Replicate [2]	0.76	0.59 – 0.99	**4.090e-02**	435.00
Replicate [3]	0.57	0.44 – 0.75	**7.061e-05**	435.00
Replicate [4]	0.44	0.33 – 0.57	**4.534e-09**	435.00
Replicate [5]	0.33	0.25 – 0.44	**1.607e-14**	435.00
Replicate [6]	0.36	0.27 – 0.47	**2.426e-12**	435.00

The relative total distance moved (RTDM) doubled between replicate 1 (1,35 cm) and 6 (2, 65 cm) for both lineages, which means that the fish became more active during their time out of the shelter. This increase is greater for the DC4s (mean RTDM = 2,54 cm) than for the EPPs (mean RDTM = 2,48 cm) (**figure 9**). **Table 5** shows that there is no significant effect of the lineage, but the replicate and the interaction between lineage and replicates are significant.

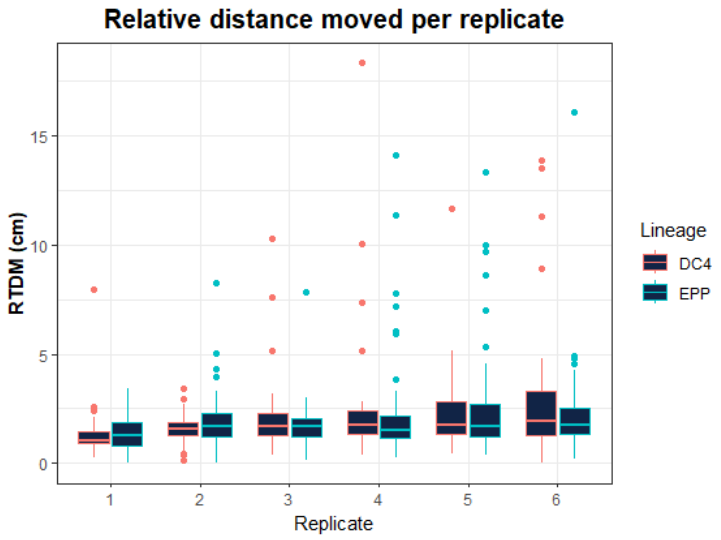

Figure 9: *Relative distance moved for the two lineages according to the replicate.*

Table 5: *Outputs of the LMER model used to monitor rivulus boldness. Model was: (RTDM~Lineage+Lineage:Replicate+(1|ID)). Bold numbers indicate significant p-value.*

	Relative total distance moved			
Fixed effects	*Estimates*	*CI*	*p*	*df*
Lineage [EPP]	0.07	-0.33 – 0.47	7.401e-01	488.00
Lineage [DC4] × Replicate2	0.44	0.07 – 0.81	**2.075e-02**	488.00
Lineage [EPP] × Replicate2	0.43	0.06 – 0.80	**2.218e-02**	488.00
Lineage [DC4] × Replicate3	0.76	0.38 – 1.15	**1.236e-04**	488.00
Lineage [EPP] × Replicate3	0.47	0.09 – 0.85	**1.569e-02**	488.00
Lineage [DC4] × Replicate4	0.68	0.29 – 1.07	**6.331e-04**	488.00
Lineage [EPP] × Replicate4	0.65	0.28 – 1.02	**6.257e-04**	488.00
Lineage [DC4] × Replicate5	0.74	0.34 – 1.14	**3.199e-04**	488.00
Lineage [EPP] × Replicate5	0.77	0.40 – 1.14	**5.714e-05**	488.00
Lineage [DC4] × Replicate6	0.75	0.35 – 1.14	**2.287e-04**	488.00
Lineage [EPP] × Replicate6	0.59	0.20 – 0.97	**2.794e-03**	488.00

Conditional repeatability of the response variables was also tested and is presented in **table 6.** For prop_out and TTA values R and P are the same, because they both represent the same parameter, but prop_out is a proportion (TTA/1800, 1800 is the total duration of the test in seconds). DC4s and EPPs have the same R and different P that are both significant. For LZE value, EPPs have a very low R that is not significant, and DC4s have a higher R that is significant. For RTZI value, EPPs have a high R that significant, and DC4s have a lower R that is barely significant. For RTDM value, both lineages have a low R that is not significant.

Table 6: *Value of repeatability (R) and their p-value (P), for each response variable studied and their model for both lineages. The number of "*" indicate the degree of significance of the p-value.*

		EPP		DC4	
Variable	**Model**	**R**	**P**	**R**	**P**
prop_out	prop_out ~ Replicate+(1|ID), grname=c("ID","fixed")	0,27	8,96e-10***	0,27	9,6e-10***

LZE	LZE~ Replicate+(1|ID), grname=c("ID","fixed")	0,05	0,09	0,28	3,22e-10***
TTA	TTA~ Replicate+(1|ID), grname=c("ID","fixed")	0,27	8,96e-10***	0,27	9,6e-10***
RTZI	RTZI~ Replicate+(1|ID), grname=c("ID","fixed")	0,23	8,02e-7***	0,11	0,01*
RTDM	RTDM~ (1|ID), grname="ID"	0,09	0,06	0,02	0,37

Plasticity was observed in both lineages (**figure 10**). **Figure 10.A** shows that EPPs are significantly more plastic than DC4s (p-values in **annex 1.C**) for the proportion of time spent out of shelter. **Figure 10.B** shows that both lineages are plastic for the latency before first emergence, but there is no significant difference between lineages. **Figure 10.C** shows reduced plasticity and no significant difference between lineages. **Figure 10.D** shows very little plasticity and no significant difference between lineages. The steepest slopes are assigned to the most plastic fish. Fish with a flat slope have changed their behavior very little over time and are therefore not plastic.

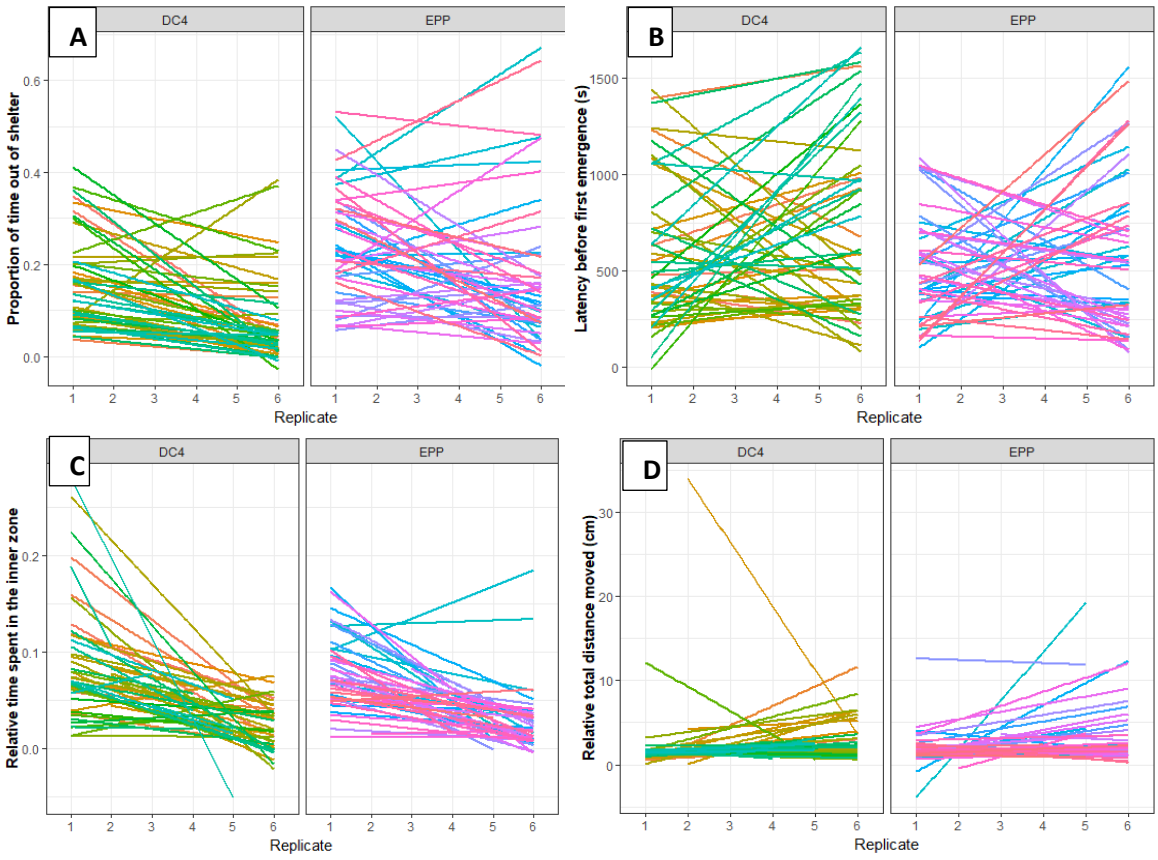

Figure 10: *Graph illustrating fish personality and its variation over time in relation to lineage for* ***A****: the proportion of time out of shelter,* ***B****: the latency before first emergence,* ***C****: the relative time spent in the inner zone* ***D****: the relative total distance moved. Each line represents a fish.*

Discussion

Boldness behavior of 50 DC4 and 49 EPP aged 50 to 92 days, was tested with six replicates of a shelter test, to assess whether these two isogenic lineages showed personality and whether this personality was plastic over time. But also, whether they were different from each other in the expression of the boldness behavior and in their plasticity.

The proportion of time spent outside the shelter is significantly different for EPPs and DC4s and the repeatability of this value for both lineages is highly significant, indicating that the lineages differ in personality for this trait, and that individuals within the lineages are different from each other. In their natural habitat, their size makes them a potential prey for a number of mangrove animals, such as the mangrove water snake, *Nerodia fasciata compressicauda*, herons, as well as larger fish (Taylor, 2012). Boldness is seen as the willingness to take a risk for potential gain (food, breeding partner, etc.) (Conrad *et al.,* 2011). But in the case of the rivulus, it doesn't have to look for a partner as it is self-fertilizing, except perhaps for the males who have to look for unfertilized eggs laid by hermaphrodites, to fertilize (Avise & Tatarenkov, 2015). Therefore, their low emergence from the shelter could be a potential consequence of being hermaphroditic. However, to reproduce, these fish need to eat more to be able to produce eggs with sufficient reserves to allow the embryos to develop (Genade, 2016). In 2012, Edenbrow & Croft, did not find significant differences between males and hermaphrodites in boldness behavior, but only had two replicates one week apart. Studies have shown that individuals tend to adapt their degree of boldness, as a response to predation risks, which is compatible with adaptative phenotypic plasticity (Arnett & Kinnison, 2017). It might be interesting to test boldness behavior with a dummy predator. To determine if boldness behavior is similar with or without a predator, for the proportion of time spent outside the shelter across replicates. In 2010, Harris *et al.,* found reduced boldness in guppies *Poecilia reticulata*, and in 2020, Mitchell *et al*., found no difference in boldness behavior between predator exposed and non-exposed guppies.

Nevertheless, despite their limited time spent outside the shelter, differences in individual personality between the two lineages are visible. EPPs spent almost twice as much time on average outside the shelter as DC4s. As the DC4 lineage contains a higher level of genetic variation than EPP, one might have expected personality differences to be more

significant for DC4s and display more phenotypic plasticity than the EPPs. However, in rivulus, the most homozygous lineages demonstrate the highest phenotypic plasticity (Earley *et al.,* 2012). Phenotypic plasticity is an outcome of the interactions between genotype and environment (Turko & Rossi, 2022). However, in the present study, fish were isolated since birth, never interacted with predators and kept in the most constant conditions possible (circadian rhythm, feeding time, and temperature), meaning that phenotypic plasticity could be passed down to next generations. This assumption that more genetically diverse individuals are more plastic may in fact be species-dependent (Bell & Stamps 2004; Sinn *et al.* 2008). In Jolles *et al.,* 2019, bolder three-spined stickleback are less plastic than shy ones, who tended to get bolder with each replicate. In Sinn *et al.* 2008, dumpling squid, *Euprymna tasmanica*, bold squids were more plastic and became bolder, while shy individuals were less plastic and stayed shy. Rivulus lineages with the most genetic diversity seem to be those with the most males. Lineages with fewer males, and therefore less genetic diversity, would then compensate with greater phenotypic plasticity to cope with the regular disturbances that are inerrant to life in mangroves (Earley *et al.,* 2012; Tatarenkov *et al.,* 2012; Taylor, 2012). In the event of a major disturbance in their environment, a high level of phenotypic plasticity would enable them to adapt quickly and maximize their chances of survival (Castillo *et al.*, 2018). In 2018, Chapelle showed that rivulus decreased their time spent in the arena and their LZE with three replicates, which is coherent with the present results.

The latency before first emergence (LZE) decreases between replicate 1 and 6, with EPPs exiting the shelter faster than DC4s. Repeatability of this value vas not significant for EPPs, but was highly significant for DC4s, indicating that EPPs don't show individual differences for this trait, but DC4s do. Both lineages presented plasticity for this value, but did not show significant differences in plasticity linked to lineage. In 2010, Harris *et al.*, found that the predation pressure in geographical location of origin of the fish had an impact on the latency before first emergence. Where the predation pressure was higher, the fish exited the shelter later than where the predation pressure was lower. In the present study, it would mean that the predation pressure was higher for DC4s than EPPs in their natural environment. This reduction in latency could also come from the fact that the fish learn that there is no risk of any sort in emerging from the shelter (Villegas-Ríos *et al.,* 2018). Since there is no potential gain ether, they would preferably stay hidden in the shelter, once they are done exploring the arena.

RTZI decreases between replicate 1 and 6 indicating positive thigmotaxis. The repeatability for RTZI was highly significative for EPPs. The value was lower for DC4s, which

indicates that individuals in this lineage showed very few individual differences for this trait. Most fish exhibit positive thigmotaxis, choosing to swim along the walls rather than in the center of the arena (Norton, 2012). A minority of fish swim towards the center of the arena and are considered the boldest. DC4s have the highest RTZI, which means that when they exit the shelter, they spend more time in the inner zone than EPPs, but their total time spent out of the shelter is still less than EPPs. Fish that swim along the edge of the arena are considered anxious (Lucon-Xiccato *et al.,* 2020). The fact that the RTZI decreases may also be due to the fact that during the first tests, the fish explore their environment and then show a preference for the outside of the arena where they would feel less exposed to potential predation compared to the center (Lucon-Xiccato *et al.,* 2022). This would mean that DC4s are less anxious because they spend more time in the center when they are in the arena, but not as bold as EPPs because they don’t spend as much time outside the shelter.

As for the decrease in time spent in the arena and the increase in RTDM, this may represent a habituation syndrome to the experimental procedure. The repeatability for RTDM value was not significant for both lineages indicating no differences in personality for this trait. Plasticity was low for both lineages for this trait. EPPs leave the shelter faster and stay in the arena longer than DC4s, but DC4s increase their RTDM more than EPPs as replicates progress. Most of the fish's movements consist of swimming around the arena alongside the wall, then re-entering the shelter. Habituation is a response to recurrent and/or prolonged stress, by reducing the stress reaction to the stressor (Houslay *et al.,* 2019). In this case, the fish seem to have become habituated to the procedure, which has reduced their stress and probably caused them to lose interest in spending time outside the shelter, while being more active when exiting the shelter. Being too bold for a juvenile can also be dangerous, as it can adversely affect the fitness of individuals by exposing them to predators that they haven't yet learned to recognize (Ballew *et al.,* 2017). This could also explain why rivulus spend less and less time in the arena, juveniles would be more cautious than adult, as they would be more subjected to predation in the wild because they haven’t learned about predator-cues yet (Mangel & Stamps, 2001).

As DC4s were bred in the lab before EPPs, one can also wonder if this habituation isn’t a consequence of domestication. Domestication can be seen as the adaptation of an animal to an artificial environment. This process begins with the first generation born in captivity (Price, 1999). Studies have shown that as domestication process progresses, captive fish become more mobile than wild fish (Horká *et al.,* 2015). Fish personalities also change with domestication, and captive fish are no longer as bold as wild individuals. This is probably related to the fact

that their captive environment is less complex than their natural one, making it simpler and less dangerous to obtain food, leading to a loss of boldness (Pasquet, 2018). Which seems to be the case here with DC4s, who increased their RTDM more than EPPs. The number of generations of captivity breeding is important in the domestication process (Douxfils *et al.,* 2011). With DC4s ranging from F7 to F11 and EPPs being F4 and F5, it therefore seems logical to think that domestication is affecting fish behavior in this case. It would have been interesting to have all the generations studied present in both lineages to visualize if the domestication process has a more significant impact on the behavior of one lineage.

Conclusion

Despite having very little genetic variation, mangrove rivulus expressed personality and phenotypic plasticity. This species makes it possible to consider behavior and phenotypic plasticity independently of the genetics of an individual. These differences in personality associated to their plasticity are coherent with their natural environment. The mangrove is a constantly changing environment, the behavioral plasticity of rivulus would therefore, have played a role in its establishment and survival in this environment. Although there was a reduction of time spent outside the shelter, it is nonetheless possible to establish the existence of boldness behavior in the mangrove rivulus tested. EPPs were generally the first to emerge from the shelter and stayed longer in the arena, but swam less and presented more thigmotaxis than DC4s. The existence of a significant lineage effect on boldness has also been revealed with the proportion of time spent out of shelter. Meaning that EPPs tend to be bolder than DC4s. The fish have shown phenotypic plasticity over the six replicates, though they were kept in the most constant conditions possible. Indicating that phenotypic plasticity is possibly inherited. Because the reproduction mechanisms of this species allow to disregard the genetic sequence of an individual. The next step could be to combine personality and plasticity assessment to epigenetics to verify if specific epigenome association with genes could be responsible for these differences in personality and their plasticity.

Despite the measures taken prior to the experiment to ensure that the fish do not become habituated to the experimental design, the reduction of TTA shows that it happened. Domestication is believed to be the cause of this habituation. To verify this hypothesis, subjects from the two lineages belonging to the same generation could be tested alongside each other.

Annexes

Annex 1: Model selections and anovas

1.A: Emerging probability and time spent outside the shelter

Table 7: GLMER logistic models

Is_out~Lineage + Replicate + (1|ID), family = binomial (link = "logit")	Model taking all the fish into account
TTA~Lineage + (1|ID), family = gamma (link = "log")	Model taking only the fish that emerged into account

Table 8: Outputs of the GLMER models used to monitor rivulus probability of emergence (left) and time spent out of shelter (right). Bold numbers indicate significative p-value.

	Probability of emergence		
Fixed effects	*Odds Ratios*	*CI*	*p*
(Intercept)	14.33	5.80 – 35.41	**8.024e-09**
Lineage [EPP]	1.81	0.91 – 3.60	8.873e-02
Replicate [2]	0.87	0.30 – 2.48	7.885e-01
Replicate [3]	0.35	0.13 – 0.90	**2.871e-02**
Replicate [4]	0.40	0.15 – 1.06	6.439e-02
Replicate [5]	0.32	0.12 – 0.82	**1.722e-02**
Replicate [6]	0.25	0.10 – 0.64	**3.974e-03**
Random Effects			
σ^2	3.29		
$\tau_{00\ ID}$	1.25		
N_{ID}	99		
Observations	594		
Marginal R^2 / Conditional R^2	0.073 / 0.328		

	Pobability of spending more time out of shelter		
Fixed effects	*Estimates*	*CI*	*p*
(Intercept)	256.55	222.10 – 296.35	**5.238e-275**
Lineage [EPP]	1.67	1.39 – 2.00	**4.364e-08**
Random Effects			
σ^2	0.93		
$\tau_{00\ Replicate}$	0.00		
$N_{Replicate}$	6		
Observations	502		
Marginal R^2 / Conditional R^2	0.065 / 0.070		

1.B: Boldness assessment

Table 9: LMER prop_out models

M1	prop_out~ Lineage + Generation + Replicate + Lineage:Generation + Lineage:Replicate + Replicate:Generation + (1|ID) + (1|Parent)
M2	prop_out~ Lineage + Generation + Replicate + Lineage:Generation + Lineage:Replicate + (1|ID) + (1|Parent)
M3	prop_out~ Lineage + Replicate + Lineage:Generation + Lineage:Replicate + (1|ID) + (1|Parent)
M4	prop_out~ Lineage + Replicate + Lineage:Replicate + (1|ID) + (1|Parent)
M5	prop_out~ Lineage + Replicate + (1|ID) + (1|Parent)

Table 10: Anova comparing all models. Model selection was based on AIC.

AOV

	npar	AIC	BIC	logLik	deviance	Chisq	Df	Pr(>Chisq)
M5	10	-390.8965	-347.0277	205.4482	-410.8965	NA	NA	NA
M4	15	-387.5598	-321.7566	208.7799	-417.5598	6.663310	5	0.2469084
M3	20	-382.5517	-294.8142	211.2759	-422.5517	4.991955	5	0.4168628
M2	20	-382.5517	-294.8142	211.2759	-422.5517	0.000000	0	NA
M1	45	-345.0750	-147.6655	217.5375	-435.0750	12.523297	25	0.9818260

Table 11: Outputs of the LMER models used to assess boldness with proportion of time out of shelter. Stars indicate significative p-value.

		prop_out	
Predictors	*Estimates*	*CI*	*df*
(Intercept)	0.16 ***	0.11 – 0.21	29.04
DC4	*Reference*		
EPP	0.10 **	0.04 – 0.15	20.22
Replicate2	-0.02	-0.06 – 0.03	490.00
Replicate3	-0.05 *	-0.10 – -0.01	490.50
Replicate4	-0.07 **	-0.11 – -0.03	490.50
Replicate5	-0.07 **	-0.11 – -0.02	490.00
Replicate6	-0.07 **	-0.11 – -0.03	490.00
Random Effects			
σ^2	0.02		
$\tau_{00\ ID}$	0.01		
$\tau_{00\ Parent}$	0.00		
ICC	0.28		
N_{ID}	99		
N_{Parent}	41		
Observations	594		
Marginal R^2 / Conditional R^2	0.085 / 0.337		

*$p<0.05$ **$p<0.01$ ***$p<0.001$

Table 12: LMER LZE models

M15	LZE~ Lineage + Replicate + Lineage:Replicate + (1|ID)
M16	LZE~ Lineage + Replicate + (1|ID)
M17	LZE~ Replicate + (1|ID)

Table 13: Anova comparing all models. Model selection was based on AIC.

AOV

	npar	AIC	BIC	logLik	deviance	Chisq	Df	Pr(>Chisq)
M17	8	1686.061	1721.156	-835.0303	1670.061	NA	NA	NA
M16	9	1687.347	1726.829	-834.6736	1669.347	0.7133633	1	0.3983295
M15	14	1692.834	1754.250	-832.4170	1664.834	4.5132109	5	0.4781178

Table 14: Outputs of the LMER models used to assess boldness with latency before first emergence. Stars indicate significative p-value.

		LZEyj	
Predictors	*Estimates*	*CI*	*df*
(Intercept)	5.07 ***	4.88 – 5.27	560.97
Replicate2	-0.19	-0.46 – 0.08	490.00
Replicate3	-0.06	-0.33 – 0.20	491.00
Replicate4	-0.03	-0.29 – 0.24	491.00
Replicate5	-0.15	-0.41 – 0.12	490.00
Replicate6	-0.01	-0.28 – 0.25	490.00
Random Effects			
σ^2	0.90		
$\tau_{00\ ID}$	0.10		
ICC	0.10		
N_{ID}	99		
Observations	594		
Marginal R^2 / Conditional R^2	0.005 / 0.103		

*$p<0.05$ **$p<0.01$ ***$p<0.001$

Table 15: LMER TTA models

M7	TTA~ Lineage + Replicate + Lineage:Replicate + (1|ID)
M8	TTA~ Replicate + Lineage:Replicate + (1|ID)
M9	TTA~ Replicate + (1|ID)

Table 16: Anova comparing all models. Model selection was based on AIC.

AOV

	npar	AIC	BIC	logLik	deviance	Chisq	Df	Pr(>Chisq)
M9	8	1362.571	1396.32	-673.2855	1346.571	NA	NA	NA
M7	14	1351.489	1410.55	-661.7447	1323.489	23.0816	6	0.0007696
M8	14	1351.489	1410.55	-661.7447	1323.489	0.0000	0	NA

Table 17: Outputs of the LMER models used to assess boldness with total time in arena. Stars indicate significative p-value.

	TTAyj		
Predictors	*Estimates*	*CI*	*df*
(Intercept)	2.78 ***	2.50 – 3.07	381.46
DC4	*Reference*		
EPP	0.53 **	0.14 – 0.93	373.11
LineageEPP:Replicate2	-0.22	-0.71 – 0.26	370.82
LineageEPP:Replicate3	-0.28	-0.79 – 0.22	374.69
LineageEPP:Replicate4	0.22	-0.29 – 0.74	379.03
LineageEPP:Replicate5	0.42	-0.10 – 0.94	378.78
LineageEPP:Replicate6	0.07	-0.45 – 0.59	380.86
Replicate2	0.16	-0.19 – 0.51	372.70
Replicate3	0.08	-0.28 – 0.45	375.51
Replicate4	-0.33	-0.70 – 0.04	381.15
Replicate5	-0.43 *	-0.80 – -0.06	382.35
Replicate6	-0.21	-0.59 – 0.17	387.05
Random Effects			
σ^2	0.67		
$\tau_{00\ ID}$	0.25		
ICC	0.27		
N_{ID}	99		
Observations	471		
Marginal R^2 / Conditional R^2	0.100 / 0.343		

** p<0.05 ** p<0.01 *** p<0.001*

Table 18: GLMER RTZI models

M19	RTZI ~ Lineage + Replicate + Replicate:Lineage + (1|ID)
M20	RTZI ~ Lineage + Replicate + (1|ID)
M21	RTZI ~ Lineage + (1|ID)

Table 19: Anova comparing all models. Model selection was based on AIC.

AOV

	npar	AIC	BIC	logLik	deviance	Chisq	Df	Pr(>Chisq)
M21	4	-1576.584	-1560.200	792.2918	-1584.584	NA	NA	NA
M20	9	-1654.492	-1617.629	836.2458	-1672.492	87.908070	5	0.0000000
M19	14	-1651.698	-1594.357	839.8491	-1679.698	7.206503	5	0.2057298

Table 20: Outputs of the GLMER models used to assess boldness with the relative time spent in inner zone. Stars indicate significative p-value.

	RTZI	
Fixed effects	*Estimates*	*CI*
(Intercept)	0.09 ***	0.07 – 0.12
Lineage [EPP]	0.92	0.71 – 1.20
Replicate [2]	0.76 *	0.59 – 0.99
Replicate [3]	0.57 ***	0.44 – 0.75
Replicate [4]	0.44 ***	0.33 – 0.57
Replicate [5]	0.33 ***	0.25 – 0.44
Replicate [6]	0.36 ***	0.27 – 0.47
Random Effects		
σ^2	0.66	
$\tau_{00\ ID}$	0.19	
N_{ID}	98	
Observations	444	
Marginal R^2 / Conditional R^2	0.163 / 0.353	

** p<0.05 ** p<0.01 *** p<0.001*

Table 21: LMER RTDM models

M11	RTDM~ Lineage + Replicate + Lineage:Replicate + (1|ID)
M12	RTDM~ Lineage + Lineage:Replicate + (1|ID)
M13	RTDM~ Lineage + (1|ID)

Table 22: Anova comparing all models. Model selection was based on AIC.

AOV

	npar	AIC	BIC	logLik	deviance	Chisq	Df	Pr(>Chisq)
M13	4	1418.046	1434.920	-705.0228	1410.046	NA	NA	NA
M12	14	1395.755	1454.815	-683.8773	1367.755	42.29102	10	6.7e-06
M11	14	1395.755	1454.815	-683.8773	1367.755	0.00000	0	NA

Table 23: Outputs of the LMER models used to assess boldness with the relative total distance moved. Stars indicate significative p-value.

	RTDMyj		
Predictors	*Estimates*	*CI*	*df*
(Intercept)	3.42 ***	3.13 – 3.71	444.37
DC4	*Reference*		
LineageDC4:Replicate2	0.53 **	0.14 – 0.92	375.56
LineageDC4:Replicate3	0.79 ***	0.38 – 1.20	380.77
LineageDC4:Replicate4	0.71 ***	0.30 – 1.12	388.43
LineageDC4:Replicate5	0.95 ***	0.54 – 1.37	390.17
LineageDC4:Replicate6	0.75 ***	0.33 – 1.16	393.51
EPP	0.21	-0.20 – 0.61	442.35
LineageEPP:Replicate2	0.45 *	0.07 – 0.84	373.05
LineageEPP:Replicate3	0.38	-0.02 – 0.77	380.60
LineageEPP:Replicate4	0.23	-0.18 – 0.63	385.42
LineageEPP:Replicate5	0.54 **	0.14 – 0.94	382.19
LineageEPP:Replicate6	0.64 **	0.24 – 1.03	380.16
Random Effects			
σ^2	0.85		
$\tau_{00\ ID}$	0.10		
ICC	0.11		
$N_{\ ID}$	99		
Observations	471		
Marginal R^2 / Conditional R^2	0.071 / 0.173		

** p<0.05 ** p<0.01 *** p<0.001*

1.C: Plasticity assessment

Table 24: LMER prop_out plasticity models

prop_out ~ Replicate + Lineage + (1|ID)	Model used to calculate personality random intercepts
prop_out ~ Replicate + Lineage +(1+Replicate|ID)	Model used to calculate plasticity random slopes according in relation to random intercepts

Table 25: Outputs of the LMER models used to calculate rivulus random intercepts (**left**) and random slopes (**right**) for prop_out value. Bold numbers indicate significative p-value.

	Personality		
Fixed effects	*Estimates*	*CI*	*p*
(Intercept)	0.16	0.12 – 0.21	**1.927e-13**
Replicate [2]	-0.02	-0.06 – 0.03	4.702e-01
Replicate [3]	-0.05	-0.10 – -0.01	**1.404e-02**
Replicate [4]	-0.07	-0.11 – -0.03	**1.786e-03**
Replicate [5]	-0.07	-0.11 – -0.02	**2.350e-03**
Replicate [6]	-0.07	-0.11 – -0.03	**1.535e-03**
Lineage [EPP]	0.09	0.05 – 0.14	**5.921e-05**
Random Effects			
σ^2	0.02		
$\tau_{00\ ID}$	0.01		
N_{ID}	99		
Observations	594		
Marginal R^2 / Conditional R^2	0.081 / 0.332		

	Plasticity		
Fixed effects	*Estimates*	*CI*	*p*
Replicate [2]	-0.02	-0.07 – 0.04	5.394e-01
Replicate [3]	-0.05	-0.10 – -0.01	**2.458e-02**
Replicate [4]	-0.07	-0.12 – -0.02	**6.131e-03**
Replicate [5]	-0.07	-0.12 – -0.02	**7.017e-03**
Replicate [6]	-0.07	-0.11 – -0.03	**1.368e-03**
Lineage [EPP]	0.09	0.04 – 0.13	**1.018e-04**
Random Effects			
σ^2	0.01		
$\tau_{00\ ID}$	0.03		
$\tau_{11\ ID.Replicate2}$	0.05		
$\tau_{11\ ID.Replicate3}$	0.04		
$\tau_{11\ ID.Replicate4}$	0.04		
$\tau_{11\ ID.Replicate5}$	0.04		
$\tau_{11\ ID.Replicate6}$	0.03		
ρ_{01}	-0.71		
	-0.74		
	-0.77		
	-0.71		
	-0.65		

Table 26: LMER LZE plasticity models

LZE~ Replicate + Lineage + (1|ID)	Model used to calculate personality random intercepts

LZE ~ Replicate + Lineage + (1+Replicate|ID)	Model used to calculate plasticity random slopes according in relation to random intercepts

Table 27: Outputs of the LMER models used to calculate rivulus random intercepts (**left**) and random slopes (**right**) for LZE value. Bold numbers indicate significative p-value.

	Personality LZE		
Fixed effects	*Estimates*	*CI*	*p*
(Intercept)	654.67	505.53 – 803.80	**6.180e-17**
Replicate [2]	-100.11	-266.91 – 66.69	2.390e-01
Replicate [3]	12.81	-154.07 – 179.69	8.802e-01
Replicate [4]	12.77	-154.11 – 179.66	8.806e-01
Replicate [5]	12.31	-154.49 – 179.11	8.848e-01
Replicate [6]	71.40	-95.40 – 238.20	4.008e-01
Lineage [EPP]	-110.29	-256.96 – 36.38	1.403e-01
Random Effects			
σ^2	357035.30		
$\tau_{00\ ID}$	78501.51		
N_{ID}	99		
Observations	594		
Marginal R^2 / Conditional R^2	0.013 / 0.191		

	Plasticity LZE		
Fixed effects	*Estimates*	*CI*	*p*
(Intercept)	650.15	509.09 – 791.20	**2.239e-18**
Replicate [2]	-100.11	-232.84 – 32.62	1.390e-01
Replicate [3]	10.63	-145.58 – 166.83	8.938e-01
Replicate [4]	12.77	-151.33 – 176.88	8.785e-01
Replicate [5]	12.31	-170.44 – 195.06	8.948e-01
Replicate [6]	71.40	-110.01 – 252.82	4.398e-01
Lineage [EPP]	-101.16	-246.71 – 44.39	1.728e-01
Random Effects			
σ^2	221336.92		
$\tau_{00\ ID}$	156062.22		
$\tau_{11\ ID.Replicate2}$	9380.46		
$\tau_{11\ ID.Replicate3}$	180825.20		
$\tau_{11\ ID.Replicate4}$	246748.19		
$\tau_{11\ ID.Replicate5}$	414373.95		
$\tau_{11\ ID.Replicate6}$	401869.79		
ρ_{01}	-0.28		
	-0.31		
	-0.53		
	-0.61		
	-0.52		
N_{ID}	99		
Observations	594		
Marginal R^2 / Conditional R^2	0.012 / 0.498		

Table 28: LMER RTZI plasticity models

RTZI~ Replicate + Lineage + (1|ID)	Model used to calculate personality random intercepts
RTZI ~ Replicate + Lineage + (1+Replicate|ID)	Model used to calculate plasticity random slopes according in relation to random intercepts

Table 29: Outputs of the LMER models used to calculate rivulus random intercepts (**left**) and random slopes (**right**) for RTZI value. Bold numbers indicate significative p-value.

	Personality RTZI		
Fixed effects	*Estimates*	*CI*	*p*
(Intercept)	0.09	0.08 – 0.11	**1.797e-34**
Replicate [2]	-0.02	-0.04 – -0.01	**3.412e-03**
Replicate [3]	-0.04	-0.06 – -0.02	**3.631e-06**
Replicate [4]	-0.05	-0.07 – -0.03	**1.711e-09**
Replicate [5]	-0.06	-0.08 – -0.04	**1.284e-12**
Replicate [6]	-0.06	-0.07 – -0.04	**2.740e-11**
Lineage [EPP]	-0.00	-0.02 – 0.01	6.722e-01
Random Effects			
σ^2	0.00		
$\tau_{00\ ID}$	0.00		
N_{ID}	99		
Observations	502		
Marginal R^2 / Conditional R^2	0.119 / 0.258		

	Plasticity RTZI		
Fixed effects	*Estimates*	*CI*	*p*
(Intercept)	0.09	0.08 – 0.11	**8.274e-22**
Replicate [2]	-0.02	-0.05 – -0.00	**2.775e-02**
Replicate [3]	-0.04	-0.06 – -0.02	**6.966e-05**
Replicate [4]	-0.05	-0.07 – -0.03	**1.330e-07**
Replicate [5]	-0.06	-0.08 – -0.04	**6.784e-10**
Replicate [6]	-0.06	-0.08 – -0.04	**3.782e-09**
Lineage [EPP]	-0.00	-0.01 – 0.01	6.999e-01
Random Effects			
σ^2	0.00		
$\tau_{00\ ID}$	0.01		
$\tau_{11\ ID.Replicate2}$	0.01		
$\tau_{11\ ID.Replicate3}$	0.01		
$\tau_{11\ ID.Replicate4}$	0.01		
$\tau_{11\ ID.Replicate5}$	0.01		
$\tau_{11\ ID.Replicate6}$	0.01		
ρ_{01}	-0.79		
	-0.86		
	-0.86		
	-0.96		
	-0.91		
N_{ID}	99		
Observations	502		
Marginal R^2 / Conditional R^2	0.347 / NA		

Table 30: LMER RTDM plasticity models

RTDM~ Replicate + Lineage + (1|ID)	Model used to calculate personality random intercepts
RTDM ~ Replicate + Lineage + (1+Replicate|ID)	Model used to calculate plasticity random slopes according in relation to random intercepts

Table 31: Outputs of the LMER models used to calculate rivulus random intercepts (**left**) and random slopes (**right**) for RTDM value. Bold numbers indicate significative p-value.

	Personality RTDM		
Fixed effects	*Estimates*	*CI*	*p*
(Intercept)	1.41	0.31 – 2.51	**1.184e-02**
Replicate [2]	0.62	-0.77 – 2.00	3.818e-01
Replicate [3]	2.02	0.60 – 3.45	**5.427e-03**
Replicate [4]	1.67	0.26 – 3.09	**2.036e-02**
Replicate [5]	1.49	0.06 – 2.92	**4.049e-02**
Replicate [6]	1.29	-0.16 – 2.73	8.030e-02
Lineage [EPP]	-0.07	-0.98 – 0.84	8.748e-01
Random Effects			
σ^2	22.39		
$\tau_{00\ ID}$	0.83		
N_{ID}	99		
Observations	502		
Marginal R^2 / Conditional R^2	0.020 / 0.055		

	Plasticity RTDM		
Fixed effects	*Estimates*	*CI*	*p*
(Intercept)	1.32	1.04 – 1.60	**3.981e-19**
Replicate [2]	0.60	0.21 – 0.99	**2.890e-03**
Replicate [3]	2.08	-0.01 – 4.17	5.072e-02
Replicate [4]	1.74	0.78 – 2.71	**4.089e-04**
Replicate [5]	1.51	0.72 – 2.29	**1.933e-04**
Replicate [6]	1.32	0.67 – 1.96	**7.356e-05**
Lineage [EPP]	0.09	-0.28 – 0.46	6.368e-01
Random Effects			
σ^2	0.48		
$\tau_{00\ ID}$	0.50		
$\tau_{11\ ID.Replicate2}$	2.63		
$\tau_{11\ ID.Replicate3}$	91.93		
$\tau_{11\ ID.Replicate4}$	19.11		
$\tau_{11\ ID.Replicate5}$	12.31		
$\tau_{11\ ID.Replicate6}$	7.38		
ρ_{01}	0.74		
	0.32		
	-0.05		
	-0.29		
	0.22		
N_{ID}	99		
Observations	502		
Marginal R^2 / Conditional R^2	0.022 / 0.980		

Introduction

I. Evolution of ethology

The study of animal behavior, or ethology, is an important component of scientific research. Although, in its early days it was rather a matter of approximate descriptions, the interpretation of which varied according to the observer, ethology has now become a science of its own (Lehner, 1987). The word ethology comes from the Greek "ethos" which refers to morals and "logos" which is the word for knowledge. This word was used for the very first time during the 18th century, in a publication issued by the French Academy of Sciences. But at the time, it referred more to the psychology of an animal more than to the study of its behavior. But what is a behavior? A behavior can be defined as the way of moving, feeding, communicating, body positioning or any change in these patterns due to an external, or an internal factor. Behavior does not always define a movement, or an action done by the animal, doing nothing is also a behavior in itself. A lion napping in a place is apparently doing nothing, but its presence

indicates to other lions that this place is part of its territory (Immelmann, 1981). The pioneers of modern ethology are Konrad Lorenz and Niko Tinbergen, their collaboration has established a basis for the biological study of the behavior of insects, birds, and fishes, in their natural living conditions. The Second World War interrupted their work, but they still won the Nobel Prize in Medicine and Physiology in 1973 (Moreno & Muñoz-Delgado, 2007).

Nowadays, behavior is considered to be the result of natural selection that has allowed the carriers of certain behavioral traits to survive and reproduce. The beginnings of ethology date back to before the 18th century, it is even older than the written language. Indeed, 12,000 years ago, humans began to domesticate the wolf, first to guard livestock and then as a companion animal. To do so, humans had to learn how wolves hunted and behaved in order to gain their trust. It was then that their domestication began, humans chose behavioral traits, coat colors and different physical traits that led to the dog breeds known today. All of that with absolutely no knowledge of what DNA, genes and chromosomes are. This domestication has considerably changed the behavior of the wolf, over generations its behavior has become more gentle, protective towards the one it considers as its caregiver and a relationship of codependence between the human and the dog has been developed (Breed, 2017).

Charles Darwin had a profound influence on this field of science. During his journey aboard the Beagle between 1831 and 1836, he became very interested in the behavior of the animals he studied. He established three categories of behavior: instinct, learning ability and the capacity to reason in a basic way. He showed that the behavior could vary over time and generations, because they are subject to natural selection. By studying the insular species, he realized that over generations, they lost some anti-predatory behaviors, if their continental predators had not followed them to the island (Fericean *et al.,* 2015).

It was only after the second world war that ethology was really considered as a science and started to be taught in universities. But depending on the animals and the context, everyone had a different interpretation of how to proceed and interpret the results. Therefore, each university had its own way of conducting studies and there were no standardized procedures. Everyone had a different view on the fact that an animal adopts a certain behavior at a certain time, some thought it was heritable with "specific genes" for the behavior and others thought it was acquired, through the parental teaching and the environmental context. But if the post-war period has allowed so much expansion of science, it is thanks to the fact that some fields of science were highlighted during the war and received a lot of funding. Because at the time,

investing $2700 in a spectrograph was considered a huge investment and not many people were willing to put their money into it (Griffiths, 2008).

In the 1970s, ethology experienced a certain withdrawal. There was a deep division between the European and American view of ethology. In Europe, the animal was studied in its environment and its instinctive behaviors were the purpose of the study. In the United States, they focused on the laboratory approach, putting the animals in mazes in which some paths led to rewards and others to punishments. It is at this moment that ethologists like Klopfer, Bateson or Hinde, respectively, German, American and British, appealed to the whole community for a standardization of the methods used. It was first at Cambridge, then at Oxford that the reform of ethology began. This new point of view, based on mathematical models taken from ecology and population genetics, fed by the theory of evolution and population dynamics, led to modern ethology, as known nowadays (Stuhrmann, 2022).

II. Ethology: practical aspects and limitations

After this brief overview of the history of ethology, it is now time to discuss the practical side. Ethology can be studied in the laboratory, but when most people hear the word ethology, they tend to think of field studies. The study of an animal's behavior in its natural environment provides more information on the nature of the behaviors observed. Behaviors will be a direct response to a change in the environment and not influenced by the experiment, if the observer can remain unnoticed. Even in field studies, the experimenter may need to handle the animals being observed, for example to collect blood or to tag an animal. These manipulations cause stress and must be done as rapidly as possible. The experimenter can also induce a behavior, for example by placing a decoy that resembles a predator or prey and observe the reaction of the animal being studied (Cuthill, 1991). To study behaviors, researchers use an ethogram. It is a description of the behaviors witnessed in the animal of interest. In this way, observations can be compared for several individuals and even between closely related species (Coelho *et al.,* 2018). There is no standard ethogram for all species. Instead, they are grouped by categories such as birds, carnivores, herbivores, fishes, etc. Behavioral data can be collected using several methods. For example, in interval sampling, time intervals are predefined by the researcher and behaviors are documented in the ethogram only during these regularly spaced time intervals. Focal sampling is another method in which the researcher focuses on a single individual within the group and reports the behaviors during the defined time period (Graszer *et al.,* 2012).

Once the behavior of an animal in its natural environment is known, it can be used as a reference for animal research in the laboratory. The animals are used for studies testing a broad range of topics, such as ecotoxicology, new medications, or even neurodegenerative diseases, etc. The purpose is to build models that can ultimately be applied to humans. To ensure that the behaviors observed in the laboratory are as close as possible to those that would be observed in natural conditions, scientists must consider the needs of the species being studied. The habitat must be as analogous as possible to the natural habitat of the species and its social needs must be accommodated. If a species is gregarious, therefore, the housing must contain several animals. These measures are used to reduce the stress of the animals, as this can alter the results of a study (Peters *et al.,* 2015). In studies involving the use of animals, it must be ensured that a sufficient number of animals are tested for the study to be considered statistically valid (Sert *et al.,* 2020). But the more animals a study has, the higher is the probability of having individuals that are significantly different from each other. Indeed, the mixed linear models used for such analyses assume the homogeneity of the residual variances. If some individuals are more variable than others, they differ in their level of predictability; therefore, this homogeneity condition is no longer respected, and the results cannot be taken into account (Biro *et al.,* 2018).

III. Personality, plasticity, and predictability

It is at this point that it becomes important to consider three concepts: personality, plasticity, and predictability **(figure 1)**. Personality is the way an individual behaves in a given context (Biro *et al.,* 2018). Plasticity is the way in which an individual will adapt their behavior over time and contexts (Jolles *et al.,* 2019). Predictability is defined as the degree of variability of an individual in a given context (O'Dea *et al.,* 2022). Originally, the differences in behavior between individuals were explained by the fact that they had differences in the way they acquired their energy. Some behaviors are more energy consuming than others and therefore, these differences came from there. The availability of the energy source and its accessibility may cause an individual to change their behavior, but it is not the reason for the differences in behavior between individuals (Mitchell & Biro, 2017).

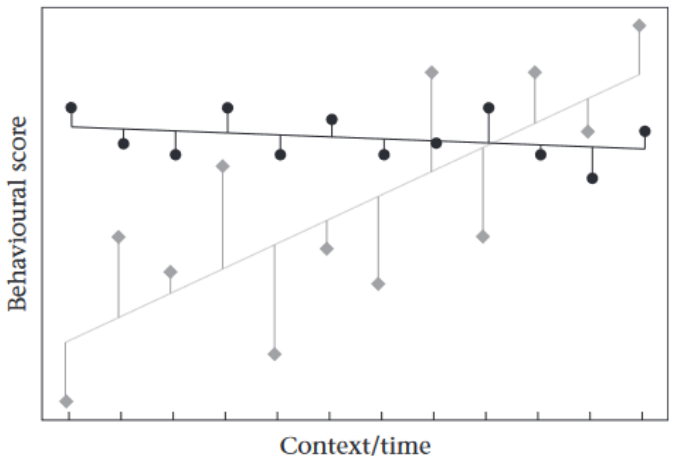

Figure 1 : *This figure is a fictitious demonstration of intra-individual variability between a grey and a black subject, they have been tested many times and exhibit certain behaviors. A linear regression of this fictional data is presented for each of the two subjects. The y-intercept on the y-axis indicates the personality of the subjects, the slope of the line their plasticity and the vertical lines the residuals per time point, together they attest the intra-individual behavioral variability of the subject. here, the black subject exhibits a high personality score, low plasticity, and low intra-individual behavioral variability, while the gray subject exhibits a low personality score, high plasticity, and high intra-individual behavioral variability (Jolles et al., 2019).*

Plasticity is usually considered as intra-individual variation, but it is not. It refers to differences between individuals in intercepts and slopes compared to a given gradient. Once changes in time and contexts have been considered, in addition to factors such as hunger, thirst, and light, in other words, factors that are not experimentally controlled. The only remaining source of variation is intra-individual variation. intra-individual variation can change over time and contexts, depending on the subject's learning abilities. This variation could be considered as a brain generated behavioral flexibility to enhance the non-predictability of the subject in order to increase its chances of survival. Depending on the context, some individuals are much more predictable than others (Biro & Adriaenssens, 2013). Unlike plasticity, which can be modulated according to context, there is nothing the scientist can do to change the degree of predictability of a behavior (Cleasby *et al.,* 2015). Some studies have revealed that behavioral predictability is important in sexual selection by females. Indeed, females tend to choose males whose behavior is predictable. For example, females of *Pelvicachromis pulcher*, a fish belonging to the *chilidae* family, have a preference for males with consistent behavior, regardless of whether a male is very aggressive or not as long as his behavior is predictable **(figure 2)** (Scherer *et al.,* 2018).

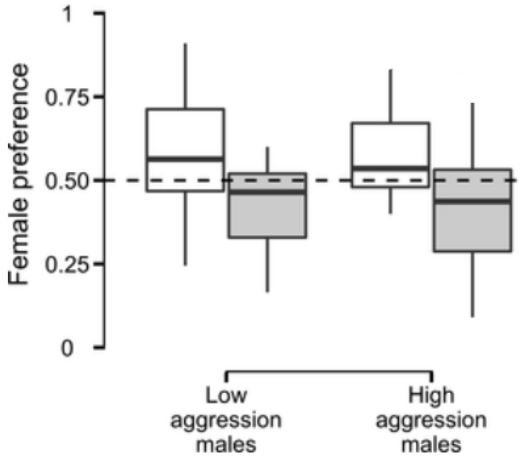

Figure 2 : *This graph shows the preference of females Pelvicachromis pulcher for the consistency of males' behavior. White boxes are assigned to males whose behavior is consistent and gray boxes to those whose behavior is not consistent (Scherer et al., 2018)*

To measure the differences in personality among individuals, five traits are mainly studied: exploration, aggressiveness, activity, boldness, and sociability. In the laboratory, these personality traits are measured by "open field tests". The advantage of being in the laboratory for this type of test is that all variables can be controlled and remain the same during the test, and thus do not influence the animal's behavioral response. The individuals of a population display different behaviors from one another. This means that out of all the behaviors observed in this population, each individual expresses only a certain number of them, which implies that each individual presents a "behavioral type" that is unique to them and that is different from the other individuals of the population (Hertel *et al.,* 2020). In general, the boldest, most aggressive and exploring individuals are less likely to change their behavior over time and are therefore more predictable than shy and non-aggressive individuals. In hierarchical species, the animal's status within the group influences the plasticity of behavior (Guayasamin *et al.,* 2017). Behavioral plasticity is potentially heritable, as plastic individuals are more likely to be able to successfully adapt to an environmental change and thus survive to reproduce (Cornwell *et al.,* 2019).

IV. Boldness behavior assessment

Among the most studied behavioral traits is boldness, this is the trait that will be studied in the present document. Boldness can be seen as the willingness to take a potential risk in an

effort to find something that will enhance the fitness of an individual, whether it be the search for food, a mating partner, or a new habitat (MacGregor *et al.,* 2021).

To evaluate this personality trait, the paper written by Jolles *et al.* (2019) was used as a foundation for developing the experimental design of the research led. They tested 80 three-spined sticklebacks, born in the year around the same period of time, their age was approximated by measuring the body length. These fish were not laboratory fish, they were caught in a river. Therefore, it was necessary to wait 1 month, so that they would get accustomed to the environment of the laboratory before being able to start the tests. During this time, they were placed in collective aquariums, because it is a gregarious species. Three-spined sticklebacks were placed in individual aquariums for identification two days before the testing. On the day of the test, they were transferred from the individual aquarium to a transport box that could be positioned on top of the test aquarium. In this test aquarium were placed a fake plant that acted as a shelter and gravels at the bottom, the aquarium was sloped with the deepest part measuring 13 cm and the shallowest 3 cm. The aquarium was illuminated from below in the shallowest part and it was placed in a closable wooden box to be totally isolated. Once all the transport boxes were placed on top of their respective aquariums, they were opened, and the fish slid into the deep end of their test aquarium. After that the wooden box was closed and the fish had 30 minutes to accustom itself to the test area and then, the video recording was initiated from a distance to avoid scaring the fish. The fish were tested for six weeks in a row, then were given a four-week rest period, and tested one last time. For the seven tests, the fish were recorded 30 minutes per testing session, these recordings were then analyzed by a software specialized in animal tracking (AnimTrack©). These data were analyzed using a linear mixed model. They analyzed the proportion of time spent in exposure during the six consecutive weeks per fish and compared it to the proportion of time spent in exposure after the four-week break. For each individual, the plasticity of boldness behavior was quantified. Their results showed that fish that were initially less bold were the most plastic **(figure 3)**. As the tests progressed, they spent a greater proportion of time uncovered. Fish that were already bold at the start did not spend significantly more time uncovered than at the beginning. The last test after the four

weeks break to "unaccustom" the fish to the test procedure and the time spent uncovered was similar to the first test for most fish (Jolles *et al.,* 2019).

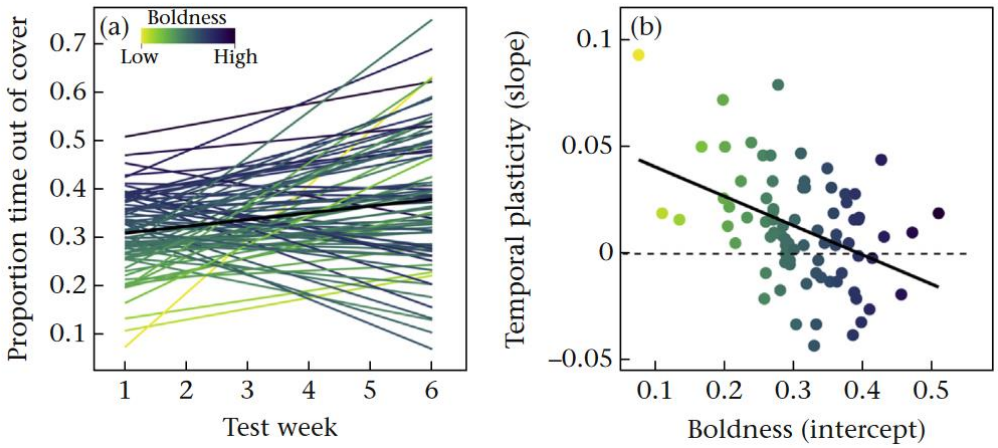

Figure 3 : *(a) Proportion of time spent uncovered per fish per week. (b) Relation between boldness trait and temporal plasticity of fish (Jolles et al., 2019).*

But in this study, Jolles *et al.* (2019) raised a potential bias due to the housing in common aquariums because of the gregarious nature of this species. Indeed, group housing can modify the behavior of the individuals and thus cause fluctuations in the results of the experiment. One can also wonder if the fact that individuals are genetically different does not modulate the expression of behavioral traits and thus the personality itself. For this reason, it seems interesting to conduct the same study on individuals with minimal genetic differences.

V. The mangrove rivulus

The mangrove rivulus (*Kryptolebias marmoratus*), is a fish living in the mangroves of America and South America has a particularity that only one other vertebrate shares with him, it reproduces by self-fertilization. The second species to reproduce by self-fertilization is also a rivulus species, *Kryptolebias hermaphroditus*. Self-fertilization can lead to a genetic bottleneck, because the genotypes adapted to an environment will increase in frequency and at the slightest disturbance, the vast majority of individuals may disappear. This is probably one of the reasons why this mode of reproduction has not been conserved in vertebrates (Tatarenkov *et al.,* 2012). But in this case, it is very interesting because individuals from the same genitor are genetically identical, and it offers an ideal opportunity to examine the link between the genetics of an individual and its personality.

In this species the wide majority of individuals are hermaphrodites possessing an ovotestis and according to the populations there may be between 1 and 25 % of males, no female individual exists. Even if the dominant mode of reproduction is self-fertilization, it happens that cross-fertilization occurs between a male and a hermaphrodite. The frequency at which it occurs is assumed to be low but is not known (Tatarenkov *et al.,* 2012). This cross-fertilization occurs when a hermaphrodite lays an unfertilized egg and a male releases his sperm on it. Males are that are born male without ovarian tissue, are called primary males. Males can also come from hermaphrodites whose ovarian tissue is no longer functional, they are then called secondary males (Avise & Tatarenkov, 2015).

The eggs laid by the rivulus measure 2 mm in diameter, they can take between 20 and 40 days to hatch unless the egg enters diapause. Indeed, if the environmental conditions are not favorable for hatching, the embryo stops momentarily its development and resumes it when the conditions are more favorable, the eggs can remain in diapause more than 90 days. The monitoring of the embryonic development can be done by visualization with a microscope because the egg is transparent and allows to see the embryo **(figure 4)**. As the embryo develops, the egg darkens (Genade, 2016). At the time of hatching, the juvenile measures on average 6, 1 mm, it will grow until it reaches a size of 3 to 4 cm and will be considered as adult when it reaches the age of 4 to 5 months, when the development of its gonads will be completed (Lee *et al.,* 2008; Sakakura & Noakes, 2000).

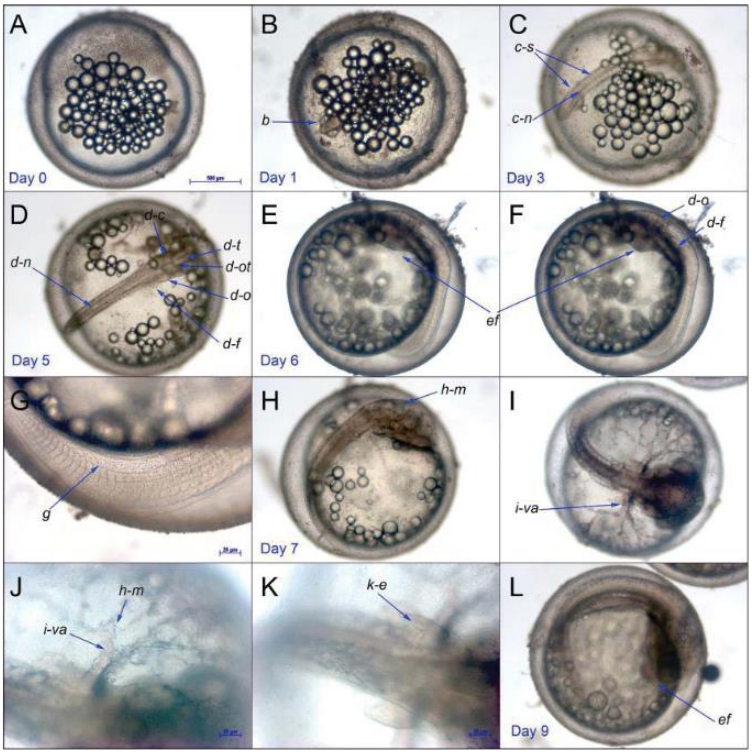

Figure 4 : *Development of an egg of Kryptolebias marmoratus visualized under the microscope, a scale at 500 µm was placed. A day 0 of development. B Day 1 of development, it is at this stage that the eyes start to be visible (b), it is also at this stage that the brain starts to develop. C On day 3 of development the somites become visible (c-s) as well as the notochord (c-n). D Day 5 of development, the development of the nervous system becomes more evident; (d-n) indicates the notochord, (d-c) the cerebellum, (d-t) the telencephalon, (d-ot) the optic tectum, (d-o) the otic vesicles, (d-f) the fin buds. E-G Day 6 of development, the heartbeat becomes visible (ef). H-I Day 7 of development, apparition of malanophores (h-m) and blood pigmentation (i-va). J-K zoom in from the previous two images, (k-e) indicates erythrophores on the pectoral fin in formation. L Day 9 of development the embryo becomes darker, and the heart is less visible (Genade, 2016).*

The rivulus is a solitary fish, it is rather aggressive towards the members of its own species whom it rarely meets. The parents are known to predate their own eggs and their own juveniles. Juveniles raised in small groups are more tolerant of other group members, but are still aggressive. To avoid unnecessary aggression and injury, these animals are generally kept in separate containers in laboratories (Tatarenkov *et al.,* 2010 ; Taylor, 2012). It would be tempting to assume that individuals carrying the same genotype would be reacting the same way to the same situation, but individuals carrying the same genotype sometimes show very different behaviors from each other. During their growth, these fish are much more plastic than as adults. This high plasticity during juvenile phase can potentially be explained by the fact that the mangrove is a constantly changing environment and therefore, juveniles have to adapt their behavior rapidly to survive (Edenbrow & Croft, 2011). The phenotypic plasticity of the rivulus is such that when the water in which it is immersed is too polluted by compounds like H_2S, the cells of its gills shrink and the fish is capable of emerging to breathe in the open air, without losing too much water. This change of phenotype is reversible (Earley *et al.,* 2012).

DNA methylation is one of the hypotheses investigated in an effort to explain the behavioral differences between individuals with identical genomes. Epigenetic modifications of the parent are unlikely to be transmitted to the offspring because of their reprogramming during embryogenesis. This time period during which the DNA methylation are rearranged is relatively long, during this period the environment in which the fish are as well as any change within it, influence the epigenetic modifications of the rivulus (Berbel-Filho *et al.,* 2020). This could imply that adults would be adapted to a broad variety of ecological niches through high phenotypic plasticity (Fellous *et al.,* 2018). Histone acetylation is also an epigenetic modification that can influence rivulus behavior. The enzymes involved in this process can impact processes such as gametogenesis or neurogenesis. These DNA modifications allow certain genes to be transcribed or not at given times during the development of the rivulus,

which could potentially impact its phenotypic plasticity and therefore its behavior (Fellous *et al.,* 2019).

The rivulus is therefore an excellent candidate for studies on personality, predictability, and behavioral plasticity. The advantage of being from isogenic lineages is that genotypic replicates can be easily obtained, allowing genotypic differences to be excluded as a source of variation in behavior. During the course of this research, two lines will be studied (EPP and DC4) to see if the results are similar between lineages. Epigenetic analyses will also be done on the livers and brains of the tested fish, to investigate the link between epigenetic changes and behavior. To verify the experimental design chosen for this research, pre-tests were performed. Because studies such as the one done by Rossi & Wright (2021), have shown that this fish has great learning capacities, it was necessary to ensure that the tests were sufficiently spaced to avoid the rivulus getting used to the procedure to avoid bias. The results of these tests along with their methodology are presented in the following sections.

Material and methods

The rivulus tested were selected from the reproductive stock, which are stored in individual containers to avoid aggressive behavior, which is very common among this species, and the containers are kept in a temperature-controlled chamber at 26°C. These fish are fed with freshly hatched live artemia, once a day. Eighteen of them, approximatively around the age of 90 days were taken, they are from the EPP lineage. Those fishes were divided into two groups of nine individuals, the first group was tested every week and the second every two week. The first group had five replicates, and the second had three replicates.

The test arena consists of a ten liter bucket of 18 cm in diameter. On the side of the bucket, a 5 cm diameter hole was cut, and a pipe corner was attached to it, using water resistant silicone. A slot has been cut in the part protruding into the bucket, to be able to pass a plastic trap door to block le fish into the pipe corner during the habituation period. The pipe corner is closed with a lid as soon as the fish is placed inside of it, to make sure it doesn’t jump out of it, and prevent it from seeing the experimenter, thereby avoiding causing stress that could bias the experiment.

The experiment itself consisted of putting the fish in the pipe bend for 10 minutes to get used to its new environment. Once the 10 minutes were over, the recording was started, and the trap door was lifted so that the fish had access to the entire test area for the 30 minutes of the test. When the test was completed, the recording was stopped, and the fish was transferred back to its individual container and placed back into the temperature-controlled chamber. It should be noted that the room in which the experiments were conducted was at the same temperature as the thermoregulated chamber to avoid any discomfort, or changes in behavior for the fish tested.

Figure 5 : *Calibration of the test arena, the yellow area indicates the center of the arena, the orange area the entire surface of the arena, the red area the entrance to the shelter and the blue area the shelter.*

The videos, which were stored on memory cards were then exported to the Ethovision© software. Each arena was manually calibrated to indicate the central area, the entrance area of the refuge and the refuge **(figure 5)**. The detection of each fish was also done manually to ensure that the software detects the fish and not its reflection, or a shadow. The software analyzed each video separately and provided a table with distances and times as shown in **figure 6**. The table was later imported into the R Studio software.

						Distance move	In zone	In zone	In zone	In zone	In zone	In zone	In zone	In zone	In zone
						Center-point	intern / Center	intern / Center-po	intern / Center-p	Shelter / C	Shelter / Center-p	Shelter / Center-	Entry shelter / C	Entry shelter / C	Entry shelter / Center
						Total	Frequency	Cumulative Durati	Latency to First	Frequency	Cumulative Durati	Latency to First	Frequency	Cumulative Dur	Latency to First
						cm		s	s		s	s		s	s
Trial	1	1	21-10	1	469,304	18	151,04	542,52	0	0	-	0	0	-	
Trial	2	1	21-06	1	899,024	19	63,48	0,08	13	473,2	410,04	39	183,52	0,24	
Trial	3	1	21-07	1	593,859	40	828,56	274,04	0	0	-	0	0	-	
Trial	4	1	21-08	1	665,093	18	214,28	761,2	3	757,68	0	5	2,8	757,52	
Trial	5	1	21-09	1	635,984	19	323,28	118,48	4	583,56	261,76	9	7,4	91,76	
Trial	6	1	21-11	1	119,22	4	14,16	1489,12	5	1010,12	644,16	11	29,72	0,12	
Trial	7	2	21-37	1	1129,21	32	130,84	0,92	11	827,88	279,92	28	50,04	0	
Trial	8	2	21-39	1	1156,27	41	257,28	0,12	7	229	50,92	17	30,28	46,32	
Trial	9	2	21-40	1	803,357	34	344,56	338,8	0	0	-	2	0,84	333,92	
Trial	10	2	21-41	1	348,42	8	32,56	1289	9	265,84	1269,68	17	31,88	1269,64	
Trial	11	2	21-42	1	3223,59	121	302,48	0,04	23	194,32	357	45	56,32	7	
Trial	12	2	21-43	1	546,459	26	136	374,6	11	343,96	424,08	36	7,96	370,16	
Trial	13	2	21-44	1	2769,69	104	484,84	238,16	30	146,08	221,04	68	57,4	203,36	
Trial	14	1	21-45	1	1035,65	33	174,64	0	7	96,36	1021,84	18	14,72	0,28	
Trial	15	1	21-46	1	738,264	33	197,96	242,72	16	597,44	12,44	27	23,08	0	
Trial	16	1	21-47	1	443,283	11	60,08	1495,6	1	75,96	1671,6	3	13,52	1473,12	
Trial	17	2	21-48	1	449,034	20	140,12	262,56	4	0,2	1419,4	8	4,56	517,28	

Figure 6 : *Raw data table from the Ethovision© software.*

A correlation matrix of the different parameters analyzed by ethovision© was produced. The data were then normalized to be exploited. A Bartlet test and a Shapiro test were performed to verify the normality of the residues. Different models were done with the data to determine if there were any significant differences between the groups. The results and their interpretation are presented in the following section.

Results and discussion

The matrix presented in **Figure 7** shows the different parameters analyzed as well as the correlation between the variables. Given that most of these variables are dependent on each other, they are significantly (*) to very significantly (***) correlated when this is the case.

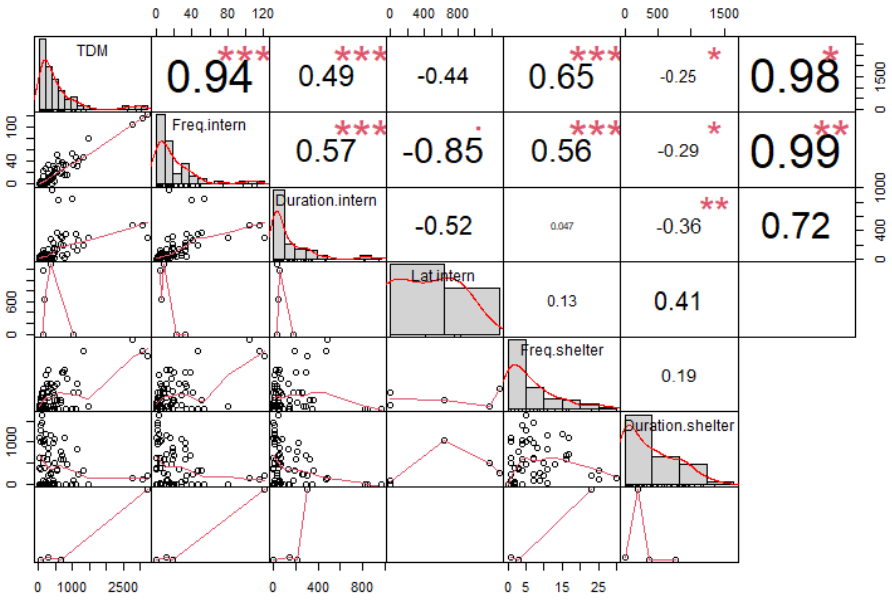

Figure 7 : *Correlation matrix of the different data analyzed. TDM stands for total distance moved, Freq.intern the number of times the individual passes through the internal area, Duration.intern the time spent in the internal area, Lat.intern the time passed before the fish went for the first time to the internal zone, Freq.shelter the number of times the individual enters the shelter, Duration.shelter the time spent in the shelter.*

The boxplot presented in **figure 8** shows the differences between the time spent in the shelter by fish. Even if the graph does not show a significative result, it is still visualizable that the majority of the fish of group 2 spend less time in the shelter, although these fish are tested only every two weeks. The room in which these tests are performed is not soundproof, therefore there are numerous external parameters that can influence the results of these tests.

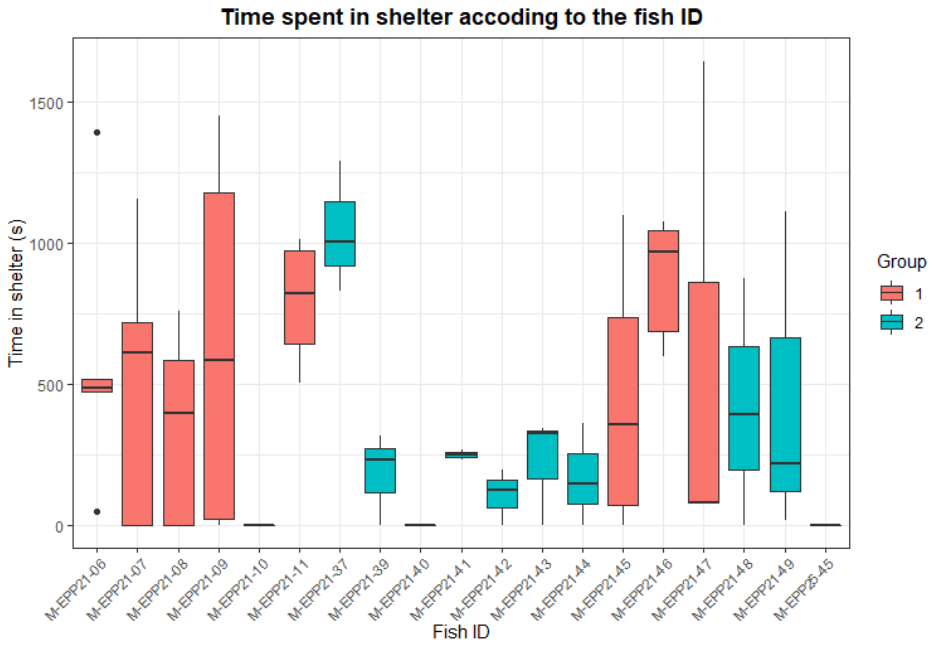

Figure 8 : *This graph shows the total time spent by each fish in the refuge during testing.*

Lastly, the linear models performed did not show that the frequency of the tests had an influence on the results.

Conclusion

The results obtained during these pre-tests validated the experimental protocol of this research, the replicates will be separated by one week and this will not affect the habituation of the fish to the experimental procedure. These pre-tests were also an opportunity to become familiar with the protocol to help save time during the manipulations.

Supplementary materials

S1: Data set

Table of the results of the shelter test provided by Ethovision™.
Additional file titled: Letexier_85562100_2024_Annexe1.csv

S2: Script

The script used to analyze the dataset.
Additional file titled : Letexier_85562100_2024_Annexe2.R

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