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Epigenetic and behavioural variability in an isogenic lineage of *Kryptolebias marmoratus* during domestication under stable laboratory condition

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Abstract

Domestication begins with the transfer of individuals from their natural environment to a captive environment. These individuals, coming from a complex heterogeneous environment with fluctuating abiotic conditions, must adapt to a new environment with much more stable conditions and the presence of humans. In this context, phenotypic changes, such as behavioural variations, may appear from the first generation reared in captivity, raising the hypothesis that epigenetic mechanisms are involved in this process. We investigate this behavioural and epigenetic variability in the Emerson Point Preserve (EPP) population of the self-fertilising hermaphrodite fish *Kryptolebias marmoratus*, the mangrove rivulus, in wild individuals (F0) and in individuals reared under stable laboratory conditions for five generations (F5). This isogenic line offers the opportunity to work with individuals presenting low genetic diversity in order to study the involvement of epigenetic mechanisms in this species under captive conditions. An open field behavioural test to measure boldness, activity and thigmotaxis in fish was performed in duplicate, showing a decrease in boldness, activity, and anxiety, as well as individuality with captivity. DNA methylation at CpG sites was studied and showed significant differences in methylation between the two groups, with overall hypermethylation and low methylation variability in wild individuals compared to captive individuals. The stable conditions of captivity induce a loss of this methylation but with more variability within the group. The next step will be to identify the genes and their function that are influenced and regulated by these methylome variations.

KEYWORDS: Domestication, Captivity, Mangrove rivulus, *Kryptolebias marmoratus*, Behavioural, Boldness, Individuality, Epigenetic, DNA methylation

1 | Introduction

Humans have contributed to the domestication of many species around them. In this process, a population of animals can adapt to captivity and the humans' presence, resulting in domesticated species that have undergone modifications compared to the wild parent species (Milla et al., 2020; Teletchea and Fontaine, 2014). Domestication begins with the transfer of individuals from their natural environment to a captive environment. These wild individuals (F0) must adapt to the presence of humans and to a new, much more stable and controlled environment, with less abiotic constraints and predation compared to the fluctuating natural environment, resulting in phenotypic changes (Taylor, 2012; Turko et al., 2014). Biological traits changes can be observed in domesticated species, from a behavioural, physiological, and morphological standpoint, also through genetic changes (Milla et al., 2020). In all described domesticated species, several specific physiological and behavioural phenotypes are observed. In mammals, for example, smaller teeth, smaller skulls, depigmentation, early sexual maturation, reduced aggressive behaviour or increased growth are found. This is described as the "domestication syndrome" (Lord et al., 2020; Milla et al., 2020).

This phenomenon has been described for a long time in many mammalian species, but not yet in fish. However, several traits that are described in the mammalian domestication syndrome are also found in fish, such as a decrease in brain size, a decrease in fear behaviour or early puberty. The phenotype of fish also appears to be altered by captivity and the presence of humans, in the same way as observed in mammals (Milla et al., 2020). Behavioural variations between wild and domesticated individuals of different fish species have been identified, such as fear behaviour, predator avoidance (Drew et al., 2012), risk-taking (Millot et al., 2009), or boldness and aggressiveness (Brignon et al., 2018; Moretz et al., 2007). Behaviour is an interesting variable to study the interaction between an organism and its environment. When faced with a new or changing environment, such as during domestication, individuals must be able to adapt in order to survive and this can result in behavioural changes (Sih et al., 2010). As a result, behavioural variations between groups studied under different environmental conditions can be observed, but also variations within groups where conditions are the same for all individuals in the group (Mustafa et al., 2019), highlighting the individuality of individuals (Sih et al., 2015), which is the behavioural difference between individuals that is constant and reproducible over time and in all situations (Long et al., 2021).

This collective occurrence of traits that define a domesticated species raises questions about the mechanisms that cause this syndrome and are associated with phenotypic variations. Phenotypic changes can occur rapidly during domestication, even in the first generation bred in captivity (Podgorniak et al., 2022). Since DNA sequence mutations are a slow process, this raises the question of the involvement of epigenetic mechanisms in the development of these early domestic phenotypes (Ahmad et al., 2020; Podgorniak et al., 2022). While genetics is the study of hereditary changes in gene activity or function and due to mutation, deletion, insertion, or translocation alterations in the DNA sequence, in comparison, epigenetics is the study of hereditary changes in gene activity or function that are not associated with changes in the DNA sequence (Moore et al., 2013), that may lead to phenotypic variation between genetically identical individuals (Jablonka and Lamb, 2002; Moore et al., 2013). These changes can be passed on to daughter cells within the individual through cell divisions during mitosis but can also be passed on transgenerationally through meiosis (Jablonka and Raz, 2009), playing an important role in evolution (Fellous et al., 2017), although many epigenetic mutations (epimutations) are neutral or deleterious (Heard and Martienssen, 2014). The phenomenon of reprogramming can however prevent this transgenerational inheritance of epimutations. This reprogramming is necessary to remove epigenetic marks that have been acquired during development or by the effect of the environment so that the embryo can develop correctly. If reprogramming fails, the epigenetic marks will be retained and passed on from one generation to the next (Burggren, 2016; Fellous et al., 2017; Heard and Martienssen, 2014).

The three main epigenetic mechanisms that have been most studied, such as post-translational histone modification, non-coding RNA activity and DNA cytosine methylation, are involved in remodelling and maintaining the state of chromatin and will result in a change in expression activity of genes, by activating, inhibiting, or reducing these activities (Fellous et al., 2017). Methylation of cytosines is the most studied mechanism. It involves the addition of a methyl group (-CH3) to the C5 (carbon 5) position of the cytosines in the DNA sequence to form 5-methylcytosine (5-mC), catalysed by a group of enzymes, the DNMTs (DNA methyltransferases) (Fellous et al., 2017). The DNMT3a and DNMT3b enzymes are responsible for methylation, whereas the DNMT1 enzymes are responsible for maintaining methylation profiles after replication (Hubert, 2010). It is mainly the so-called CpG groups that are methylated in vertebrates and consist of a group with a cytosine directly followed by a guanosine. Methylation in regions highly concentrated in CpG (CpG islands) is a mechanism

that allows the regulation of gene expression generally in the form of repression when methylation is located at a promoter region (Fellous et al., 2017).

During domestication, epigenetic changes can be observed directly after the exposure of individuals to a breeding environment and could explain the rapid phenotypic variations observed within a few generations during domestication (Ahmad et al., 2020). Indeed, it has recently been shown that epigenetic mechanisms such as DNA methylation could strongly affect the phenotype of fish during domestication, showing differentially methylated CpG sites between wild individuals and their offspring under rearing conditions (Podgorniak et al., 2022). However, this field of study is still very poorly understood, requiring a deeper understanding of the involvement of epigenetic mechanisms in the domestication process.

As environment and genotype can influence DNA methylation, the genetic variability between individuals is an obstacle in the study of the role of epivariation in phenotypic heritability. To fully understand its evolutionary role, it is necessary to ignore the DNA sequence variability between individuals and therefore work with genetically identical individuals (Berbel-Filho et al., 2019; Heard and Martienssen, 2014).
Therefore, in this master thesis, we have focused on a model species of great interest for this characteristic, the mangrove rivulus, *Kryptolebias marmoratus*. As its name suggests, this fish of the family Rivulidae (Turko and Wright, 2015) occurs in mangrove forest waters throughout the tropical and subtropical Atlantic basin from Florida to southeastern Brazil (Avise and Tatarenkov, 2015; Taylor, 2012). It is one of the only known vertebrates, along with its close relative *K. hermaphroditus*, to be hermaphroditic and to reproduce by self-fertilisation (Avise and Tatarenkov, 2015), allowing highly homozygous offspring with an quasi-identical genome (Avise and Tatarenkov, 2015; Tatarenkov et al., 2010). *K. marmoratus* is therefore a very interesting model organism for this study. Being a self-fertilising hermaphrodite, the genetic variability of mangrove rivulus is very low within a single lineage. The effect of the environment on the phenotype can be assessed by ignoring the genetic effect, allowing to focus on other sources to explain the phenotypic variability (Earley et al., 2012). In addition, data regarding genomic sequence and epigenetic regulation through DNA methylation in mangrove rivulus are available (Berbel-Filho et al., 2020; Ellison et al., 2015; Fellous et al., 2017; Kelley et al., 2016; Rhee and Lee, 2014). Furthermore, the natural environment of the mangrove rivulus is a fluctuating environment with stochastic conditions. Their habitat has variable abiotic conditions such as intermittent tides and seasonal drying, poor water quality with

sometimes extreme temperatures, low oxygen concentration, high hydrogen sulphide levels and variable salinity. The mangrove rivulus must be able to adapt to these variable and sometimes difficult conditions. In addition, its food availability can also be variable due to lack of resources and periods of drought. When conditions become too extreme, the fish can emerge and adapt its physiology to live out of the water (Taylor, 2012; Turko et al., 2014). The natural environment may also present significant predation pressure and disease development (Podgorniak et al., 2019). In contrast, captive-bred individuals are faced with much more stable environmental conditions, especially in the laboratory where water temperature and salinity conditions are controlled. They are fed the same homogenous diet every day at the same time, ad libitium. Furthermore, they are not subject to any selection pressure and adapt to the daily human presence (Podgorniak et al., 2022). Thus, in this study, the objective is to investigate behavioural phenotypic variability between wild and domesticated adult mangrove rivulus fish and to focus on epigenetics as a potential source.

In a previous experiment conducted in July 2019 in Florida (27°31'N 82°38'W), wild fish from the Emerson Point Preserve (EPP) population were subjected to an open field behavioural test measuring boldness, activity, and anxiety and their DNA methylation was studied (Chapelle et al., unpublished data). Our aim is to study these parameters in fish of the same lineage, but which have been reared in the laboratory for several successive generations. We will investigate whether rearing fish in a captive environment impacts the behavioural traits of adult fish compared to wild individuals, and whether these phenotypic variations between wild and domesticated individuals are the result of epigenetic mechanisms such as DNA methylation that are transgenerationally heritable and could explain a domestication process.

2 | Material and methods

2.1 | Experimental fish and experimental design

The individuals used for this experiment are adult hermaphroditic mangrove rivulus (*Kryptolebias marmoratus*) of the Emerson Point Preserve (EPP) lineage from the F5 generation to compare with the F0 generation.

In July 2019, 42 wild mangrove rivulus from the EPP population were collected directly from their natural environment in Florida (GPS location: 27°31'N 82°38'W) and subjected to duplicate behavioural tests, two days apart. These individuals were then euthanised, photographed, weighed, measured and their brains, liver and gonads removed for genome

methylation studies. These individuals correspond to the F0 generation. Among these 42 wild individuals, individual EPP1078 provided 16 viable eggs that hatched on 3rd of August 2019, corresponding to the F1 generation. These fish were housed at the University of Namur (Belgium) (Annex 3 *more information on experimental fish and breeding*). From these 16 fish of the F1 generation, year after year, successive generations were obtained in the laboratory giving F2, F3 and F4 generations.

In this study, eggs were collected from the F4 hermaphroditic individuals to obtain an F5 generation. The objective is to compare this generation with the wild F0 individuals. The F4 individuals used to obtain a new F5 are 6 individuals (Annex 3) which all come from the same individual of F3 generation, being itself one of the descendants of the wild fish EPP1078. All F5 individuals therefore come from the same F0. The fish have therefore all the same genome (beside mutations). The number of individuals required to obtain significant results in the behavioural test was determined with Gpower software considering a statistical power of 80%, with an effect size of 0.63. A total of N=41 fish was obtained and is similar to the number obtained for the experiment in 2019 (N=42). For generation 5, a mortality of 30% was considered during the egg collection. A total of 60 eggs were therefore collected during five weeks. As the egg collection was limited to 6 individuals of generation 4, it was difficult to obtain the necessary number of eggs quickly. However, a higher mortality rate was observed, and only 28 individuals could be obtained for this F5.

To make a comparison with the data obtained for the wild F0 fish, the fish of the F5 generation are therefore also subjected to duplicate behavioural tests at two-day intervals in pre-designed arenas under controlled conditions of water temperature and salinity. The tests are filmed for a defined period and the videos are then analysed using the same software used for the wild fish. The fish are then also euthanised, photographed, weighed, measured, and dissected to recover the brain for molecular analysis. The results of the experiments on these fish will be compared with the results obtained for F0 individuals collected in their natural environment in Florida (Fig. 1).

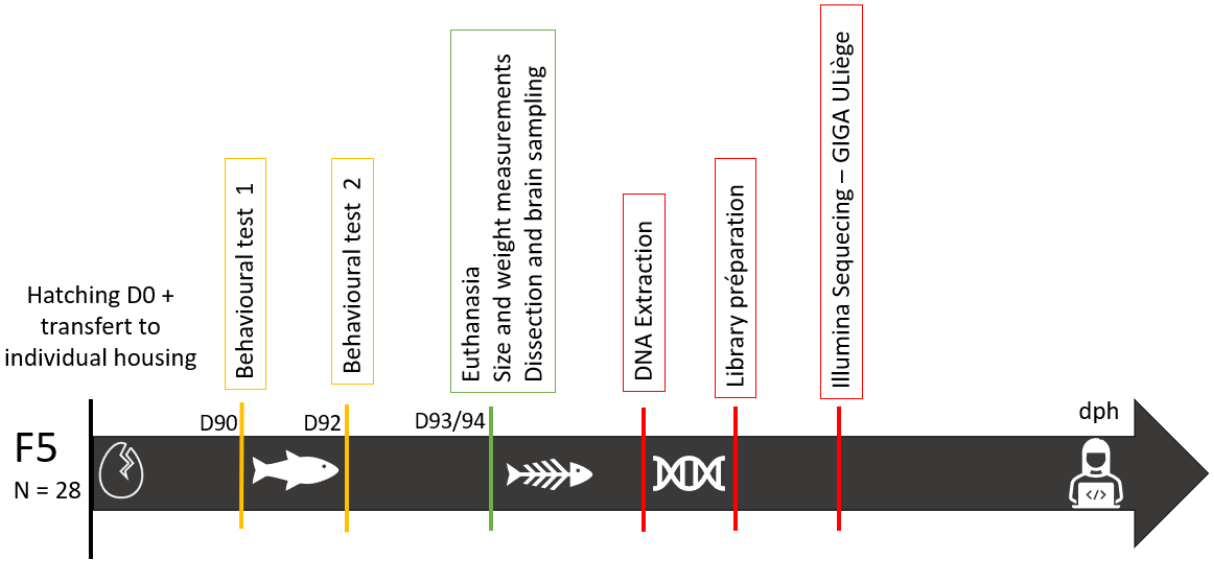


Fig. 1. Scheme of the experimental protocol for the study of the domestication and the impact of captivity conditions in the 5th generation of the mangrove rivulus (*Kryptolebias marmoratus*) in comparison with wild individuals. Similar experimental protocol for wild F0 fish (N = 42), carried out in 2019 by Valentine Chapelle. The age of the F0 fish is not known but sexual maturity is also reached.

2.2 | Behavioural analysis – the shelter test

Each fish of each generation (F0 and F5) was subjected to duplicate a behavioural test (separated by 2 days) to measure the boldness of the fish. Various tests exist to measure the boldness of an individual. The test that was chosen in this experiment is the "shelter test" or "emergence test". This test measures the propensity of the individual to leave the refuge to explore an unknown open field environment that may be potentially threatening (Burns, 2008). It allows to evaluate different parameters such as if the animal is bold and leaves the shelter voluntarily and quickly to navigate into the open field, if the animal takes risks by staying long in the open field, if the animal is anxious and tends to stay close to the walls, called thigmotaxis (Schnörr et al., 2012), or if the animal is active and moved a lot in the arena.

The F5 generation of fish passed the first replicate of the boldness test at 90 days post-hatching as this is the age when they become sexually mature. The exact age of the wild fish studied in Florida (F0 generation) is unknown. Because wild fish used in this study are of unknown age, the length of the fish was measured as a reference to compare wild (Table S1) and laboratory fish (Table S2).

The fish were all tested in the morning, before the daily feeding. None of the fish had previously undergone any behavioural tests before the first replicate

The shelter test consists of an arena containing a shelter, both separated by a sliding door (Fig. 2). It is important to note that the arenas used for behavioural tests of wild individuals were slightly different as they were rectangular.

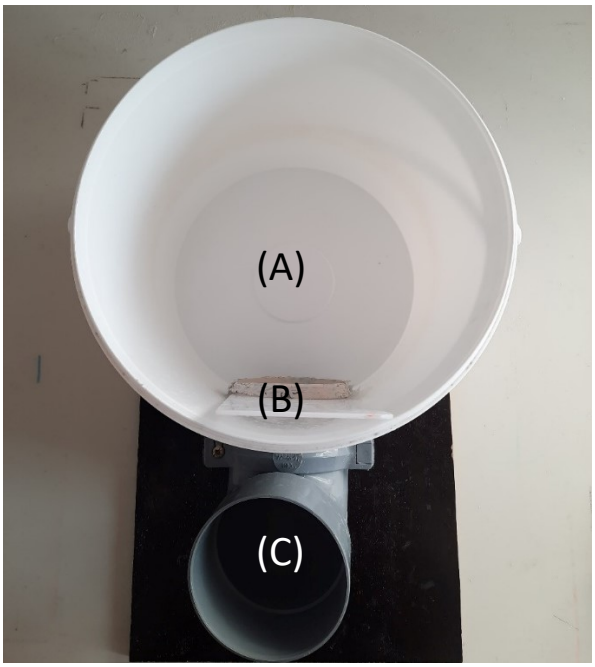


Fig. 2. Shelter test: a behavioural test arena measuring the boldness of *Kryptolebias marmoratus*. (A) Arena (open field) (B) Entrance door of the shelter (C) Shelter.

During the test in the arena, the fish were in new water with the same salinity and temperature conditions as in their original individual housing to avoid any additional stress (12 ppt salinity and temperature at 26.0 ± 1°C). As the fish may release hormones into the water due to the stress of moving and the new environment, the water was changed between each test before introducing a new fish.

The test fish were first introduced into the shelter covered with a dark grey lid for 15 min of acclimation and with the door to the open field closed. The aim is to minimise the stress of each fish to start the test. After the acclimation, the sliding door between the shelter and the open field was opened to give access to the whole arena. The behaviour of the fish during the test was filmed (SONY® HDR-CX625 camera) for 20 minutes. After the test, the fish were returned to their housing and the temperature-controlled room.

The recorded videos of each test were then analysed by video-tracking technologies (NoldusEthovision XT 13™). This software converts the video images into pixels and assigns X and Y coordinates for the center point of each fish during the 20 minutes of the video (Denoël et al., 2013), allows to obtain some parameters such as the total time in the open-field and in

the inner zone of the open-field, the total time in the shelter, the latency to first entry in open-field or the total distance moved in the arena.

The position and size of each arena was calibrated (Fig. 3A), and fish detection was determined by contrast with the background (Fig. 3B).

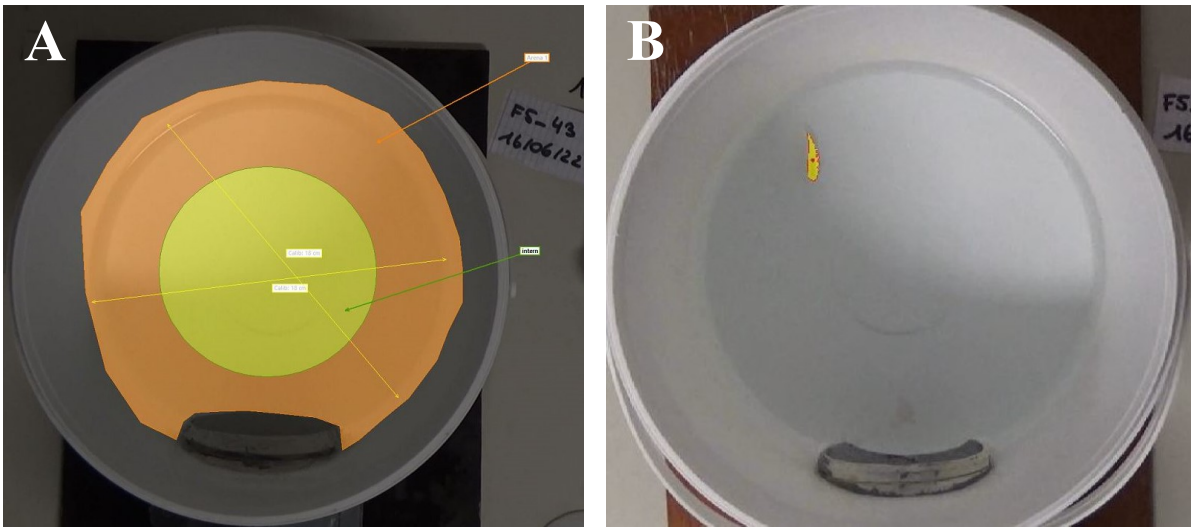


Fig. 3. Analysis of videos on Ethovision for the behavioural test. Calibration of the position and size of the drawn arena (A) and detection of the fish (B).

The analysis of the behavioural data obtained was carried out in RStudio (version R 4.1.1). The correlations between the measured variables were studied with a Spearman test (function rcorr, package Hmisc R). A principal component analysis (PCA) was performed on the measured variables of the behavioural test measuring boldness for the 42 fish of the F0 generation and for the 28 fish of the F5 generation (function dudi.pca, package ade4). The purpose of this analysis is to select the response variables to be studied. Linear mixed models (LMMs) were run (function lmer, package lme4) on the data of the boldness response variables of interest such as total time in the arena (TTA), latency to leave the shelter the first time and enter in the arena (Latarena), relative total time in the inner zone (IZ), and relative total distance moved (RTDM). The relative total time in the inner zone illustrates the total time spent in the inner zone divided by the total time in the arena to study the thigmotaxis of the fish independently of its boldness and the time it stayed. The relative total distance moved illustrates the total distance moved by the fish divided by the size and the total time spent in the arena to correct the distance moved with the differences in size between individuals, also independently of the boldness of the fish. For each variable studied, fish size, weight, generation, and test replicate are fixed-effect explanatory variables. The individual is a random effect explanatory variable.

To respect the assumptions of normality of the residuals and homoscedasticity of the variance, the variables Latarena, TTA and RTDM were transformed with a Yeo-Johnson transformation and the variable IZ with an OrderNorm transformation (bestNormalize package). To select the explanatory variables of the models studied, the top-down method was used to eliminate the variables that do not improve the model. This method consists of first considering a complex model with all possible explanatory variables and the interactions between these variables, and then eliminating the least significant variable from the model each time. The different models were compared, and the final model was selected based on the AIC values (Annex 4).

By performing two temporal replicates of the behavioural test for each individual, reproducible behavioural differences can be assessed, highlighting the individuality of the species. To do this, the repeatability present in our random effect (fish ID) was calculated for both generations and for each measured variable with 1000 bootstrap iterations (function rpt, package rptR). Repeatability is the ratio of the variability between individuals to the total variability within individuals, in this case between two replicates. If the fish are more different from each other than within the same fish between two replicates, this means that the fish express a personality.

2.3 | Euthanasia and brain collection

After the second replicate of the boldness test, fish were euthanised in 4°C water, weighed with a Mettler AE 200 precision balance and photographed with a size scale to measure their size on ImageJ (Annex 5). Fish were decapitated to ensure euthanasia. The brain of each fish was removed in its entirety during a binocular dissection. An incision of the skin and muscles was made on both sides of the cranium and a third incision was made between the two eyes to connect the first two incisions. The skull plate could then be lifted to visualise the brain to be removed (Fig. 4). The brain samples were placed directly in liquid nitrogen and stored at -80°C until DNA extraction.

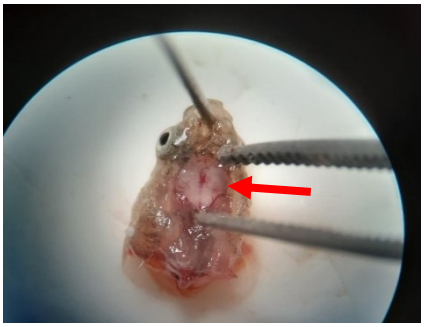


Fig. 4. Dissection and removal of the brain from the mangrove rivulus *Kryptolebias marmoratus*

2.4 | Reduced representation bisulphite sequencing (RRBS)

Genomic DNA from the brains of 27 F5 fish and 40 F0 fish was extracted using the NucleoSpin ® Tissue XS kit (Macherey-Nagel, Germany). This kit allows the rapid purification of genomic DNA from very small tissue samples. DNA quality and concentration (ng/µl) from each sample was determined using the NanoDrop™ 2000c spectrophotometer (Thermofisher Scientific) (Annex 6). The integrity of the extracted DNA for each fish was also assessed by 1% agarose gel migration (Annex 7). All molecular analysis of F0 fish was carried out in 2019 by Valentine Chapelle.

The next step in the experiment was to prepare the reduced representation bisulphite sequencing (RRBS) libraries for Illumina ® Platforms. The preparation of the library was performed for each brain sample from 50 to 100 ng of purified DNA using the Premium RRBS Kit V2 Cat. No. C02030036 (Diagenode, Belgium). The analysis for F0 fish was performed with the previous version of the protocol (Cat No. C02030033). The genomic DNA was digested with the MspI restriction enzyme at 37°C. This enzyme cuts the genome at the target sites (5' -CCGG- 3') between the two cytosines. The library was prepared by adding specific adapters, followed by size selection on the RRBS libraries using Agencourt AMPure XP beads to select fragments between approximately 40 and 220 bp in size. A bisulfite conversion converts non-methylated cytosines to uracil and retains the methylated cytosines. This conversion is then followed by amplification.

Libraries from the F5 samples were then sequenced on an Illumina NovaSeq S4 V1.5 300 cycles XP workflow-1 lane platform at the GIGA Institute in Liege.

Before performing the DNA methylation analysis, the sequencing data obtained must be processed. This step was again performed previously by Valentine Chapelle for the wild individuals and the same process was followed for the laboratory individuals. The quality of the sequencing data was assessed using FastQC (Andrews S., 2010), the adapters added during the preparation of the libraries were cut using cutadapt (Martin, 2011) and both included in the TrimGalore wrapper and run with the specific parameters for RRBS analysis --rrbs-- and --non_directional--. The different reads are aligned using Bismark (Krueger and Andrews, 2011) based on the latest version of the *Kryptolebias marmoratus* genome (Source NCBI: GCF_001649575.2) with the parameters --score_min L,0,-0.6. Two samples from F5 individuals were deleted due to lower quality. Two samples of the F0 individuals had also been deleted by Valentine Chapelle.

Further analysis is performed on R using the methylKit package (v.1.20.0) (Akalin et al., 2012) to highlight differences in methylation between the two groups studied. The bimodal distribution of methylation values was checked. The samples were then filtered using the filterByCoverage command to discard bases with very high read coverage, which could be due to PCR bias, and bases with low read coverage. Bases with a read coverage of less than 10x and bases with more than 99.9th percentile coverage in each sample were removed.

The analysis was first performed at the level of cytosine bases. The CpG sites found in all individuals of both groups were selected to compare the level of DNA methylation for each group, as well as the level of variation within each group. Clustering was used to study the grouping of samples based on their similarities in methylation, as well as principal component analysis. Significantly differentially methylated cytosines (DMCs) can be demonstrated, with a q-value less than or equal to 0.01 and a minimum difference in methylation percentage of 10% between each group.
Subsequently, a selection of 300 bp genomic regions was performed, with a jump every 300 bp and a read coverage of at least 10x, to study methylation at the region level. Significantly differentially methylated regions (DMRs) were identified, with a q-value less than or equal to 0.01 and a minimum difference in methylation percentage between the two generations of 10%.

3 | Results

3.1 | Morphological traits

As the age of wild fish was not known, size and weight were measured after the second test replicate as reference variables. The size and weight of the domesticated fish were also measured after the second test replicate, at the age of 92 days.
Differences in height (cm) and weight (g), and thus also BMI, were observed between the two groups (Fig. 5) with a mean of 3,001 ± 0,362 cm and 0,428 ± 0,150 g for F0 fish; 2,748 ± 0,095 cm and 0,242 ± 0,025 g for F5 fish (mean ± SD). The BMI of wild individuals is also higher (0.045 ± 0.004) compared to captive individuals (0.032 ± 0.002). These values may indicate that the wild fish were also mature and possibly older than the F5 fish, especially as the domestic environment tends to increase the growth of individuals.

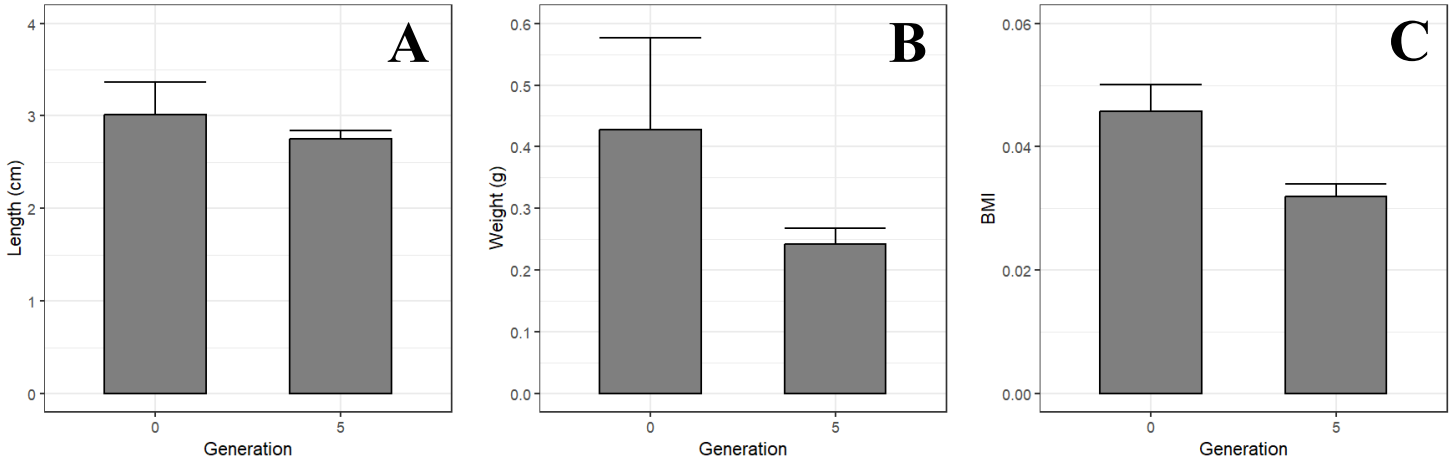


Fig. 5. Average size (cm) (A), weight (g) (B) and BMI (C) of 42 F0 and 28 F5 individuals measured after the second replication of the boldness test. Bar graphs representing the mean ± SD.

3.2 | Behavioural test of boldness

A principal component analysis (PCA) carried out on the explanatory variables measured during the two replicates of the behavioural test of boldness for the 42 individuals of the F0 generation and for the 28 individuals of the F5 generation revealed the main axes which explain a total of 77.82% of the behavioural variation (cumulative projected inertia) with 58.54% for axis 1 (horizontal) and 19.28% for axis 2 (vertical) (Fig. 6A). This type of analysis makes it possible to visualise the position of each variable on the two main axes.

Axis 1 represents most of the boldness variables along a shy-bold continuum. Axis 1 indicates that the total time in the arena (TTA) is negatively correlated to the variables of latency to enter the arena (Latarena) and the inner zone (Latint), which are themselves correlated to each other, but is correlated to the activity obtained by the relative total distance travelled in the arena (RTDM), even if it is not well represented. Axis 2 explains the variable relative total time in the internal zone (IZ) which represents the thigmotaxis and the anxious character of the fish. The correlation coefficients and associated p-values of the different variables showed strong positive correlations between the explanatory variables of weight and length (Table S3 and S4). Length was therefore selected for further analysis.

The PCA also allows us to visualise the position of individuals in relation to the variables during the two behavioural test replicas. The individuals on the right-hand side of the graph are those who stay the longest in the arena, and therefore the shortest in the shelter, who have a high level of activity in the arena, and whose latency to enter the arena and the inner zone is low. On the right-hand side of the graph are the individuals who are more daring in this test and on the left-hand side are the less daring. Axis 1 represents a continuum of bold-shy behaviour. Individuals

at the bottom of the graph are proportionally more present in the inner zone of the arena, regardless of boldness. Individuals from F5 generation show more variability than individuals from F0 generation.

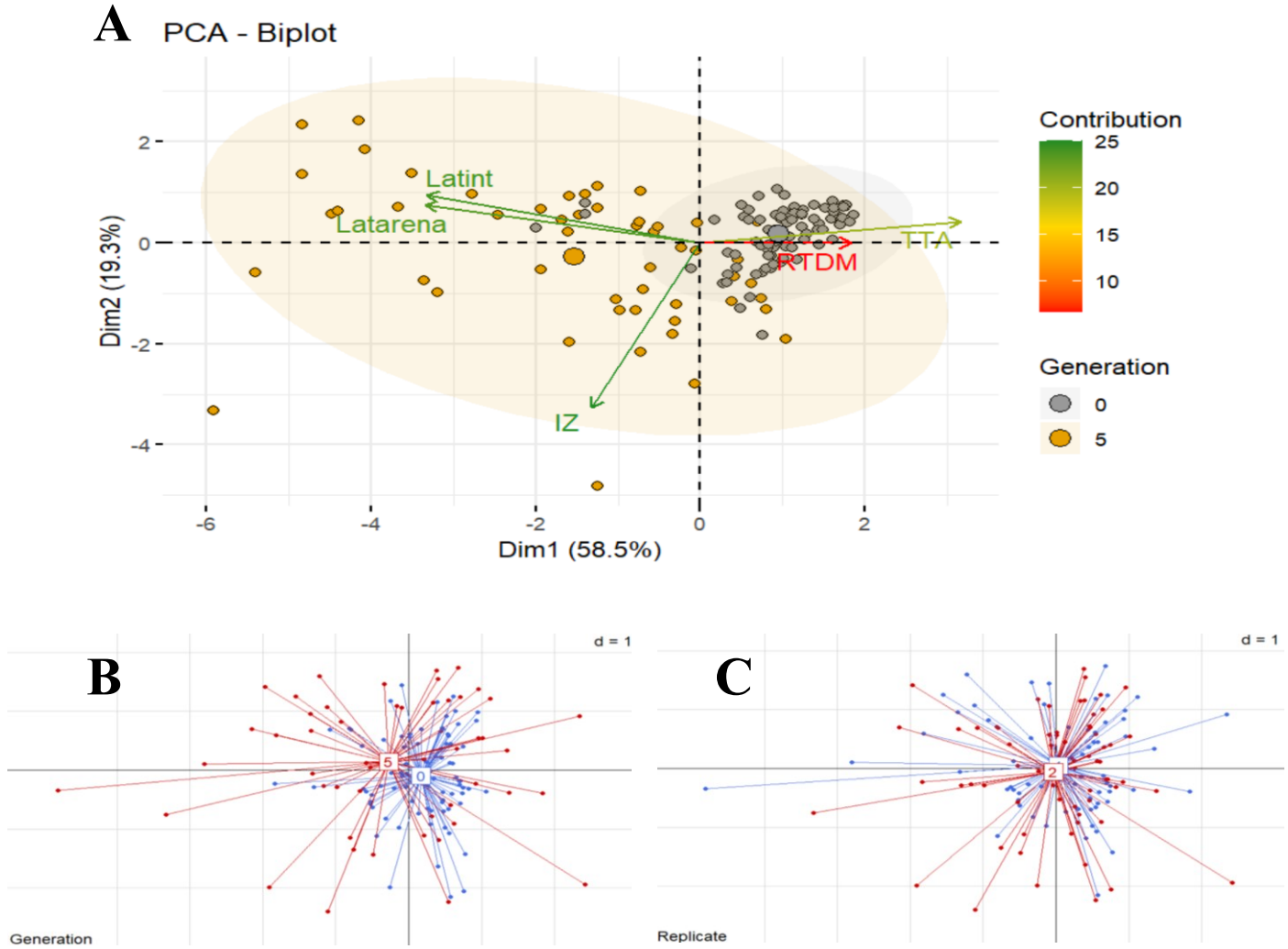


Fig. 6. Principal Component Analysis (PCA) on the behavioural data of the shelter test performed on the 42 individuals of generation 0 and for the 28 individuals of generation 5 of the mangrove rivulus, *Kryptolebias marmoratus* (A). A schematic representation of each individual for the two replicates of the behavioural tests and for the two generations shows a greater effect of generation (B) on the behavioural variables than the effect of replicate (C).

Linear mixed models (LMMs) showed highly significant effects of generation on fish boldness traits such as latency to enter the open field of the arena for the first time (Table 1), the total time in the arena (Table 2), and the relative total time in inner zone (Table 3), but no significant effect of replicate. This result also appears on the schematic representation of the PCA, there is a variation of the boldness traits of the fish according to the generation (Fig. 6B) in contrast to

the replicate where the variability is low (Fig. 6C). Generation also has a highly significant effect on the relative total distance moved, as well as the size and interaction between generation and replicate (Table 4).

Between F0 and F5, an increase of 375.85 seconds in latency to leave the shelter is observed in replicate 1 and an increase of 366.41 seconds in replicate 2 (Fig. 7A), as well as a decrease of 566.89s in total time in the arena in replicate 1 and 565.81s in replicate 2 (Fig. 7B). The activity of the fish is also impacted with a decrease of 0.43 of the relative total distance moved in replicate 1 and 0,14 in the second replicate (Fig. 8A). It also appears that smaller fish are significantly more active (Fig. 8B). Independent of the time spent by the fish in the arena, the relative total time in the inner zone increases by 0.08 for R1 and 0.06 for R2 between F0 and F5 (Fig. 9).

Table 1 Results of the linear mixed model of the variable latency to first entry in the arena.

	Latency to first entry in arena (Latarena)		
Fixed effects	*F*	*Dfn, Dfd*	*p*
Generation	35.77	1,70	**8.47e-08 *****

Linear mixed model was applied on the variable latency to first entry in arena with Generation as fixed factors and individuals as random factors (Latarena ~ Generation + (1|ID)).

Table 2 Results of the linear mixed model of the variable total time in arena.

	Total time in arena (TTA)		
Fixed effects	*F*	*Dfn, Dfd*	*p*
Generation	136.7064	1,68	**<2e-16 *****
Replicate	3.3458	1,69	0.0717

Linear mixed model was applied on the variable total time in arena with Generation and Replicate as fixed factors and individuals as random factors (TTA ~ Generation + Replicate + (1|ID)).

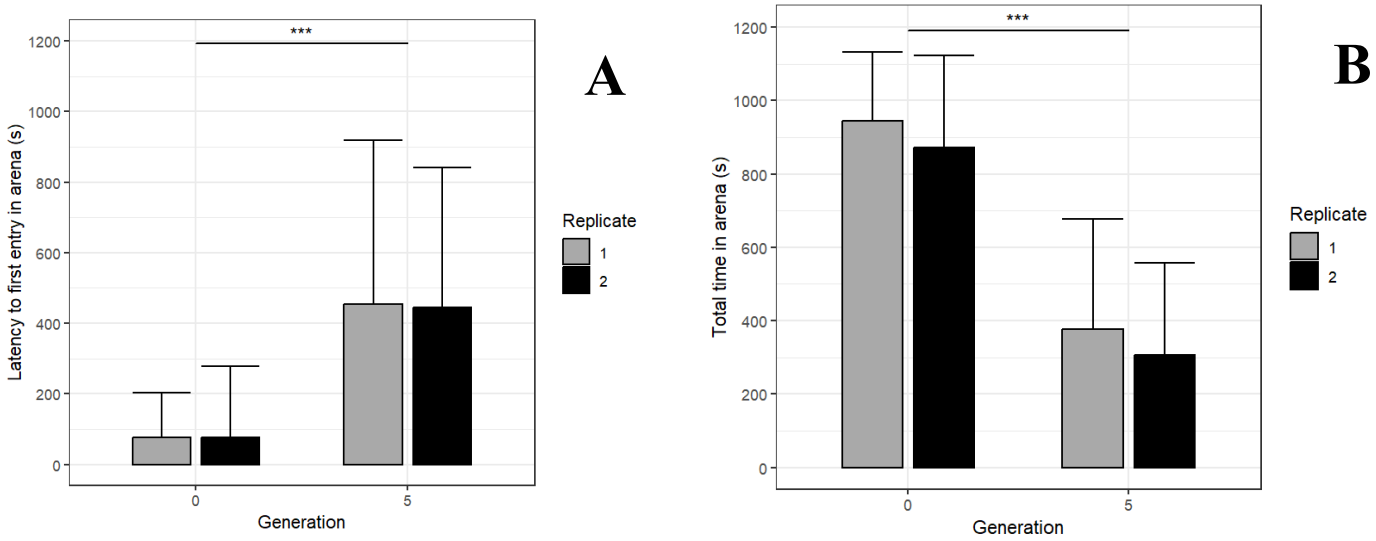


Fig.7. Results of the two replicates of the behavioural test measuring the boldness variables of 42 individuals of the F0 generation and 28 individuals of the F5 generation of *Kryptolebias marmoratus*. The variables measuring fish boldness are the latency to first leave the shelter and enter the arena (A) and the total time the fish spends in the entire open arena (B). Bar graphs representing the mean ± SD. * p<0.05; ** p<0.01; *** p<0,001.

Table 3 Results of the linear mixed model of the variable relative total distance moved.

Fixed effects	Relative total distance moved (RTDM)		
	F	*Dfn, Dfd*	*p*
Generation	47.7462	1, 65.925	**2.354e-09** ***
Replicate	2.4885	1, 65.954	0.1194629
Length	13.7355	1, 64.318	**0.0004400** ***
Generation:Replicate	14.5011	1, 65.943	**0.0003098** ***

Linear mixed model was applied on the variable relative total distance moved with Generation, Replicate, length as fixed factors and interacting, and individuals as random factors (RTDM ~ Generation + Replicate + Length + Generation:Replicate + (1|ID)).

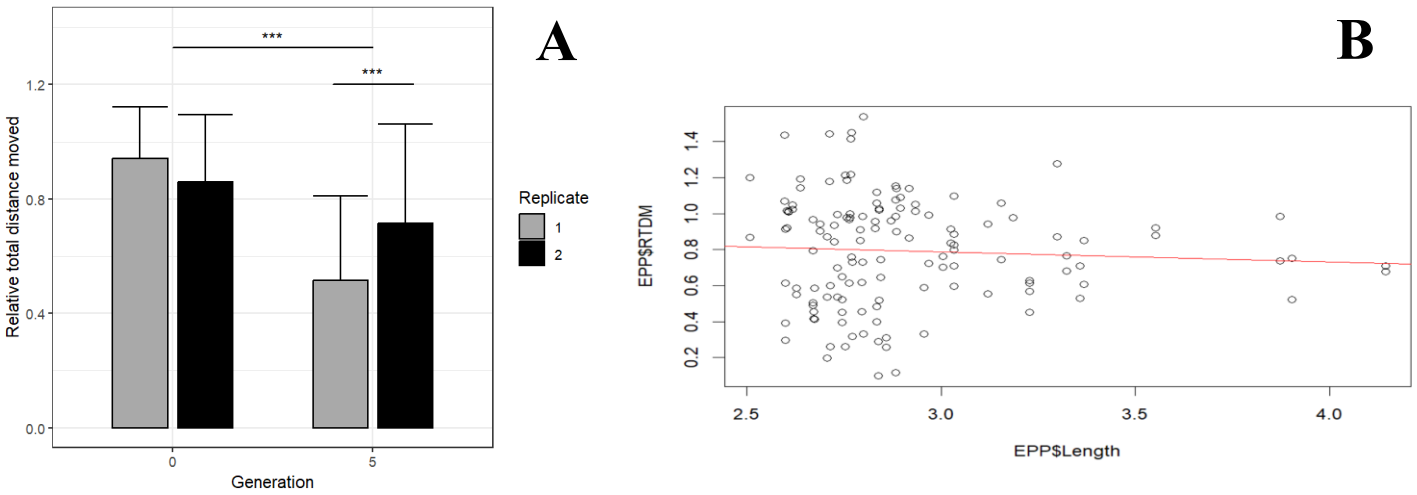


Fig.8. Results of two replicates of the activity of 42 individuals of the F0 generation and 28 individuals of the F5 generation of *Kryptolebias marmoratus* during the behavioural test. Activity is measured by the relative total moved distance (A). Bar graphs representing the mean ± SD. * p<0.05; ** p<0.01; *** p<0,001. Demonstration of the effect of size on the activity of individuals (B).

Table 4 Results of the linear mixed model of the variable relative total time in inner zone.

	Relative total time in inner zone (IZ)		
Fixed effects	*F*	*Dfn, Dfd*	*p*
Generation	13.946	1, 68.097	**0.0003865** ***

Linear mixed model was applied on the variable relative total time in inner zone with Generation as fixed factors and individuals as random factors (IZ ~ Generation + (1|ID)).

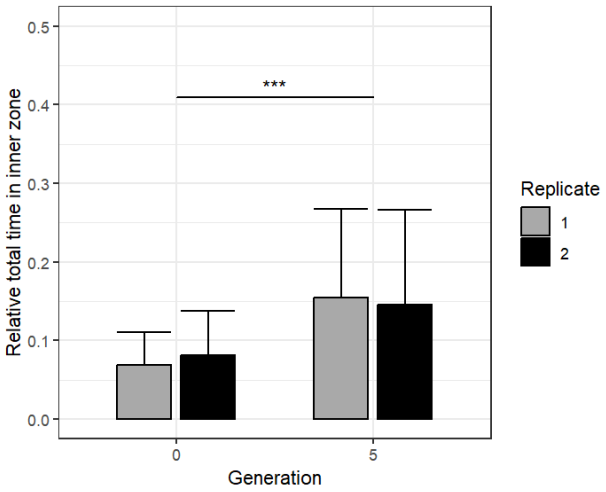


Fig.9. Results of the study of thigmotaxis and the fish anxiety trait independently of the boldness trait during the two repetitions of the behavioural test for the 42 individuals of the F0 generation and 28 individuals of the F5 generation of *Kryptolebias marmoratus*. Bar graphs representing the mean ± SD. * p<0.05; ** p<0.01; *** p<0,001.

Significant repeatability between individuals of the F0 generation was found for the variables total time in the arena ([R] = 0.428; p = 0.00179), relative total distance moved ([R] = 0.472; p = 0.000778), and relative distance in inner zone ([R] = 0.45; p = 0,00132). In comparison, individuals from the F5 generation showed little variability between them for the same variables ([R] = 0,08; p = 0,336 for TTA, [R] = 0,177; p = 0,216 for RTDM, and [R] = 0,136; p = 0,275 for IZ) (Table 5). There are therefore inter-individual behavioural differences that are repeatable over time in the F0 generation but not in the F5 generation.

Table 5 Conditional repeatability values for behavioural variables studied in the two generations of mangrove rivulus

		F0		**F5**	
Variables	**Models**	**R**	**P**	**R**	**P**
Latarena	Latarena ~ (1|ID), grname="ID"	R = 0.217	p = 0.0896	R = 0.244	p = 0.112
TTA	TTA ~ Replicate + (1|ID), grname=c("ID", "Fixed")	R = 0.428	p = 0.00179	R = 0.08	p = 0.336
RTDM	RTDM ~ Replicate + Length + (1|ID), grname=c("ID", "Fixed")	R = 0.472	p = 0.000778	R = 0.177	p = 0.216
IZ	IZ ~ (1|ID), grname="ID"	R = 0.45	p = 0.00132	R = 0.136	p = 0.275

The detailed results of the two replicates of the behavioural test measuring boldness for the F0 (Table S1) and F5 individuals (Table S2) are available in the supplementary data.

3.3 | Reduced representation bisulphite sequencing (RRBS)

A Reduced Representative Bisulfite Sequencing (RRBS) of methylomes from brain tissues of the two studied generations was performed to investigate methylation differences between the natural and laboratory populations of mangrove rivulus.

On average, 11.7 (± 4.46) Million reads were generated with RRBS for brain methylomes for both groups (13.81 ± 3.71 for F0 and 7.69 ± 2.66 for F5). This corresponds to an average number of 257.30 million cytosine analyses, 226 million for F0 and 315 million for F5.

The analysis for both groups together shows that on average 20% of the cytosines in the mangrove rivulus genome are found in a CpG context, whereas 80% of the cytosines are found

in a non-CpG context (25% in a CHG context and 55% for CHH) (Fig. 10). Similar results were obtained by doing the analysis separately for each group. However, of all these cytosines analysed, the average proportion of methylated cytosines in a CpG context in the brain samples was 66.94% (67.26% for F0 individuals and 66.33% for F5) and the proportion of non-methylated cytosines in a non-CpG context (CHG and CHH) in the brain samples was 1.43% (1.43% for F0 and 1.19% for F5) (Fig. 11). Cytosine methylation of the mangrove rivulus genome is therefore mainly at CpG sites.

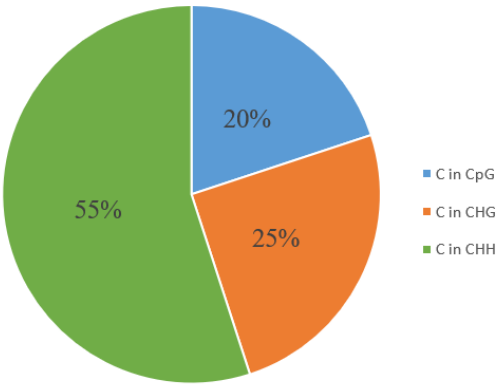


Fig. 10. Cytosine distribution in the mangrove rivulus genome (n=61) in CpG and non-CpG contexts (H= all bases except one G (A, C or T)).

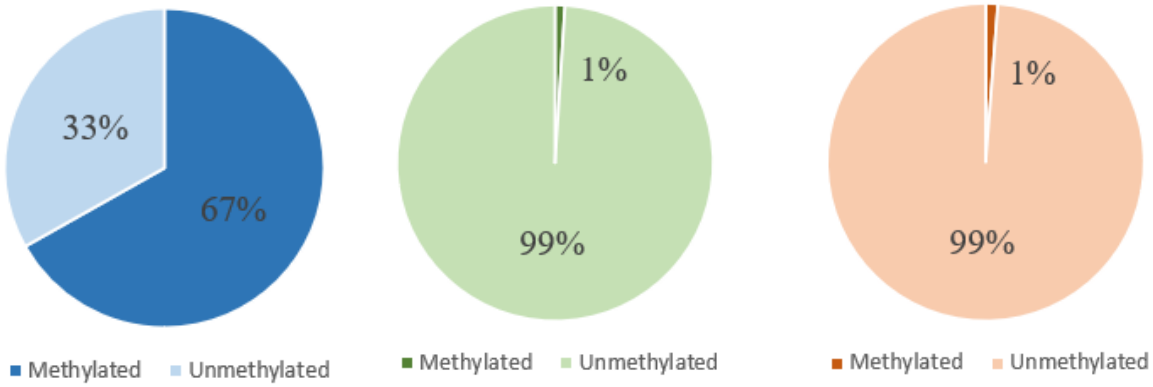


Fig. 11. Percentage of cytosine methylation in the different contexts CpG (blue), CHH (green) and CHG (orange).

The analysis of cytosine methylation variations for the brain samples of the two studied generations revealed a total of 25717 CpGs shared in all individuals of both groups (n=61), with a coverage of 10x.

Based on these 25717 CpGs, a mean coefficient of variability (CV) within each generation was calculated giving a variability of 47.86% for wild individuals (F0) and 65.51% for captive individuals (F5).

Hierarchical clustering aims to compare individuals of the two generations according to their methylation profile and has allowed to show a clear distinct grouping of individuals by generation (Fig. 12).

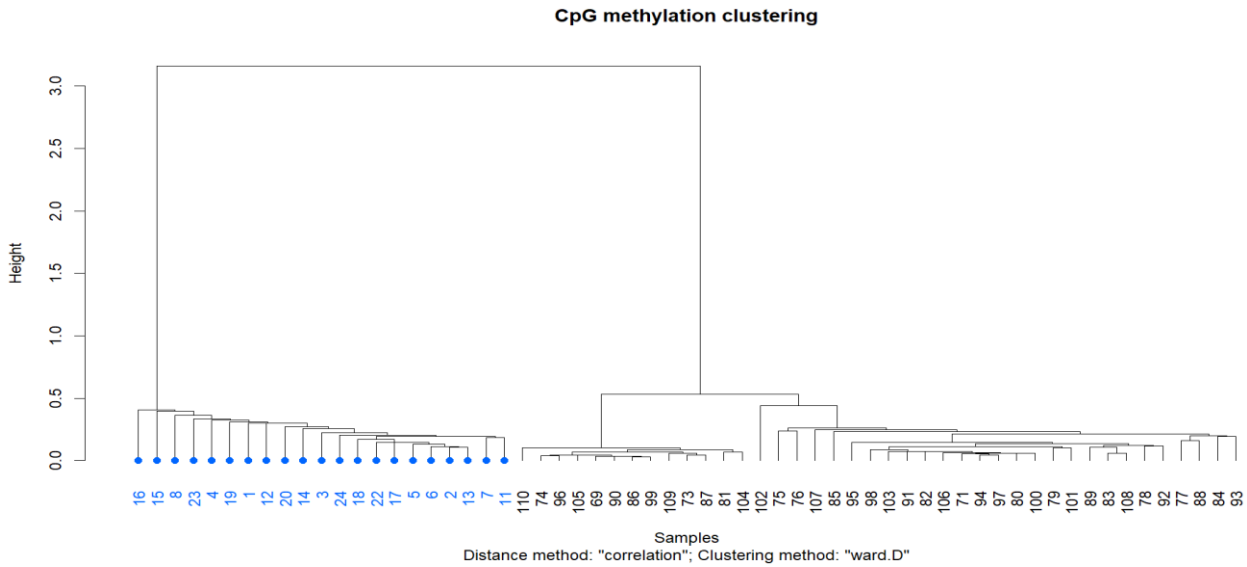


Fig. 12. Hierarchical clustering of CpG methylation in mangrove rivulus brains between the wild and laboratory generations, based on Ward's hierarchical clustering with Pearson correlation distances. Individuals from 69 to 110 (black) are individuals from the F0 generation and individuals from 1 to 24 (blue) are individuals from the F5 generation.

A principal component analysis can also be used to identify the distribution of samples along the PC1 and PC2 axes. As with the clustering dendogram, similar samples from the same generation and environment are close together on the scatterplot. The PC1 axis shows a distinct clustering of wild and laboratory individuals (Fig. 13).

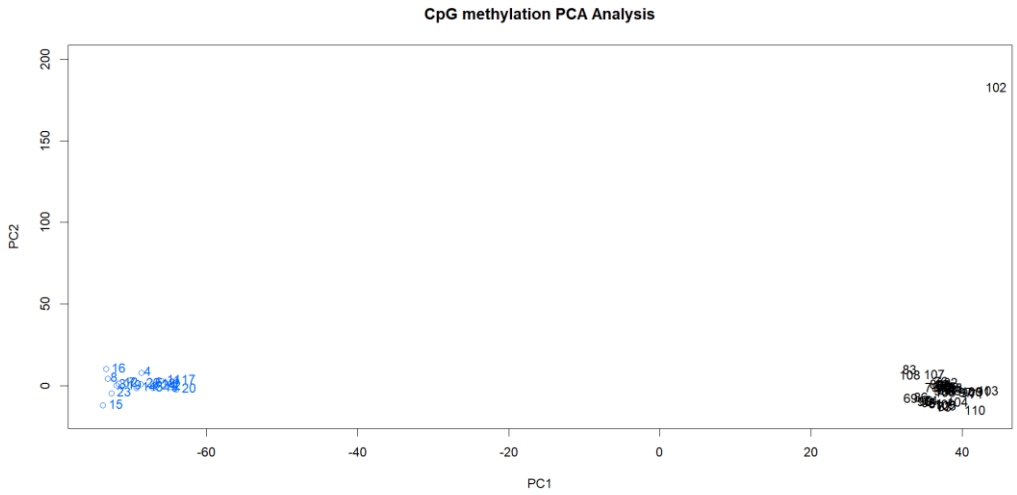
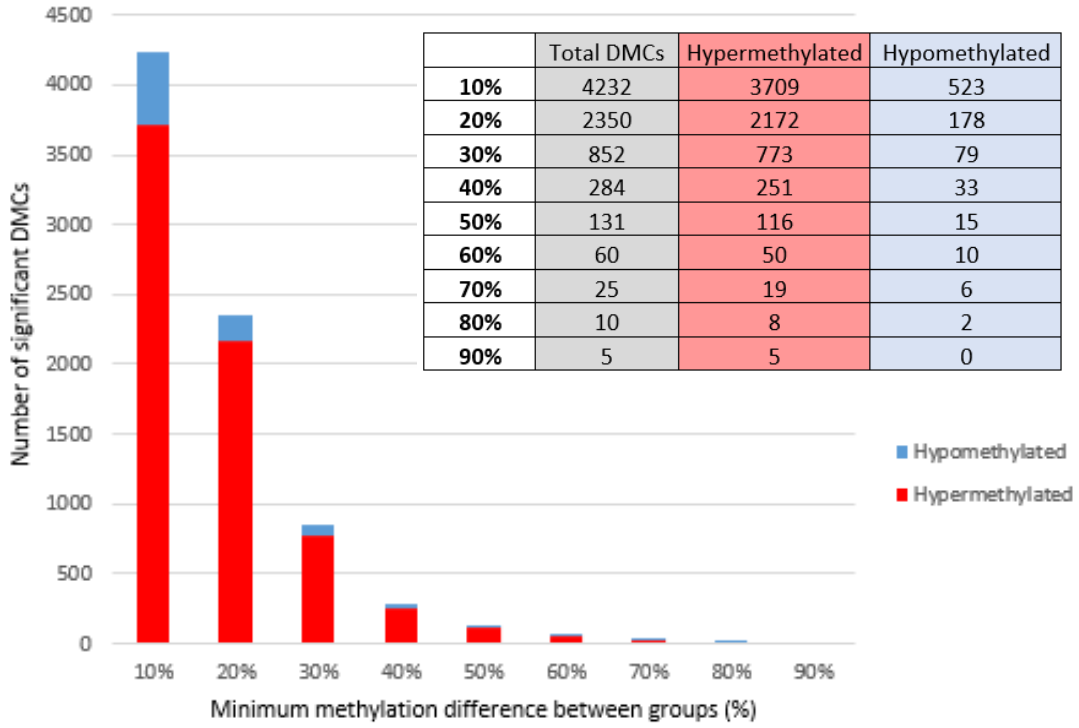


Fig. 13. Principal component analyses showing the distribution of PC1 and PC2 dimensions of mangrove rivulus individuals from the F0 (black) and F5 (blue) generations according to the methylation of brain CpGs.

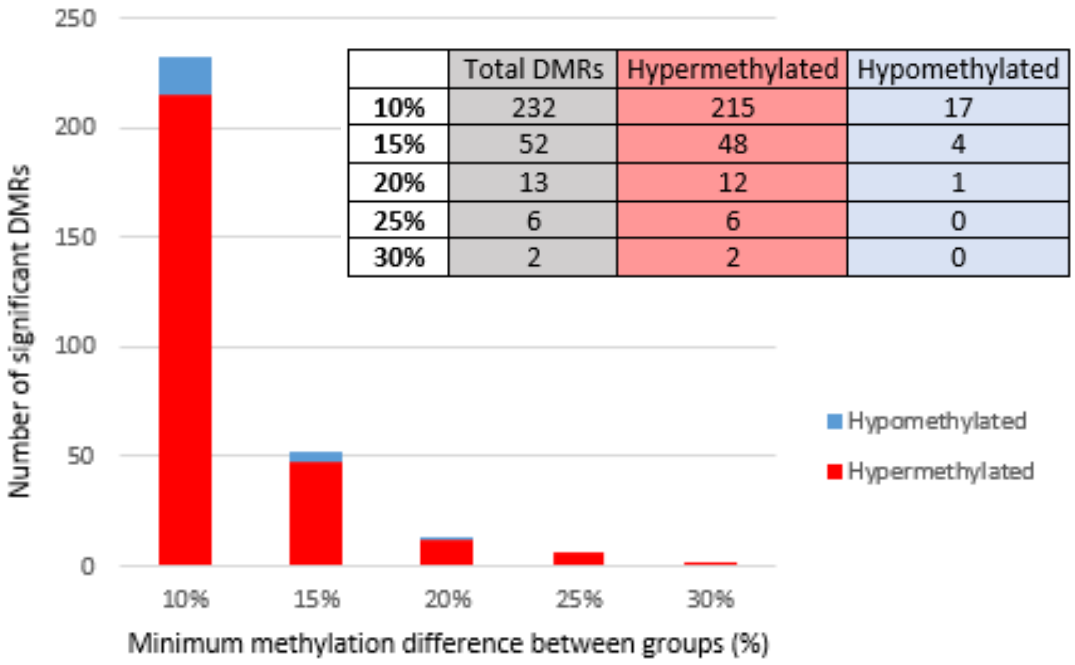
Significantly differentially methylated cytosines (DMCs) are identified between the two generations with a q-value less than or equal to 0.01. A difference of at least 10% between the two groups studied gives a number of 4232 differentially methylated cytosines between the two generations. The calculation of the methylation difference is done by doing Group 2 (F0) - Group 1 (F5). Of the 4232 DMRs, 3709 are hypermethylated (in F0 compared to F5) and 523 are hypomethylated (Fig. 14).



	Total DMCs	Hypermethylated	Hypomethylated
10%	4232	3709	523
20%	2350	2172	178
30%	852	773	79
40%	284	251	33
50%	131	116	15
60%	60	50	10
70%	25	19	6
80%	10	8	2
90%	5	5	0

Fig. 14. Number of significantly differentially methylated cytosines (DMCs), with specification of hyper and hypomethylated cytosines, between the F0 generation and the F5 generation, as a function of the minimum percentage of methylation difference selected (%).

The comparison of methylation profiles between the groups studied can be done at the level of cytosine bases but also at the level of regions. For this purpose, regions of 300 bp were selected and a total of 5799 methylated regions were identified which are shared by both generations (n=61). Based on these regions, the average coefficient of variability (CV) within each generation was again calculated and resulted in a variability of 17.80% for the F0 generation and 43.65% for the F5 generation. Considering a minimum difference of 10% between the two generations, 234 regions with significant methylation differences (DMRs) are obtained, of which 216 are hypermethylated regions in F0 compared to F5 and 18 are hypomethylated (Fig. 15). The calculation of the methylation difference is performed again by doing Group 2 (F0) - Group 1 (F5). The maximum methylation difference observed is 33.89% in the region chr NC_051448.1; start 15542401 - end 15542700.



	Total DMRs	Hypermethylated	Hypomethylated
10%	232	215	17
15%	52	48	4
20%	13	12	1
25%	6	6	0
30%	2	2	0

Fig. 15. Number of significantly differentially methylated regions (DMRs), hyper and hypomethylated, between the F0 generation and the F5 generation, according to the minimum percentage of methylation difference chosen (%).

4 | Discussion

During domestication, when animals are transferred from their natural environment to a captive environment, the individuals adapt to this captivity and to the presence of humans, resulting in different phenotypic changes in the species from the original wild species (Ahmad et al., 2020; Lord et al., 2020; Wilkins et al., 2014). The emergence of studies on the effects of domestication in fish has revealed the presence of phenotypic variation between wild and domesticated individuals (Brignon et al., 2018; Drew et al., 2012; Milla et al., 2020; Millot et al., 2009; Moretz et al., 2007; Podgorniak et al., 2022; Teletchea and Fontaine, 2014). This raises questions about the underlying mechanisms associated with these variations. The major interest of our study is to determine the involvement of epigenetic modifications in these changes during domestication. As environment and genotype can influence DNA methylation, studying the influence of this epigenetic mechanism is difficult in populations with genetic variation (Berbel-Filho et al., 2019). Therefore, working with genetically identical organisms offers an opportunity to study the role of DNA methylation independent of genomic variation (Heard and Martienssen, 2014). It has been shown that the wild individuals (F0) of the Emerson Point Preserve population had very low genetic diversity compared to other mangrove rivulus populations studied (Chapelle et al., unpublished data). The F5 individuals used in the present research are all derived from the same F0 fish of this isogenic lineage, also allowing this low genetic variability to be maintained even between the two studied generations. Therefore, this study aims to determine the effect of captivity on the behavioural responses of the mangrove rivulus, *Kryptolebias marmoratus*, and to assess whether these differences are due to epigenetic variability.

The results of our study identify behavioural phenotypic differences between the F0 and F5 generations of mangrove rivulus. Wild fish are on average 1.5 times more active than captive fish. In a same way, Berbel-Filho et al. have shown that rearing mangrove rivulus in a poor environment decreases the activity of the fish but will make them less neophobic compared to an enriched environment (Berbel-Filho et al., 2020). In our case, wild individuals are more active and bolder but, regardless of the time they spent in the arena and thus their boldness, they seem to be more anxious. F5 individuals tend to move on average twice as much in the central part of the arena compared to wild individuals. Even if fish reared in a captive environment are less active at the beginning, their activity increases with replicate, a phenomenon of habituation seems to make them more and more active. A study on the behaviour of *Dicentrarchus labrax*

also showed effects of domestication on the behaviour of selected fish compared to the wild strain, with a lower risk-taking behaviour in domesticated fish but in the same way as in our study, domesticated fish showed a gradual habituation to environmental changes unlike wild fish which showed more variable responses over time (Millot et al., 2009). This habituation could be explained by the fact that F5 individuals are less anxious. Smaller individuals tend to be also more active. However, as the age is only fixed for F5 individuals and unknown for F0 individuals, a confounded age effect could be present for wild individuals. It would be interesting to repeat the experiment with different generations but with known age to confirm this size effect.

Our study also shows that wild individuals tend to be more bold than captive individuals. They voluntarily leave the shelter on average almost 6 times faster and spend on average more than 2.5 time longer in the arena than F5 individuals. We hypothesise that these behavioural variations can be explained by differences in the complexity of the environment between the two studied generations. Indeed, studies show that the level of complexity of an individual's environment, and therefore the level of stress associated with it, influences the behaviour of species (Brantsæter et al., 2016; Brignon et al., 2018; Pasquet, 2019; Pértille et al., 2017). The stress level in a complex wilderness environment is lower and episodic, making individuals less fearful, bolder, and more exploratory. The stress level in a low-complexity confined environment is more sustained and longer (Brantsæter et al., 2016; Pértille et al., 2017). A study on Bull trout (*Salvelinus confluentus*) showed a higher level of boldness in wild fish and fish reared in captivity but in a complex habitat, compared to fish reared in a conventional captive environment, suggesting that rearing in a complex captive environment may result in wilder phenotypes compared to conventional rearing (Brignon et al., 2018). In addition, a study in cod (*Gadus morhua*) also showed that fish reared in a heterogeneous environment exhibit a more daring, curious behaviour. In a changing environment, an individual with a more daring and exploratory behaviour will be better able to cope (Braithwaite and Salvanes, 2005). In these changing conditions, as in the wild, individuals face predation, variations in food availability and variable abiotic conditions, causing species to leave their environment and settle elsewhere during their lifetime (Braithwaite and Salvanes, 2005; Conrad et al., 2011). The natural environment in which the mangrove rivulus lives is heterogeneous and fluctuating (Podgorniak et al., 2019; Taylor, 2012; Turko et al., 2014), this could explain the more adventurous, bold and exploratory tendency of wild individuals as opposed to those reared under controlled and stable conditions.

It has also been shown that environmental heterogeneity favours phenotypic diversity, as is the case for *Kryptolebias marmoratus* (Earley et al., 2012). Therefore, the heterogeneous, fluctuating and stochastic natural environment in which *Kryptolebias marmoratus* evolves could maintain the variations within the populations (Edenbrow and Croft, 2011). Conversely, breeding under stable and controlled conditions could erase these variations within populations. Indeed, in our study, the repeatability analysis shows greater variability between individuals for wild fish than for laboratory-reared fish for the behavioural variables total time in the arena, relative total distance moved and relative total time in inner zone. Fish that have lived in the wild have more individuality in terms of their boldness, activity, and anxiety. Inversely, fish bred in captivity will lose this individuality and all adopt a similar behaviour. An interesting prospect would therefore be to test the effect of the complexity of the captive environment on phenotypic variability by breeding "domesticated" individuals in a more heterogeneous environment, close to the natural environment. It would also be interesting to study the behaviour of captive individuals of the F1 generation to assess the immediate effect of captivity. It is important to distinguish whether the behavioural modifications observed are due to a phenomenon of reversible acclimatization of individuals to their changing environment or whether it is the result of an adaptive phenomenon.

DNA methylation is a mechanism that can generate phenotypic differences following environmental heterogeneity (Flores et al., 2013). It is therefore of interest to gain further insight into this epigenetic mechanism during species domestication. Methylation patterns during the early stages of domestication may explain the appearance of traits described in the domestication syndrome (Anastasiadi and Piferrer, 2019). A recent study on Nile tilapia (*Oreochromis niloticus*), demonstrated that epigenetic mechanisms such as DNA methylation could strongly affect the phenotype of fish during domestication, showing differentially methylated CpG sites between wild individuals and their offspring under rearing conditions (Podgorniak et al., 2022).

In our study, methylome variations could also be identified between F0 and F5 individuals at CpG sites, with a distinct clustering according to generation. Wild individuals seem to be globally hypermethylated compared to captive individuals, which may impact the expression of the genes involved (Flores et al., 2013). These changes in brain DNA methylation levels could explain, among other things, the observed behavioural differences between the two groups. We therefore observe here a loss of DNA methylation with captivity. We propose that

this hypermethylation in wild individuals compared to captive individuals can also be explained by the complex environmental conditions in the wild compared to stable conditions in captivity.

Epigenetic variability between wild EPP rivulus indicates that genotype-independent epimutations play a role in methylome variation given their low genetic variability (Chapelle et al., unpublish data). Indeed, epigenetic variations called pure epimutations are induced by environmental variations or stochastic events, but there are also so-called obligatory epimutations that are dependent on genetic variation (Berbel-Filho et al., 2019). A lower variability of methylation is observed in wild individuals compared to individuals in the laboratory, despite the more stable conditions in the laboratory compared to the natural environment. It is possible that a certain percentage of these epigenetic marks have been transmitted in some captive individuals, despite the reprogramming phenomenon (Fellous et al., 2017), which generates this methylation variability in captive individuals. F5 individuals therefore have more epigenetic variability, but less behavioural variability, illustrating here that behavioural variability is therefore not linked to epigenetics.

Our results therefore highlight the importance of epigenetic changes induced by the rearing environment in captive individuals compared to wild individuals during the early stages of domestication. Subsequently, it would be necessary to continue this study by visualising and identifying differentially methylated genes between generations using SeqMonk software to visualise differentially methylated regions (intergenic region, promoter sequence, etc.) and to assess the gene expressions impacted during this process.

5 | Conclusion

This study provides information on behavioural and epigenetic variations in individuals reared under stable and controlled captive conditions for five generations compared to wild individuals in an isogenetic population of mangrove rivulus *Kryptolebias marmoratus* with very low genetic variability, thus allowing the involvement of the methylome in these phenotypic changes to be studied independently of genetic variability. Behavioural variations between the two studied groups could be identified, with a decrease in boldness and activity of the fish in captivity. Captivity also leads to a loss of the behavioural individuality observed in the field. In addition, captivity appears to induce global hypomethylation, but with greater variability in methylation. It would be interesting to identify the genes involved in this methylation difference

to better understand the implication of DNA methylation on phenotypes during the domestication process and captivity. Epigenetic variability therefore appears to be important in generating behavioural variability in a domestication context.

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6 | Annexes

ANNEX 1 THE MANGROVE RIVULUS (*KRYPTOLEBIAS MARMORATUS*) AS A STUDY MODEL

Kryptolebias marmoratus (Poey, 1880), also known as mangrove rivulus or killifish, is an actinopterygian fish in the order Cyprinodontiformes and family Rivulidae (Turko and Wright, 2015). It was first described in 1880, but scientists only became interested in it in 1961 (Taylor, 2012). It was then realised that this fish was one of the only known vertebrates, along with its close relative (*Kryptolebias hermaphroditus*), to be hermaphroditic and to reproduce by self-fertilisation (Avise and Tatarenkov, 2015).

This fish was first observed in Florida in 1958 by Harrington and Rivas (Taylor, 1988) but is found throughout the tropical and subtropical Atlantic basin in mangrove forest waters (Avise and Tatarenkov, 2015), with a geographic range that extends from Florida to southeastern Brazil along the American continent. This is the widest distribution of any fish species in the coasts of the Americas, with approximately 91° longitude and 52° latitude (Taylor, 2012).

1. Habitat

As its common name suggests, the mangrove rivulus spends its entire life cycle in mangroves (Taylor, 2012). It is an environment populated by heavy vegetation such as red mangrove (*Rhizophora mangle*), black mangrove (*Avicennia germinans*) and sea oxeye daisy (*Borrichia frutescens*) (Taylor, 1988). This fish is characterised as rare because it is difficult to find due to the thick leaf litter and mangrove roots but also because it is rare in some habitats impacted by humans. However, it is quite abundant within mangroves in various microhabitats that are burrowing, intermittently dry and have poor water quality, preventing the presence of other fish species. Examples are ephemeral pools that will be intermittently dry, with stagnant and shallow water; crab burrows; in or under mangrove logs and leaves; in empty cans or coconuts. These burrowing niches provide thermal protection in cold temperatures. It is also sometimes found in terrestrial environments (Taylor, 2012).

2. Diet

In terms of diet, *K. marmoratus* is a cannibalistic predator species. It feeds on mosquito larvae, snails, crabs, gastropods, crustaceans, diptera, polychaetes, copepods, ants, and juvenile fish.

However, its feeding frequency may sometimes be rather low due to lack of resources and periods of drought (Taylor, 2012).

3. Emersion ability

It appears that this fish has almost amphibious behaviours and can be emergent, tolerating relatively long periods of drought (Turko et al., 2014). It can emerge from the water in active movements, for example to capture prey and then return to the water to swallow it (Taylor, 1988), but can also be emerged for a longer period. Being capable of skin respiration, it can survive in the open air for 66 days (Turko et al., 2014; Wright, 2012). When faced with a drying out of its habitat, it can easily travel tens of metres to return to an area of water, simply by jumping, wriggling, or crawling. Various mechanisms allow this ability to emerge. Firstly, there is a thicker epidermis with caliciform cells that produce mucus on the ventral surface of the fish's head, serving as protection, preventing desiccation, and improving adhesion to surfaces and vegetation. Their fins include blood vessels in the epidermis, contributing to air breathing. Reversible remodelling of the gills, with a decrease in gill lamellae and an increase in the mass of cells between them, is observed in emerged fish after a week out of water (Taylor, 2012; Wright, 2012).

4. Behaviour

This species is commonly reared individually as it can express aggressive behaviour when living in groups, both in the laboratory and in the wild (Edenbrow and Croft, 2012). However, it can modulate its aggressiveness according to its conspecific and be less aggressive towards individuals of the same lineage and therefore with a close genotype. Moreover, this species can be bold and adopt a "fight or flight" response to the same stimulus, or timid and adopt a "freeze and hide" response (Conrad et al., 2011). The presence of conspecifics during an individual's development may influence the boldness and aggression behaviours of the fish in that it decreases boldness and increases aggression (Edenbrow and Croft, 2013). This may also explain why these fish are routinely reared individually, especially for behavioural studies.

5. Adaptation to extreme environmental conditions

This atypical fish can tolerate and adapt to extreme environmental conditions (Kelley et al., 2016; Wright, 2012). Indeed, it is exposed in water to high temperatures, low dissolved oxygen concentrations below 1.0 ppm, high levels of hydrogen sulphide and ammonia concentrations above 10 mmol/L. It is more resistant than other tropical species to extreme temperatures. It

can be found in a temperature range of 7-38°C and the fish can even survive at 5°C when out of the water. However, as with all tropical fish species, it is endangered and may die if it is found at the limits of its range and faces extreme cold that it is not used to (Taylor, 2012; Wright, 2012).

From a salinity point of view, *Kryptolebis marmoratus* has been found in natural environments at salinity values ranging from 0 to 70 ppt but generally always at salinities above 10 ppt. In fact, this is not a freshwater species, and the samples taken at very low salinity are probably because heavy rainfall has temporarily caused these conditions. In the laboratory, this euryhaline species has already adapted to 114 ppt seawater (Taylor, 2012). Fish can adapt to these hypersaline conditions through the activity of chloride cells in the epithelium and opercular skin. As a result, it has been shown that the size of these cells, not the number, increases in rivulus reared in high salt water, ranging from 39 to 100% larger compared to fish species that live in low salt water (Taylor, 2012). *K. marmoratus* is therefore tolerant of extreme conditions, but its ability to emerge can be a convenient escape behaviour when environmental conditions exceed its tolerance level (Wright, 2012).

6. Reproduction and life cycle

As previously stated, *Kryptolebias marmoratus* is one of the only vertebrates to be self-fertilising hermaphrodites (Avise and Tatarenkov, 2015). This means that individually reared fish will be able to lay fertilised eggs. It has both testicular and ovarian tissue (Garcia et al., 2016), allowing both spermatogenesis and ovogenesis (Sakakura et al., 2006), and thus performs sexual reproduction (Sakakura et al., 2006). All populations are androdiotic. Individuals are either hermaphroditic and produce sperm and eggs or are male. There are primary males that will directly produce sperm throughout their life after development, or secondary males that will develop from hermaphrodites that have lost their ovarian tissue. However, males are rather rare in nature (Sakakura et al., 2006). The abundance of males varies from 0 to 24% depending on the population (Kelley et al., 2016). A recent field study in four different populations showed a higher percentage of males in the Belizean populations (41.8% males in Twin Cayes, 12.5% males in Long Caye) and a much lower rate of males in the Florida populations (2.8% males in Long Key and 0.4% males in Emerson Point Preserve) (Chapelle et al., unpublished data). Primary males can be obtained by incubation at low temperature (18-20°C) of hermaphrodite eggs that have been fertilised and secondary males can be obtained by exposure of immature hermaphrodites to high temperature (Mackiewicz et al., 2006). Males and hermaphrodites can be distinguished as they show sexual dimorphism (Fig. 16),

hermaphrodites are brown in colour and have an ocellus on their caudal fin while males are rather orange in colour and do not have this ocellus (Fellous et al., 2017).

Most hermaphrodite eggs are fertilised internally (Taylor, 2012), in the ovarian cavity, by sperm and egg cells from the same gonad (Sakakura et al., 2006), which is bilobed in structure (Taylor, 2012). All individuals have two gonads (Sakakura et al., 2006). After several cycles of self-fertilisation, a homozygous line with an identical genome and low allelic variability is produced (Tatarenkov et al., 2007). However, rare cases of exogamy have been observed in the laboratory. Indeed, crosses can be made between males and hermaphrodites if the males drop sperm near eggs laid by hermaphrodites that are not fertile because they have not been fertilised and they externally fertilise these eggs (Garcia et al., 2016; Taylor, 2012).

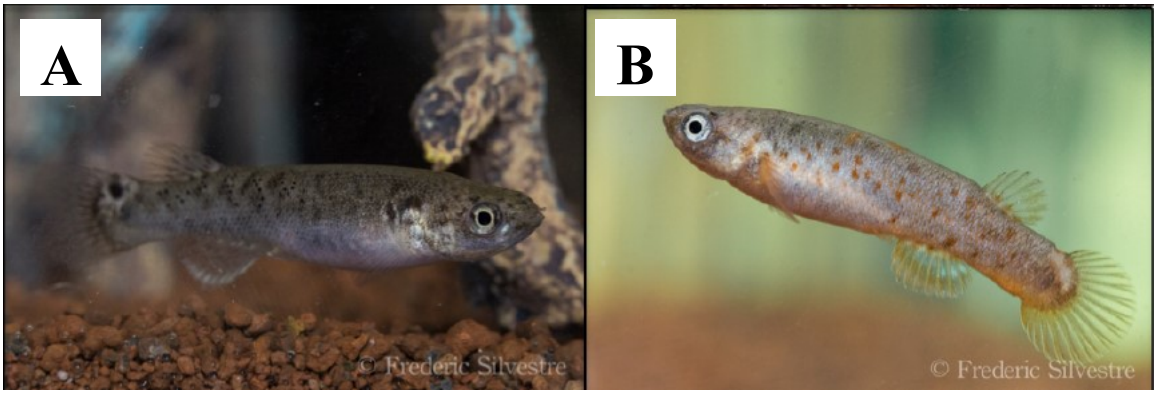


Fig. 16. Photographs of the different phenotypes of *Kryptolebias marmoratus* by Frédéric Silvestre: hermaphrodite individual (A) male individual (B).

Once the eggs are fertilised, embryonic development takes place for three days, again internally, after which the eggs are deposited (Harrington, 1963). Like many species of the genera *Rivulus* and *Kryptolebias*, these fish will then often beach their eggs out of the water, usually overnight. The embryos can develop completely without standing water if they are in a moist environment. The eggs will eventually hatch about 16 days after fertilisation, but they can also enter diapause. This diapause allows them to protect themselves from the stress of dry periods and hypoxia. It can last until the eggs are immersed in water again. Rivulus can therefore reproduce throughout the year (Taylor, 2012). Larval development will then begin, until the fish reach sexual maturity at around 90 days for hermaphrodites and 70 for males (Weeks et al., 2006). In the laboratory, the species can live for a long time, one individual has survived for over 8 years, but the longevity of the species in the wild is not yet known (Taylor, 2012).

ANNEX 2 THE DOMESTICATION PROCESS AND SYNDROME

1. The history of domestication

About 12,000 years ago, humans turned their attention from hunting and gathering wild animals and plants to domesticating them. It was thus with the beginning of agriculture that wild plants and animals began to be domesticated, resulting in the appearance of the farm animals we know today (Teletchea, 2016). This phenomenon of domestication due to agricultural practices occurred in different regions of the world, after which domesticated species were gradually introduced all over the world with the migration of farmers. Gradually, a major transition in the way of life of society, called the Neolithic transition, was observed with the domestication of plants and animals. For most of the history of domestication, animals derived from agriculture were managed to be increasingly adapted to conditions. Subsequently, around 200 years ago, well-defined breeds were created by selecting for phenotypic traits of interest in these animals, with reduced breeding between breeds (Teletchea, 2019).

Three main pathways have been proposed to explain how terrestrial animals became domesticated. The first pathway is the commensal pathway, where animals have moved to an anthropogenic environment and interact with humans, for example because they are attracted to human waste. This is the case, for example, with dogs (*Canis familiaris*), cats (*Felis catus*), pigs (*Sus scrofa domesticus*) or chickens (*Gallus gallus domesticus*). In the second pathway, the prey pathway, it was humans who brought about domestication due to the depletion of stocks of animals considered as prey, leading to a well-controlled breeding of animals to improve the quantity of the resource, in the form of meat or skin for example. This is the case for example with sheep (*Ovis*), goats (*Capra hircus*), or cattle (*Bos taurus* and *B. indicus*). The third pathway is the direct pathway where humans intentionally domesticate a species by capturing wild animals to control their reproduction, thus creating a bottleneck. This is the case for the horse (*Equus caballus*), the donkey (*Equus asinus*) and the dromedary (*Camelus dromedarius*) (Teletchea, 2019).

2. The principle of domestication

The exact definition of domestication and a domesticated species is still unclear, but we consider a domesticated species to be a group of animals that will be bred in captivity and undergo modifications from the original wild species. The term “level of domestication” is preferred. This process is a long and continuous change. There are many forms in transition, it is not just a wild species VS domestic species classification (Teletchea, 2019). Thus,

domestication is the process in which a population of animals gradually adapts to captivity and the presence of humans. It is therefore a long and endless evolutionary process of adaptation where the pressure comes from an anthropogenic niche (Teletchea, 2016). Once wild animals are in captivity and adapting to a new environment, domestication will begin (Milla et al., 2020). The whole life cycle of the species needs to be controlled in captivity to have the establishment of breeds with specific traits that will have been selected and improved. If the process stops at the beginning of the species' captivity, the wild animal is just tamed, with modification of its behaviour without genetic modification that can be transmitted to the offspring. Thus, animals in captivity become domesticated when they are completely isolated from the wild species, creating a bottleneck, which will reduce the genetic diversity of the species and make it sufficiently different from the wild species. The smaller the effective population size, the greater the genetic drift, allele loss and thus inbreeding (Teletchea, 2019, 2016). To avoid inbreeding, it is important to have species selection programmes with a sufficiently large population size and a genetically diverse population at the start, followed by a regular input of external genetic variability during breeding (Teletchea, 2016).

Over generations, the animal's environment will generate genetic, genomic, and phenotypic changes from the wild stock species, whether voluntary or not (Milla et al., 2020; Teletchea and Fontaine, 2014). This domestication therefore leads to biological changes in the domesticated species, from a behavioural, physiological, and morphological point of view, with the aim of adapting to the new captive environment and through genetic changes (Milla et al., 2020).

3. The domestication syndrome

In all described domesticated populations, there is a sequence of specific physiological, morphological, and behavioural characteristics, which is not the case in wild ancestors. This is described as the "domestication syndrome" (Lord et al., 2020). This set of inherited traits present in farmed animals and plants but absent in their wild progenitor was first described by Charles Darwin in his book "The Variation of Plants and Animals under Domestication" in 1868 (Wilkins et al., 2014) but domestication syndrome was only named this way in the 1980s, the term having already been used for plants in the early 1900s (Lord et al., 2020). Since then, this phenomenon has been described in many mammalian species (Milla et al., 2020). The observed traits related to the domestication syndrome vary according to the species domesticated. The vast majority are behavioural changes such as taming, decreased aggression and increased docility. A loss of reproductive seasonality can be observed with an increase in fecundity (Lord et al., 2020), as well as a change in the level of corticotropic hormones and in

the concentration of several neurotransmitters (Wilkins et al., 2014). Phenotypic changes are also present in most species, with changes in coat colouration, brain size, skull and jaw shape, and morphological changes in the ears, tail, and skeleton. Many domesticated species have shorter jaws and teeth, larger and sometimes floppy ears, depigmentation with the appearance of white patches, and smaller brain size with decreases in the size of some brain regions (Wilkins et al., 2014), probably due to adaptation to the captive environment which fewer cognitive demands for survival (Lord et al., 2020). Changes in species pigmentation relative to the wild form are one of the most observed and consistent changes during the domestication process observed in many mammalian species (Wilkins et al., 2014).

A Russian experiment, founded by Dr. Dimitry Belyaev, bred male and female silver foxes (*Vulpes vulpes*) from Soviet fur farms and selected the foxes that were least fearful of human presence. They observed, after several replications, that in only 10 generations of selection on these wild foxes, they obtained foxes that sought human attention (Lord et al., 2020), and that had certain behavioural traits found in domestic dogs such as licking, barking, and whining (Moretz et al., 2007). Phenotypic changes were also observed in these animals, with floppy ears, curled tails, changes in coat and breeding cycles. As a result of this experience, it was stated that domestication may result from selection on the docility of the species, which will then modify the regulation of various other interconnected traits (Lord et al., 2020).

Many hypotheses have tried to explain this phenomenon. Firstly, scientists have explained it by a mechanism of pleiotropy, where a pleiotropic gene or hormone has multiple effects and determines several phenotypic traits. However, no mutants of a "domestication gene" that could explain the entire domestication syndrome phenotype have been found. Scientists also hypothesise that it is the neural crest genes that cause domestication syndrome because of a deficit of neural crest cells during embryo development. They explain this by the fact that domestication syndrome impacts a wide range of traits in species and that all these traits have in common that their development is linked to neural crest cells. At the beginning of embryogenesis, these stem cells appear at the neural crest and then migrate to different parts of the body, both in the skull and in the trunk, to give different types of tissues and cells. They suggest that species selection in captivity, such as selection for docility, may result in a reduction in the supply of neural crest cells, explaining the impact on different phenotypic traits (Lord et al., 2020; Wilkins et al., 2014).

Scientists have also pointed out that these changes in domestic animals. Indeed, phenotypic changes can be rapidly observed during the domestication process when individuals are subjected to a new environment to which they must adapt. These rapid changes can’t be

explained exclusively by changes in DNA sequence, which may be a slower process, but rather by epigenetic effects, such as DNA methylation, which modulate gene expression and rapidly influence phenotypic traits (Ahmad et al., 2020). More and more studies show that rapid changes in the environment can induce variations in behaviour and personality, explained by epigenetic modifications (Verhulst et al., 2016). A study on Nile tilapia fish showed about 700 differentially methylated CpG sites between wild females and their direct offspring bred in captivity, even before any artificial selection on gene sequences was performed. Most of these differences are associated with genes involved in muscle growth, immunity, autophagy or feeding response (Podgorniak et al., 2022). The level of complexity of the breeding environment, and thus the level of stress associated with that environment, is an important element influencing the behaviour of individuals, with differences explained by differentially methylated regions between different environmental conditions. Indeed, a study on birds shows that birds reared in open aviaries with a high level of environmental complexity will tend to be less fearful, spend more time around humans and new objects and move around their environment more than cage-reared birds with a low level of environmental complexity (Brantsæter et al., 2016; Pértille et al., 2017). During domestication, the environmental conditions in captivity and in the wild are also very different, with more regular, homogenous, and often ad libitium feeding, stable environmental conditions, absence of predation and continuous human presence (Podgorniak et al., 2022).

Various hypotheses have therefore been put forward, but the source of the domestication syndrome and the observed changes are still unclear. This collective appearance of traits defining a domesticated species therefore raises questions about the genetic and/or environmental mechanisms that cause this syndrome (Lord et al., 2020).

4. The domestication syndrome in fish

This phenomenon has long been described in many mammalian species and this idea was reinforced when it was realised that domesticated birds and fish also share some of the traits seen in mammals (Wilkins et al., 2014). Several traits that are described in the mammalian domestication syndrome are also found in fish, but this has been much less described. The phenotype of fish also seems to be modified by captivity and the presence of humans, in the same way as it can be observed in mammals (Milla et al., 2020). For aquatic animals, the phenomenon is much more recent. Most of the fish domestication only dates to the early 1980s. However, many species are already well advanced in the domestication process. Many fish species live in captivity with a life cycle that takes place in a totally closed environment and

with genetic modification of certain traits to be improved. Therefore, this phenomenon of controlling and improving the life cycle of species in captivity, which took hundreds of years in mammals, could be achieved in just a few decades for some fish species. The domestication of the Atlantic salmon (*Salmo salar*) is one of the best examples. Its domestication started in Norway in the 1970s and less than 40 years later all farmed salmon in the world have gone through the whole domestication process. Traits often selected in fish are improved growth rate, with a genetic gain per generation of about 10-14%, which is 4-5 times more than what is observed in captive and selected terrestrial species. Greater disease resistance or improved flesh quality is also selected for. Given the high fecundity of fish, selection of farmed aquatic animals may therefore be important (Teletchea, 2016).

Studies comparing wild and domesticated strains of zebrafish have shown variations in boldness and aggressiveness between these strains, suggesting the presence of a domestication syndrome in fish (Moretz et al., 2007). Indeed, when comparing wild and domestic populations in animals from many different taxa, domestication populations show a reduction in aggressive behaviour and an increase in boldness, with a decrease in predator avoidance and a decrease in fearful behaviour (Drew et al., 2012). This observation therefore seems to be an important characteristic that could result from the domestication syndrome and would also seem to be observable in fish. Very little is yet known about the genetic polymorphisms that are associated with domestication. Studies show that domestication may also be associated with changes in gene expression. The comparative study between wild and domesticated zebrafish showed high levels of variation in the expression of certain genes between domesticated and wild strains. They found 112 genes expressed significantly differently between domesticated and wild strains, the main ones being *gpr177* (G protein-coupled receptor 177), *sepp1a* (Selenoprotein P), *acbd3* and *acbd4* (acyl-CoA binding domain) (Drew et al., 2012).

ANNEX 3 HOUSING OF EXPERIMENTAL FISH

1. Sampling and egg development

The population used in this master thesis is the EPP population. This population present in the stock originally came from Florida (Emerson Point Preserve, Florida; 27°31'N 82°38'W). The eggs corresponding to the F1 generation hatched on 8 August 2019 and the larvae were housed at the University of Namur.

In the experiment, we are interested in the eggs of the F4 individuals of the EPP lineage present in the laboratory's fish stock. To obtain the F5 generation, the eggs produced by self-fertilisation are collected from six individuals of the F4 generation: EPP21-164, EPP21-168, EPP21-169, EPP21-170, EPP21-174 and EPP21-177, all of which come from the same individual (EPP21-68 from F3). Eggs were collected approximately three times per week from May 2022 for five weeks. The wild F0 fish that produced the F1 generation (then F2, F3, F4 and finally F5) is EPP1078.

Fig. 17. Photographs of the mangrove rivulus (*Kryptolebias marmoratus*) of the EPP population from different generations. Initial wild individual EPP1078 from F0 generation (A), Individual EPP19-1 from F1 generation (B) and Individual F5_25 from F5 generation (C).

As soon as the eggs are collected, they are individualised in 12-well plates made for cell culture (Cellstar®) (Fig. 18) filled with 2mL of 12 ppt saltwater. The plate is placed in an incubator at 26.0 ± 1°C. The water in the plates is changed each week to ensure constant conditions, avoid

contamination and stimulate egg hatching. Synchronized hatchings were carried out to group hatchings of several eggs on the same day and thus obtain fish of the same age. To achieve these synchronised hatchings, the plates containing the eggs are placed in incubation Ziplock bags containing an Anaerocult™ sachet on which a few drops of water are placed. This bag contains chemicals that bind oxygen when combined with water. This creates an anaerobic environment that triggers hatching within a few hours.

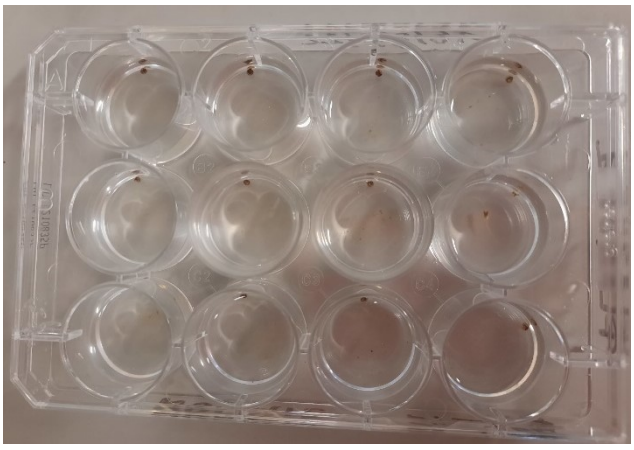


Fig. 18. Individualisation of eggs in 12-well plates (Cellstar®) filled with 2mL of 12 ppt saltwater.

2. General maintenance of fish

The different lineage of the mangrove rivulus (*Kryptolebias marmoratus*) are maintained in an approved thermoregulated room within the Laboratory of Evolutionary and Adaptive Physiology (LEAP) of the Research Unit in Environmental and Evolutionary Biology (URBE) of the University of Namur. The room has controlled conditions, i.e. an average temperature of 26 ± 1°C and a photoperiod of 12h of light during the day and 12h of darkness at night.

As soon as the eggs hatch, the larval fish are reared individually in a glass jar at a salinity of approximately 12 parts per thousand (ppt) saltwater (Instant Ocean® sea salt) and a temperature of 26.0 ± 1°C. Each fish is given a unique number to identify the individual, linked in an Excel table to its parent and the date of hatching to know its age. At the age of two months, the fish are transferred to a plastic container with a capacity of 300 mL under the conditions mentioned above. The container is closed to prevent the fish from escaping as they jump, but a small hole is present to allow daily feeding (Fig. 19). The fish are reared individually as they may express aggressive behaviour when living in groups (Edenbrow and Croft, 2012). This allows for easy identification of the fish and more obvious monitoring for experiments and for checking the general condition of the individual.

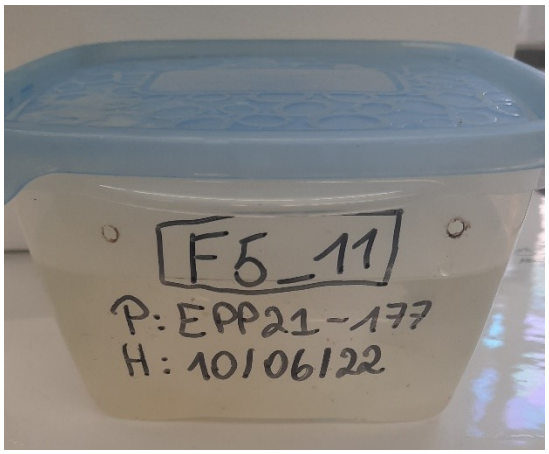


Fig. 19. The individual fish rearing container with a capacity of 300 mL of 26.0 ± 1°C and 12 ppt saltwater.

The fish are fed everyday ad libitum with living *Artemia salina*. This live food stimulates the hunting behaviour of the fish and is a good enrichment. The amount of Artemia fed will depend on the age of the fish. The fish will receive 1mL until 2 months, 2 mL until 3 months and 3 mL after 3 months. Breeding fish are given 5mL to ensure a good spawning yield.
Each week, the salinity of the water of 10 fish is randomly checked and corrected if necessary. To improve fish welfare, cotton cushion padding is placed in all individual housing to allow the fish to hide in or emerge from the water and lay eggs.

ANNEX 4 MODEL SELECTION BASED ON AIC VALUES

Data: Latency to first entry in arena (Latarena)

Models:

M5: Latarena ~ Generation + (1 | ID)

M4: Latarena ~ Generation + Replicate + (1 | ID)

M2: Latarena ~ Generation + Replicate + Length + Generation:Length + (1 | ID)

M3: Latarena ~ Generation + Replicate + Generation:Length + (1 | ID)

M1: Latarena ~ Generation + Replicate + Length + Generation:Replicate + Generation:Length + (1 | ID)

Table 6. Model selection based on AIC values for the variable *Latency to first entry in arena*

	AIC	Pr (>Chisq)
M5	**362.96**	
M4	364.20	0.3828
M2	365.13	0.2155
M3	365.13	
M1	365.98	0.2827

Data: Total time in arena (TTA)

Models:

M5: TTA ~ Generation + (1 | ID)

M4: TTA ~ Generation + Replicate + (1 | ID)

M3: TTA ~ Generation + Replicate + Length + (1 | ID)

M2: TTA ~ Generation + Replicate + Length + Generation:Replicate + (1 | ID)

M1: TTA ~ Generation + Replicate + Length + Generation:Replicate + Generation:Length + (1 | ID)

Table 7. Model selection based on AIC values for the variable *Total time in arena*

	AIC	Pr (>Chisq)
M5	290.65	
M4	**289.33**	**0.06867 .**
M3	291.10	0.62755
M2	293.10	0.98923
M1	293.94	0.28226

Data: Relative total distance moved (RTDM)

Models:

M5: RTDM ~ Generation + (1 | ID)

M4: RTDM ~ Generation + Length + (1 | ID)

M3: RTDM ~ Generation + Replicate + Length + (1 | ID)

M2: RTDM ~ Generation + Replicate + Length + Generation:Replicate + (1 | ID)

M1: RTDM ~ Generation + Replicate + Length + Generation:Replicate + Generation:Length + (1 | ID)

Table 8. Model selection based on AIC values for the variable *Relative total distance moved*

	AIC	Pr (>Chisq)
M5	349.53	
M4	339.19	0.0004434 ***
M3	340.79	0.5264012
M2	**329.08**	**0.0002137 *****
M1	331.06	0.8800549

Data: Relative total time in inner zone (IZ)

Models:

M5: IZ ~ Generation + (1 | ID)

M4: IZ ~ Generation + Length + (1 | ID)

M3: IZ ~ Generation + Replicate + Length + (1 | ID)

M2: IZ ~ Generation + Replicate + Length + Generation:Replicate + (1 | ID)

M1: IZ ~ Generation + Replicate + Length + Generation:Replicate + Generation:Length + (1 | ID)

Table 9. Model selection based on AIC values for the variable *Relative total time in inner zone*

	AIC	Pr (>Chisq)
M5	**364.83**	
M4	366.66	0.6744
M3	368.60	0.8067
M2	369.53	0.3024
M1	370.43	0.2936

ANNEX 5 ImageJ

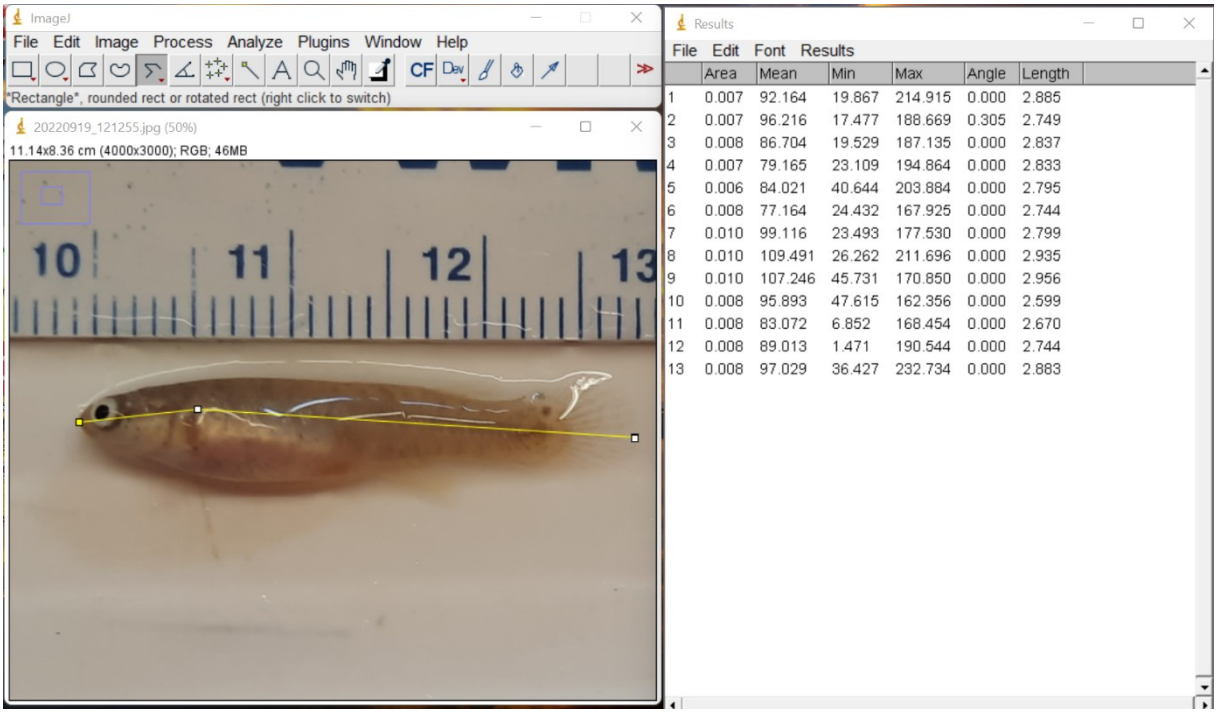


Fig. 20. Using ImageJ software for fish size measurements.

ANNEX 6 NanoDrop™ 2000c spectrophotometer (Thermo Scientific™)

The NanoDrop provides the concentration (ng/µl) of extracted genetic material. It also allows the quality of this DNA to be assessed by highlighting problems of solvent or protein/RNA contamination based on two ratios. It measures absorbances at three different wavelengths (260, 280 and 230 nm) and performs two ratios (260/280 and 260/230) which should be close to 2 if the sample is pure and not contaminated.

Protein contamination can be checked with an absorbance ratio of 260 nm/280 nm. DNA absorbs at a wavelength of 260 nm and protein at a wavelength of 280 nm. If the 260/280 ratio is less than 1.8, there may be protein/RNA contamination or other substances that absorb at 280 nm. The second 260/230 ratio is therefore used to check for contamination with solvents that absorb at 230 nm.

The assessment of the concentration of genetic material and potential contamination of this material allows consideration of whether molecular analysis can be pursued on the entire sample. A sample that is too low in concentration or with too much contamination may not be used for further experiments.

Table 10. NanoDrop results of F0 samples of *Kryptolebias marmoratus* giving the concentration and quality of the extracted DNA.

Sample ID	Conc (ng/µl)	260/280	260/230
EPP1082	43,7	2,03	1,80
EPP1092	71,7	2,01	1,76
EPP1452	71,6	2,01	1,70
EPP1077	40,5	2,01	1,59
EPP1123	58,9	1,98	1,75
EPP1099	53,7	1,99	1,61
EPP1137	23,4	2,06	1,04
EPP1110	91,4	2,02	2,07
EPP1456	23,3	2,16	1,13
EPP1106	182,1	1,83	1,61
EPP1100	122,2	2,01	2,05
EPP1438	93,3	2,04	2,07
EPP1441	108,1	1,97	2,02
EPP1463	77,1	2,03	2,08
EPP1448	303,4	1,88	1,86
EPP1073	113,6	1,94	1,81
EPP1139	92,8	1,99	2,18
EPP1461	104,7	1,98	2,03
EPP1083	96,6	2,05	1,82
EPP1103	121,6	2,06	1,97
EPP1127	127,5	2,05	1,84
EPP1451	102,4	2,06	1,91
EPP1101	171,3	1,97	2,03
EPP1091	77,8	1,98	1,93
EPP1125	134,7	1,96	1,97
EPP1464	109,0	1,94	1,51
EPP1071	85,3	2,00	2,07
EPP1067	360,7	1,94	2,22
EPP1079	143,0	1,97	2,00
EPP1056	144,9	1,99	2,13

EPP1064	130,1	1,99	1,99
EPP1114	83,9	1,99	2,04
EPP1107	172,4	1,94	1,88
EPP1113	82,7	2,01	2,05
EPP1133	148,4	1,95	2,11
EPP1443	125,1	1,97	2,15
EPP1450	91,2	1,98	1,84
EPP1453	86,3	2,02	2,04
EPP1057	203,8	1,95	2,19
EPP1130	51,5	2,01	1,81

Table 11. NanoDrop results of F5 samples of *Kryptolebias marmoratus* giving the concentration and quality of the extracted DNA.

Sample ID	**Conc (ng/µl)**	**260/280**	**260/230**
F5_2	94,3	2,01	2,07
F5_3	151,1	2,03	1,99
F5_7	142,3	2,01	1,97
F5_17	55	1,96	1,87
F5_34	226,6	1,97	2,05
F5_29	152,8	2,03	1,91
F5_40	180,8	2,12	1
F5_23	194,1	2	2,12
F5_14	97,8	1,94	1,29
F5_19	116	1,75	0,38
F5_26	143	1,81	0,71
F5_48	141,1	1,99	1,56
F5_22	177,5	1,93	0,98
F5_4	228,2	2,01	2,31
F5_32	132,6	2,03	2,25
F5_11	234,8	2,05	2,43
F5_38	102,2	1,91	0,57
F5_33	131,2	1,91	0,75
F5_50	127,4	2,05	2,37

F5_35	26,8	1,79	0,39
F5_43	139,1	2,09	2,47
F5_6	119,3	2,04	2,41
F5_18	105,1	1,68	0,45
F5_25	72,7	2,02	1,78
F5_12	208,6	1,79	0,71
F5_21	102,8	1,97	2,26
F5_10	181,3	2,02	2,17

ANNEX 7 MIGRATION ON 1% AGAROSE GEL

Agarose gel migration is used to ensure that the extracted DNA is not degraded and fragmented. This could interfere with further molecular analysis. The migration was performed on a few randomly selected samples.

To perform the gel, 0.4g of agarose was added to 40 mL of 0.5% Tris-Acetate Electrophoresis (TAE) buffer and the mixture was boiled. The percentage of agarose determines the mesh size of the gel. As the genetic material extracted in this experiment is whole genomic DNA, the gel should not have mesh sizes that are too small and would block the migration of this large DNA. 2 µL of SYBR™ Safe intercalating agent is added to the mixture to allow visualisation of the genetic material. The mixture is poured into a caster with a comb with the correct number of wells for the number of samples.

After 40 minutes of polymerisation in the dark, the gel is placed in an electrophoresis tank which is filled with 0.5% TAE buffer. The first well is loaded with 3 µL of 1000 base pair calibration buffer (1kb ladder buffer) and 1µL of loading buffer. This first well will provide a reference scale to get an idea of the DNA size of the samples. The remaining wells are loaded with 1µL of loading buffer and 3µL of each of the samples previously mixed on a sheet of parafilm.

After 35 minutes of migration at 100V, the gel can be revealed using a Biorad® camera and ImageLab® software.

Fig. 21. DNAs extracted from the brains of *Kryptolebias marmoratus* of the F0 generation that have migrated on a 1% agarose gel.

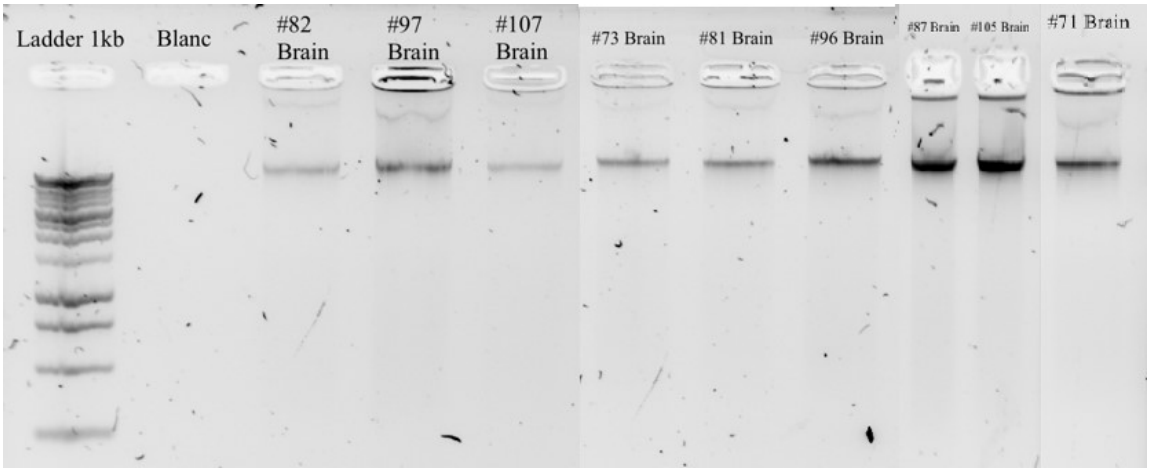
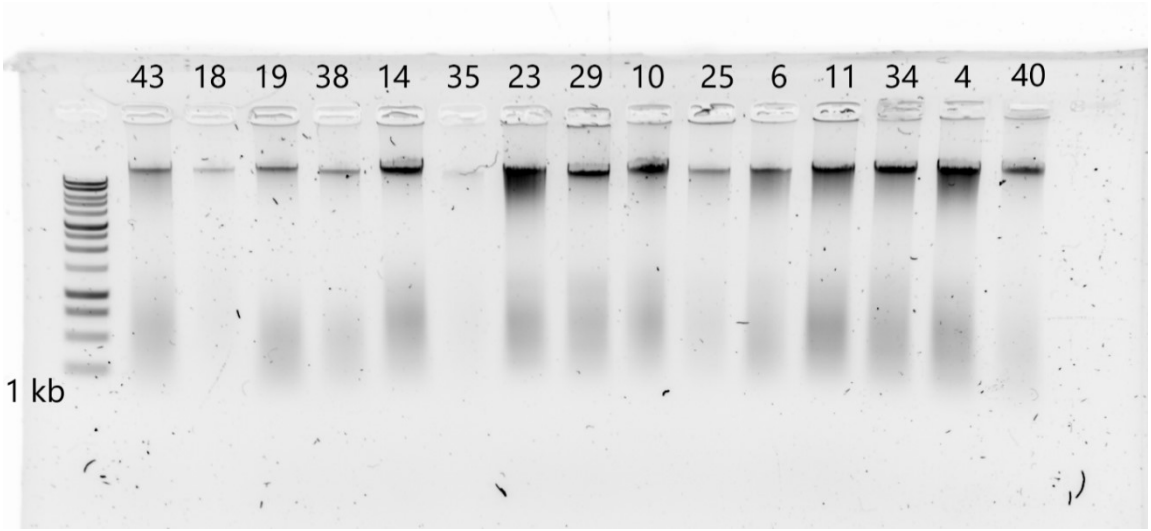


Fig.22. DNAs extracted from the brains of *Kryptolebias marmoratus* of the F5 generation that have migrated on a 1% agarose gel.



ANNEX 8 BEHAVIOURAL STATISTICS

R script with the statistics of the behavioural analyses of my master thesis.
Additional file entitled: Madarasz_18731700_2023_Annex1.Rmd

ANNEX 9 RRBS STATISTICS

R script with the statistics of the RRBS analyses of my master thesis.
Additional file entitled: Madarasz_18731700_2023_Annex2.R

7 | Supplementary data

S1 Table of results of the behavioural tests for boldness of *Kryptolebias marmoratus* of the F0 generation.

Fish ID	Generation	Replicate	Weight (g)	Length (cm)	Total time in open field (s)	Latency to first entry in open field (s)	Total time in external zone (s)	Total time in internal zone (s)	Latency to first entry in internal zone (s)	Total distance moved (cm)
EPP1071	0	1	0,541	3,322	1038,52	0	999,52	39	103,04	2643,66
EPP1078	0	1	0,814	3,873	1146,68	0	1051,96	94,72	14,4	4370,38
EPP1082	0	1	0,628	3,552	807,08	50,6	731,52	75,56	51,04	2521,23
EPP1075	0	1	0,966	4,144	873,88	116,84	830,2	43,68	156,48	2573
EPP1077	0	1	0,306	2,841	1167,16	0	1142,16	25	63,84	3402,91
EPP1083	0	1	0,767	3,903	950	16,64	894,64	55,36	230,48	2787,61
EPP1057	0	1	0,446	3,184	1152,56	0	1090,4	62,16	5,28	3589,35
EPP1067	0	1	0,337	2,885	1168,52	9	1123,2	45,32	29,48	3842,45
EPP1079	0	1	0,564	3,367	1088,44	8,88	1031,8	56,64	13,08	3112,86
EPP1056	0	1	0,267	2,603	1126,4	1,4	1103,28	23,12	10,64	2977,38
EPP1064	0	1	0,356	2,882	1049,28	15,6	1004,6	44,68	28,16	3252,76
EPP1073	0	1	0,473	3,154	1138,56	7,28	1116,44	22,12	32,68	3802,12
EPP1123	0	1	0,346	2,763	738,76	27,08	639,88	98,88	40,32	1972,84
EPP1137	0	1	0,441	3,12	913,28	31,04	892,6	20,68	42,04	2682,09
EPP1139	0	1	0,369	3,033	932,76	73	844,52	88,24	85,8	2510,75
EPP1101	0	1	0,558	3,358	1085,28	63,32	1035,24	50,04	92,76	2582,37
EPP1106	0	1	0,397	2,967	1033,88	21,12	969,92	63,96	29,92	3037,57
EPP1114	0	1	0,347	2,792	753,96	147,56	666,36	87,6	155,2	1914
EPP1092	0	1	0,362	2,829	859,04	20	757,44	101,6	37,44	2326,97
EPP1100	0	1	0,479	3,227	1110,12	20,2	999,76	110,36	44,76	1624,4
EPP1103	0	1	0,515	3,228	972,56	114,92	960,32	12,24	136,72	1930,09
EPP1091	0	1	0,406	3,005	1153,24	14,56	1068,12	85,12	31,12	2437,97
EPP1130	0	1	0,418	2,764	961	156,48	956,64	4,36	168,12	2595,71
EPP1127	0	1	0,324	2,766	1047,56	26,04	1004,64	42,92	43,44	3531,1
EPP1107	0	1	0,413	3,033	1110,48	82,68	1099,08	11,4	100,88	2015,9
EPP1113	0	1	0,383	2,917	1123,56	22,8	1072,8	50,76	41,92	3737,58
EPP1125	0	1	0,307	2,712	858,08	105,32	816,28	41,8	201,52	2744,18
EPP1099	0	1	0,293	2,616	1019,52	124,32	983,48	36,04	135,52	2798,22

EPP1110	0	1	0,302	2,733	1179,68	1,08	1151,28	28,4	6,84	2254,98
EPP1133	0	1	0,358	2,845	1108,84	12,28	974,72	134,12	23,24	2350,33
EPP1438	0	1	0,276	2,599	854	16,44	778,4	75,6	25,64	2027,03
EPP1441	0	1	0,424	3,032	899,88	47,4	847,24	52,64	67,12	2994,08
EPP1443	0	1	0,317	2,725	644,36	207,92	546,72	97,64	217,08	1640,33
EPP1448	0	1	0,38	2,798	893,52	64,84	771,04	122,48	75,72	2466,03
EPP1450	0	1	0,408	2,934	865,36	11,28	838,28	27,08	112,32	2567,92
EPP1451	0	1	0,369	2,834	793,24	37,88	704,32	88,92	51	2515,85
EPP1452	0	1	0,305	2,607	682,48	103,84	590,36	92,12	115,84	1802,41
EPP1453	0	1	0,367	2,894	961,48	101,28	879,72	81,76	118,8	3035,85
EPP1456	0	1	0,278	2,597	491,68	567,72	451,84	39,84	591,88	1365,83
EPP1461	0	1	0,45	3,024	702,68	107,92	598,56	104,12	112,48	1940,44
EPP1463	0	1	0,301	2,637	748,76	91,2	673,8	74,96	96,24	2259,04
EPP1464	0	1	0,6	3,298	417,92	592,36	376,16	41,76	597,16	1761,6
EPP1071	0	2	0,541	3,322	786,04	27,8	724	62,04	38,12	1780,66
EPP1078	0	2	0,814	3,873	988,84	10,08	957,64	31,2	14,56	2820,21
EPP1082	0	2	0,628	3,552	726,28	37,56	579,28	147	49,32	2372,62
EPP1075	0	2	0,966	4,144	1043,12	71,12	991,12	52	74,48	2933,57
EPP1077	0	2	0,306	2,841	1170,44	4,52	1146,32	24,12	135,32	3387,5
EPP1083	0	2	0,767	3,903	1080,76	41,12	1044,6	36,16	54,24	2205,97
EPP1057	0	2	0,446	3,184	0	1200	0	0	1200	0
EPP1067	0	2	0,337	2,885	1122,24	0	1088,68	33,56	13	2918,18
EPP1079	0	2	0,564	3,367	740,68	103,04	700,88	39,8	194,8	1517,76
EPP1056	0	2	0,267	2,603	1130,96	20,16	1057	73,96	33,76	2708,87
EPP1064	0	2	0,356	2,882	1157,56	42,48	1124,96	32,6	46,24	3280,82
EPP1073	0	2	0,473	3,154	880,96	40,8	797,52	83,44	85,92	2072,34
EPP1123	0	2	0,346	2,763	794,96	64,2	665,48	129,48	78,28	1352,05
EPP1137	0	2	0,441	3,12	1089,12	0	1030,36	58,76	73,84	1882,05
EPP1139	0	2	0,369	3,033	998,4	45,64	909,92	88,48	60,52	2151,82
EPP1101	0	2	0,558	3,358	1086,4	31,36	962,04	124,36	46,32	1926,83
EPP1106	0	2	0,397	2,967	1105,2	31,12	994,36	110,84	48,92	2370,83
EPP1114	0	2	0,347	2,792	668,68	79,64	597,44	71,24	83,96	1585,44
EPP1092	0	2	0,362	2,829	913,28	1,68	680,32	232,96	24,68	2372,58
EPP1100	0	2	0,479	3,227	740	36,08	625	115	55,24	1502,28
EPP1103	0	2	0,515	3,228	776,32	48,72	758,24	18,08	67,48	1420,53
EPP1091	0	2	0,406	3,005	976,4	10,72	904,8	71,6	37,28	2235,07
EPP1130	0	2	0,418	2,764	1061,68	6,76	966,44	95,24	24,64	2927,02
EPP1127	0	2	0,324	2,766	997,12	30,28	949,56	47,56	34	3906,81
EPP1107	0	2	0,413	3,033	1115,24	25,92	1091,12	24,12	59,36	2694,96

EPP1113	0	2	0,383	2,917	993,68	18,44	958,48	35,2	76,08	2504,83
EPP1125	0	2	0,307	2,712	517,08	38,24	463,8	53,28	117,12	2021,64
EPP1099	0	2	0,293	2,616	942,12	21,68	905,24	36,88	26,96	2520,27
EPP1110	0	2	0,302	2,733	1143,08	5,08	1118,52	24,56	21,12	3112,32
EPP1133	0	2	0,358	2,845	947,92	26,48	769,56	178,36	44,2	1746,03
EPP1438	0	2	0,276	2,599	1028,32	52,48	984,88	43,44	58,2	1645,4
EPP1441	0	2	0,424	3,032	1024,64	45,24	972,28	52,36	96,96	2570,87
EPP1443	0	2	0,317	2,725	745,08	9,16	706,12	38,96	24	1710,55
EPP1448	0	2	0,38	2,798	944,44	59,56	802,32	142,12	66,68	1934,59
EPP1450	0	2	0,408	2,934	950,28	37,68	872,84	77,44	40,72	2935,38
EPP1451	0	2	0,369	2,834	634,8	22,12	600,84	33,96	81,68	1906,83
EPP1452	0	2	0,305	2,607	554,32	80,04	534,2	20,12	83,52	1459,01
EPP1453	0	2	0,367	2,894	746,72	41,6	649,84	96,88	46,92	2226,58
EPP1456	0	2	0,278	2,597	297,16	51,08	294,16	3	229,68	1109,46
EPP1461	0	2	0,45	3,024	871,12	58,88	792,4	78,72	62,16	2205,8
EPP1463	0	2	0,301	2,637	704,08	47,24	618,28	85,8	59,12	2217,79
EPP1464	0	2	0,6	3,298	417,28	618,84	362,68	54,6	633,96	1200,51

S2 Table of results of the behavioural tests for boldness of *Kryptolebias marmoratus* of the F5 generation.

Fish ID	Generation	Replicate	Weight (g)	Length (cm)	Total time in open field (s)	Latency to first entry in open field (s)	Total time in external zone (s)	Total time in internal zone (s)	Latency to first entry in internal zone (s)	Total distance moved (cm)
F5_2	5	1	0,2652	2,858	69,68	1094	63,04	6,64	1132,12	51,4345
F5_11	5	1	0,261	2,795	465,16	468,12	420,76	44,4	475,68	591,917
F5_3	5	1	0,2256	2,732	78,4	1000,92	75,56	2,84	1051,68	114,974
F5_7	5	1	0,2476	2,837	9,72	1021	9,72	0	1200	2,71267
F5_10	5	1	0,2489	2,833	673,84	106,8	603,76	70,08	122,04	921,658
F5_6	5	1	0,2475	2,744	609,04	195,88	470,68	138,36	199,08	754,2
F5_12	5	1	0,2686	2,799	678,84	319	654,28	24,56	581,2	632,472
F5_17	5	1	0,2981	2,956	307,92	337,64	289,4	18,52	341,44	303,527
F5_21	5	1	0,2376	2,599	245,32	667,64	227,84	17,48	686	249,165
F5_22	5	1	0,2431	2,67	415,32	141,92	343,2	72,12	148,24	560,382
F5_23	5	1	0,2377	2,744	589,72	245,36	464	125,72	254,32	637,84
F5_25	5	1	0,2716	2,883	27,88	736,28	25,16	2,72	743,8	9,54134

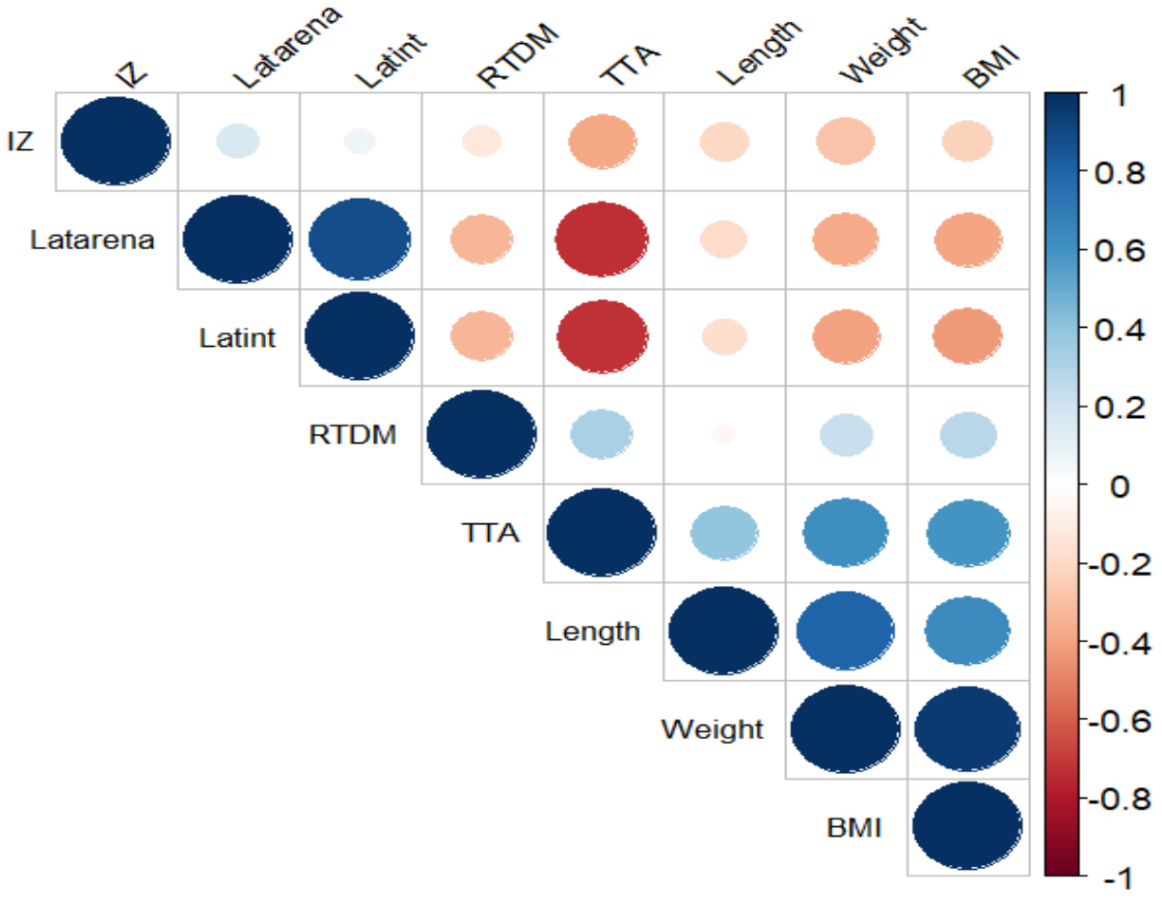
F5_32	5	1	0,2341	2,772	609	45,68	491,52	117,48	53,6	538,524
F5_35	5	1	0,24	2,756	508,6	23,48	433,92	74,68	36,64	1660,86
F5_43	5	1	0,2422	2,77	570,48	0,48	380,12	190,36	3,2	1199,83
F5_4	5	1	0,2311	2,672	905,48	0	798,88	106,6	89,84	1006,65
F5_18	5	1	0,2068	2,706	371,88	262,32	356,68	15,2	299,2	198,97
F5_29	5	1	0,2458	2,706	1033,08	53,12	809,48	223,6	63,44	2435,64
F5_33	5	1	0,2017	2,674	631,92	61,16	488,52	143,4	67,68	697,652
F5_34	5	1	0,234	2,752	22,04	1124,36	15,88	6,16	1130	15,9437
F5_38	5	1	0,2258	2,687	480,04	0	416,92	63,12	36,84	1213,75
F5_48	5	1	0,2555	2,715	480,92	9,4	362,56	118,36	97,32	783,056
F5_50	5	1	0,1858	2,507	593,52	106,32	565,56	27,96	119,44	1784,83
F5_14	5	1	0,277	2,84	0	1200	0	0	1200	0
F5_19	5	1	0,2579	2,77	0	1200	0	0	1200	0
F5_26	5	1	0,2731	2,87	0	1200	0	0	1200	0
F5_40	5	1	0,1952	2,627	124,36	1062,96	103,88	20,48	1084,24	180,455
F5_2	5	2	0,2652	2,858	745,64	124,4	703,4	42,24	126,92	664,484
F5_11	5	2	0,261	2,795	42,52	370,88	35,8	6,72	376,16	73,533
F5_3	5	2	0,2256	2,732	484,92	238,88	444,48	40,44	259,92	925,279
F5_7	5	2	0,2476	2,837	226,28	282,28	223,04	3,24	332,32	187,167
F5_10	5	2	0,2489	2,833	615,36	270,96	566,04	49,32	284,4	696,456
F5_6	5	2	0,2475	2,744	320,92	157	252,76	68,16	173,24	461,128
F5_12	5	2	0,2686	2,799	755,8	149,52	603	152,8	161,88	3252,56
F5_17	5	2	0,2981	2,956	89,8	645,04	84,36	5,44	1114,24	156,987
F5_21	5	2	0,2376	2,599	455,84	188,92	430,44	25,4	198,16	350,8
F5_22	5	2	0,2431	2,67	253,08	10,24	235,44	17,64	25,68	331,846
F5_23	5	2	0,2377	2,744	64,12	1042,2	54,16	9,96	1075,68	114,613
F5_25	5	2	0,2716	2,883	322,12	272,04	274,28	47,84	278,8	1072,1
F5_32	5	2	0,2341	2,772	52,52	253,96	51	1,52	558	106,542
F5_35	5	2	0,24	2,756	351	250,76	325,8	25,2	282,48	947,018
F5_43	5	2	0,2422	2,77	779,64	17	577,76	201,88	26,2	3132,18
F5_4	5	2	0,2311	2,672	49,68	1150,36	22,64	27,04	1154,24	60,648
F5_18	5	2	0,2068	2,706	0	1200	0	0	1200	0
F5_29	5	2	0,2458	2,706	401,24	81,52	288,8	112,44	92,24	581,056
F5_33	5	2	0,2017	2,674	206,24	698,76	153,08	53,16	704,88	323,028
F5_34	5	2	0,234	2,752	50,92	711	45,36	5,56	714,96	170,192
F5_38	5	2	0,2258	2,687	767,24	0	614,16	153,08	176,8	1861,12
F5_48	5	2	0,2555	2,715	166,52	159,08	122,08	44,44	162,64	117,965
F5_50	5	2	0,1858	2,507	208,04	805,72	154,92	53,12	850,24	452,252
F5_14	5	2	0,277	2,84	347,24	516	320,04	27,2	530,36	512,372

F5_19	5	2	0,2579	2,77	0	1200	0	0	1200	0
F5_26	5	2	0,2731	2,87	494,04	439,72	426,8	67,24	714,24	1361,08
F5_40	5	2	0,1952	2,627	299,44	57,64	298,36	1,08	439,16	462,296
F5_57	5	2	0,2238	2,669	15,6	1128,8	15,6	0	1200	40,3324

S3 Table of correlation coefficients and p-values of morphological and behavioural variables of *Kryptolebias marmoratus*. * p<0.05; ** p<0.01; *** p<0,001.

	Weight	Length	BMI	Total time in arena	Latency to 1st entry in arena	Latency to 1st entry in inner zone	Relative total distance moved	Relative total time in inner zone
Weight	1,00	0,81***	0,95***	0,62***	-0,38***	-0,40***	0,23**	-0,28***
Length		1,00	0,64***	0,39***	-0,18*	-0,18*	-0,05	-0,21*
BMI			1,00	0,59***	-0,39***	-0,43***	0,28**	-0,22**
Total time in arena				1,00	-0,73***	-0,72***	0,33***	-0,39***
Latency to first entry in arena					1,00	0,88***	-0,33***	0,16
Latency to first entry in inner zone						1,00	-0,34***	0,08
Relative total distance moved							1,00	-0,13
Relative total time in inner zone								1,00

S4 Graphical representation of the correlation coefficients of the morphological and behavioural variables of *Kryptolebias marmoratus*.



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