

Research article

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## Water-borne cortisol levels show individuality and predict bold/shy behaviors in the self-fertilizing fish *Kryptolebias marmoratus*

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**Abstract.** Differences in personality traits can have important consequences for ecological and evolutionary processes because they can either provide fitness benefits (e.g., better access to food or reproductive success) or lower responsiveness to changes in the environment (i.e., lower behavioral flexibility). Since the last decade, there is a growing interest in studying the mechanisms that generate and maintain consistent individual differences in animal behavior such as inter-individual differences in endocrine traits. In fish, little is known about how repeatable, among-individual variance in endocrine traits such as cortisol levels may predict inter-individual variability in behavioral expression. Based on a common-garden experiment, we investigated whether cortisol could predict expression of behaviors associated with bold/shy personality in three naturally isogenic lineages of the self-fertilizing mangrove rivulus, *Kryptolebias marmoratus*. First, we explored both inter-individual and inter-lineage variability of water-borne cortisol levels over time. Cortisol levels were different between lineages: fish that originate from Emerson Point Preserve population (EPP) had significantly lower cortisol levels than individuals from the Dove Creek population. Moreover, while fish cortisol levels can vary over time, we observed repeatability in inter-individual variation within each lineage. We also obtain evidence for a slight but significant effect of interaction between cortisol levels and lineages on fish probability of exiting from a shelter and proportion of time before exiting from a shelter. Mangrove rivulus from the EPP lineage had lower probability of exiting from shelter and spent more time before exiting from shelter when they had low cortisol levels, compared to DC4 and DC11 individuals. Our study supports that cortisol levels have a genetic basis in the mangrove rivulus and suggests that water-borne cortisol levels predict behaviors associated with fish personality traits.

**Keywords.** Boldness, repeatability, hormone, isogenic lineage, mangrove rivulus.

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## Introduction

Personality traits refer to stable, predictable and repeatable differences among individuals in their behaviors (Réale *et al.* 2007). This behavioral individuality is a ubiquitous phenomenon in humans and in non-human animal species (Nettle 2006; Gosling 2008). Within the animal kingdom, personality traits can have an important impact on the fitness of organisms (Gosling 2001; Sih *et al.* 2004; Réale *et al.* 2007). For example, boldness (i.e., consistency in risk-taking behavior) can provide benefits such as better access to food and reproductive success (Short & Petren 2008), but it also may imply a higher risk of predation (Hulthén *et al.* 2017) and a lower responsiveness to changes in the environment (i.e., lower behavioral flexibility; Coppens *et al.* 2010). Differences in personality can therefore have important consequences for ecological and evolutionary processes (Wolf & Weissing 2012), which explains the growing interest in the study of the mechanisms generating and maintaining such consistent individual differences in behavior (Dall *et al.* 2012; Wolf *et al.* 2008; Stamps 2007; Schuett *et al.* 2010; Dingemanse & Wolf 2010; Sih *et al.* 2015).

The variability of personality expressed in a population can come from inter-individual differences in endocrine traits which depend on the genotype of the organism, the environment to which it is exposed throughout its life (or specifically during its development), and interaction between genotype and environment (Bierbach *et al.* 2017; Honegger & de Bivort 2018). In vertebrates, the endocrine system generally responds to stressful environmental variations with the release of glucocorticoids (from the hypothalamic-pituitary-adrenal axis) such as cortisol (Wingfield 2013). Cortisol has both organizational effects (i.e., that occur during a specific critical period in early development and create permanent changes in neural responses that underlie behaviors) and activational effects (i.e., that are transient and occur throughout life) that could allow them to act as mediators of personality (Crespi *et al.* 2013). To our knowledge, most studies about the link between hormones and personality traits come from humans and non-human primates (Sellers *et al.* 2007; Anestis 2011), and more recently from birds (Holtmann *et al.* 2017). A relatively recent meta-analysis (Niemelä & Dingemanse 2018) showed that “intrinsic state” (e.g., circulating hormones) explained weak but significant variation in behavior among individuals: bold individuals tend to be characterized by high cortisol titers. In fish, if cortisol levels have been shown to link with behavioral traits such as dominance (Clement *et al.* 2005; Gesto 2019), boldness/shyness (Raoult *et al.* 2012; Fürtbauer *et al.* 2015) or fearfulness (Martins *et al.* 2011), most studies focused on the responsiveness of the cortisol levels after an exposure to stress rather than on how cortisol levels may predict the variability of behavior expression. Moreover, previous works have also provided evidence of genetic variation in fish plasmatic cortisol (Barton 2002; Pottinger 2010), and there is no trait heritability without repeatability. Nonetheless, few studies have directly tested for repeatable, among-individual variance in stress-related endocrine traits such as cortisol levels (rainbow trout: Schjolden *et al.* 2005; Largemouth bass: Cook *et al.* 2011; bluegill sunfish: Cook *et al.* 2012; Trinidadian guppy: Houslay *et al.* 2019; European sea bass: Samaras *et al.* 2016, 2021).

As both hormone levels and behaviors generally have a genetic component, among-individual genetic variation needs to be decreased to only study within-individual association between hormone levels and the expression of behaviors associated with personality traits. Investigating these questions in vertebrates is challenging because natural populations are genetically diverse, which complicates the distinction between genetic and non-genetic effects, such as epigenetic. Studying twins in humans, or isogenic (i.e., genetically identical) populations in non-human animals makes this challenge easier to handle. The mangrove rivulus, *Kryptolebias marmoratus*, belongs to the latter category. This fish is a member of the *Rivulidae* family (order: Cyprinodontiformes) and inhabits mangrove ecosystems from Brazil to Florida, Central America and the Bahamas (Taylor 2012). Populations are exclusively composed of male and hermaphrodite individuals in different proportions (Taylor *et al.* 2001). Although self-fertilization is more common in plants and invertebrates, *K. marmoratus* and its sister species *K. hermaphroditus*, are the only known hermaphrodite vertebrates commonly breeding by self-fertilization (Taylor *et al.* 2001;

Chang *et al.* 2012; but see Lampert & Schartl 2008 for example on the self-fertilizing Amazon molly). Selfing allows the mangrove rivulus to express a very low genetic variability within the lineages (Razin & Shemer 1995; Mhanni & McGowan 2004; Fellous *et al.* 2018), thus making it possible to work with highly homozygous and isogenic natural lineages and, therefore, to reduce the genetic component within the strains during the experiments (Taylor 2000; Avise & Tatarenkov 2015; Tatarenkov *et al.* 2017), and thus allows investigating non-genetic variability. Importantly, different isogenic lineages can be found in their natural environment whose individuals are genetically quasi-identical within each strain (Lins *et al.* 2018), but genetically different among lineages. Moreover, this species expresses individuality and a high level of phenotypic variation (such as behavior), both within and among lineages. For example, previous studies have shown that boldness is repeatable both at the individual level and at the genotype level (Edenbrow & Croft 2013; Carion 2020). These characteristics provide a unique opportunity to better understand the factors that play a role in the evolutionary emergence and variability of behavior associated with personality traits in fishes. Mangrove rivulus is therefore a good model to study the importance of hormones in predicting behavioral phenotypes (Fellous *et al.* 2018).

In this study, we aimed to explore the putative hormonal mechanisms that underlie expression variability of behavior associated with boldness in three naturally isogenic lineages of the mangrove rivulus. Although shy/bold personality and cortisol levels appear to predict similar traits in this species (e.g., behaviors during dyadic contests; Earley *et al.* 2008; Li *et al.* 2020), relationships between hormone levels and shy/bold behaviors have not been directly determined. Moreover, significant differences in cortisol levels have been found between different isogenic lineages (Garcia *et al.* 2015). This suggests that the correlation between cortisol levels and the expression of bold behaviors can vary between lineages, and that covariation between both traits has genetic component, but this hypothesis still needs to be tested.

Here, we set up a common-garden experiment that allowed us to study over time (1) intra- and inter-individual variability and (2) intra- and inter-lineage variability of water-borne cortisol levels in adult mangrove rivulus from three isogenic lineages. Two of these isogenic lineages (DC4 and DC11) originally came from fish of the same population at Dove Creek (South Florida) whereas rivulus of the EPP lineage originated from fish collected at the Emerson Point Preserve (West Florida). Fish were then raised in the laboratory in the same controlled conditions for at least three generations. We also quantified behaviors associated with bold/shy personality after determining cortisol levels to verify if behavior expression correlated with cortisol level variation at both individual and lineage levels. If cortisol levels and their effects on rivulus behavior differed between lineages that were kept under the same environmental conditions during several generations (minimum 5 generations in the laboratory), this would imply that such differences came from genes rather than environmental factors (de Villemereuil *et al.* 2016). As such, we expected to observe higher inter-lineages variability between DC and EPP fish than between the two DC lineages.

## **Material and methods**

### **Ethics statement**

All mangrove rivulus husbandry and experimental procedures were in accordance with the Belgian animal protection standards and approved by the University of Namur Local Research Ethics Committee (UN KE 21/374). Agreement number of the laboratory for fish experiments: LA1900048.

### **Laboratory breeding**

The individuals used for this experiment came from hermaphrodite fish of the DC4 (N=11), DC11 (N=10) and EPP (N=12) lineages that are currently housed at the University of Namur (Belgium).

These hermaphrodites are individually maintained in saltwater tanks at  $12 \pm 1$  ppt (Instant Ocean™ sea salt), under controlled conditions (temperature:  $26 \pm 1^\circ\text{C}$ , light cycle: 12 dark: 12 light) and fed daily *ad libitum* with live brine shrimp *Artemia salina*. The stock population of DC4 and DC11 were originally obtained from fish sampled by Ryan L. Earley and Scott Taylor in 2010 in the Florida Keys (Dove Creek; Florida;  $25^\circ 01' 45.64''$  N,  $80^\circ 29' 49.24''$  W) and transferred to the University of Alabama (USA) to produce the F1 and F2 generations by self-fertilization (Voisin *et al.* 2016). F2 individuals were sent and acclimatized in the lab of the University of Namur. Fish from the EPP population were collected at the Emerson Point Preserve (Florida;  $27^\circ 53' 29.80''$  N,  $82^\circ 62' 55.01''$  W) by Valentine Chapelle and Frédéric Silvestre in 2019 and then acclimatized in the facilities of the University of Namur. Previous studies have shown that these three isogenic lineages are homozygous at all loci tested by microsatellite analysis (Mesak *et al.* 2014; Mackiewicz *et al.* 2006; Chapelle 2023).

### Egg collection and laboratory rearing

Mangrove rivulus eggs (N=21, 27 and 40, respectively for DC4, DC11 and EPP lineages) were collected from hermaphrodite adults (section 2.2.) and were placed individually in 24-well microplates designed for cell culture (Cellstar®) filled with 2 mL of sea water ( $12 \pm 1$  ppt). Plates were maintained at  $26 \pm 1^\circ\text{C}$  and a day: night cycle of 12 h: 12 h until hatching (i.e., 15 to 30 days after fertilization). Newly hatched larvae were transferred into 318 ml glass jars filled with 200 mL of salt water ( $12 \pm 1$  ppt,  $26 \pm 1^\circ\text{C}$ ) until sexual maturity (between 60 and 80 days post-hatching (dph)). When embryos are reared at relatively high temperatures (e.g.,  $25\text{--}30^\circ\text{C}$ ), males, if any, are numerically less abundant than at low temperatures (e.g.,  $18\text{--}20^\circ\text{C}$ ; Harrington 1967). From hatching until the end of the experiments, all individuals were fed daily *ad libitum*.

At 80 dph, the adults were sexed and then placed individually in 1 L polypropylene tanks (H×W×D:  $7.7 \times 12.0 \times 17.5$  cm) filled with 400 mL of salt water ( $12 \pm 1$  ppt; water depth: 3.5 cm depth) under controlled conditions ( $26 \pm 1^\circ\text{C}$ , day: night cycle of 12 h:12 h). Male and hermaphrodite mangrove rivulus are identifiable from external characteristics: males express orange color, faded ocellus, and black margins on anal/caudal fins, whereas hermaphrodites exhibit silver to brown skin with a black ocellus on their caudal fins (Harrington 1971; Fig. 1). Sexing was also ensured at the end of the study (see below). All mature individuals were determined as hermaphrodites. A piece of cotton was also added in each tank to allow fish to lay eggs and/or hide. No fish died during the whole experiment so final sample sizes for hormone quantification, behavioral observations and size measurements remained 21, 27 and 40, respectively for DC4, DC11 and EPP individuals.



Figure 1 – Mangrove rivulus *Kryptolebias marmoratus*. Left: hermaphrodite adult rivulus. Right: male adult rivulus. Photo credits: Frédéric Silvestre.

## Hormone collection, extraction, and assay

### Water-borne hormone collection

Hermaphrodite individuals were removed from their housing tank at 137, 154 and 171 dph to proceed to water-borne hormone collection (Fig. 2). Collection and extraction of water-borne cortisol were performed following the protocol developed by Earley & Hsu (2008). This technique enables measurement of hormones in water rather than plasma, based on hormone diffusion through gills, urine and feces (Ellis *et al.* 2004; Félix *et al.* 2013). It is thus non-invasive and well suited for repeated hormone collection in small fish species for which sufficient plasma cannot be obtained non-lethally. While it has not been confirmed in mangrove rivulus, water-borne cortisol levels have been shown to be a good predictor of plasma cortisol levels in other species such as convict cichlid fish (Earley *et al.* 2008). At 137, 154, and 171 dph, each adult was carefully transferred (handling time < 10 s) from its housing tank to 318 ml glass beakers (one fish/beaker) filled with 100 ml clean 12 ppt saltwater ( $26 \pm 1^\circ\text{C}$ ) and was kept there for 4 h. To visually isolate individuals from each other, beakers were separated by opaque partitions. After 4 h, mangrove rivulus were returned to their respective tanks. Water samples were stored in a  $-20^\circ\text{C}$  freezer for hormone extraction.

### Water-borne hormone extraction and assays

Cortisol was extracted from water with a vacuum pump and passed through a C18 solid-phase column (Waters Sep-Pak 500 mg 3 cc) fitted to a 20-port manifold. Before use, the columns were primed twice with 2 mL HPLC grade methanol washes followed twice by washes with 2 mL distilled water each. After use, the columns were purged of salt with two washes of 2 mL distilled water. Cortisol was eluted from the columns by two washes with 2 mL HPLC grade methanol. The eluted solvent was evaporated with a vacuum centrifuge (Thermo/Jouan RC10-10;  $45^\circ\text{C}$ ). The resulting hormone residue was resuspended in 600  $\mu\text{L}$  of enzyme-immunoassay (EIA) buffer supplied with the Cayman Chemicals Inc. EIA kits, and then stored at  $-20^\circ\text{C}$  until hormone assays.



Figure 2 – General experimental design of the study. Eggs from three *Kryptolebias marmoratus* isogenic lineages (n = 21, 27 and 40 respectively for DC4, DC11 and EPP lineages) were collected and maintained in clean saltwater from hatching (0 dph) to 173 dph. At 80 dph, sexually mature fish were sexed and transferred individually to adult tanks under control conditions. To investigate how cortisol levels varied over time at both individual and lineage levels, cortisol levels were determined at 137, 154 and 171 dph. Finally, shelter tests were set up at 138, 155 and 172 dph to explore if expression of shy/bold behaviors correlated with cortisol levels, age, lineage and their interactions. In accordance with the University of Namur Local Research Ethics Committee (UN KE 21/374), all fish were sacrificed at 173 dph for further analysis.

Hormone assays were performed using Cayman Chemicals Inc. EIA kits (Item N°500360-480) following the procedures recommended by manufacturers. Previous studies validated the use of Cayman Chemicals Inc. EIA kits for cortisol assays in mangrove rivulus (Earley & Hsu 2008; Earley *et al.* 2013; Voisin *et al.* 2016). Cortisol levels were assayed in duplicate for each individual on ten 96-well plates. A standard curve was run in duplicate on each plate. An additional pooled sample (N=88 water-borne extracted samples from all experimental fish) was also run in duplicate at the beginning and the end of each plate to determine intra- and inter-assay coefficient variation (CV). Absorbance was measured on a BMG LABTECH microplate reader (FLUOstar® Omega) at 405 nm. Final average hormonal levels were obtained using a Five Parameter Logistic (5PL) curve fit from ‘MyAssays’ website (MyAssays Ltd.) suitable for calculating concentrations from asymmetrical sigmoidal calibrators. 5PL function allows to significantly improve the accuracy of asymmetric assays as compared to symmetric models such as the Four Parameter Logistic function, which is important for EIA tests whose dose response curves are more asymmetric than other types of assays (<https://www.brendan.com/5pl-curve-fitting/>). All data were presented as pg per sample (i.e., per fish). Values of intra-assay CVs ranged from 1.81-12.28% (mean: 5.50%; N=10 plates) and the estimated inter-assay average CV was 15.78%.

### Behavioral observations

The day after each hormone collection (i.e., at 138, 155 and 172 dph; Fig. 2), bold/shy behaviors were assessed with the shelter test (also known as emergence test; GARCIA *et al.* 2016; CARION *et al.* 2020). Shelter test arenas consisted of a grey shelter covered with a dark grey lid giving access to a white round open zone (diameter: 21 cm) filled with 1.5 L of saltwater ( $12 \pm 1$  ppt;  $26 \pm 1^\circ$  C; Fig. 3). Each fish was individually placed in the shelter that was closed from the open zone for 5 min of acclimation. After acclimation, the door between shelter and open zone was lifted and each trial was recorded for 15 min by video (60 frames/s HDR-CX625 Sony camera).

Videos were then analyzed using Noldus Ethovision XT<sup>15</sup>™. This software automatically converts the fish images into pixels and therefore allows to accurately quantify movement patterns (Delcourt *et al.* 2013). For each video, we determined whether fish exited from the shelter or not and the latency before exiting the shelter for the first time.

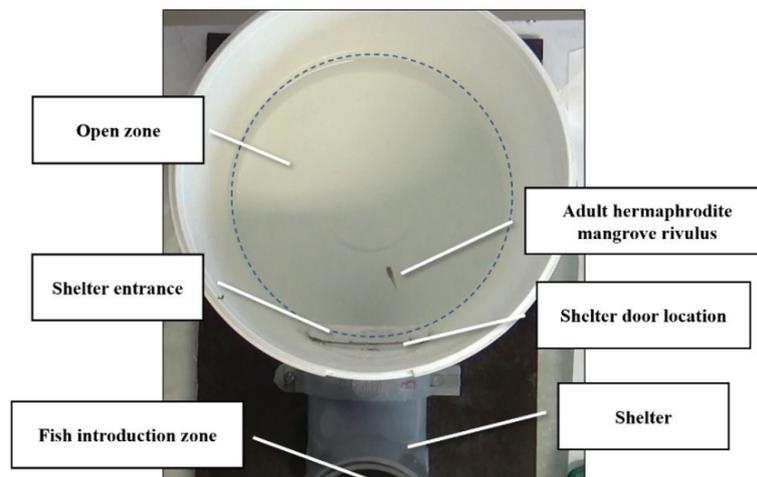


Figure 3 – Experimental design of shelter tests performed at 138, 155 and 172 dph. At the beginning of each test, fish were introduced individually for 5 min of acclimation in a grey shelter that was covered with a dark grey lid and closed from a round open zone filled with 1.5 l of saltwater ( $12 \pm 1$  ppt;  $26 \pm 1^\circ$  C). After acclimation, the door between shelter and the open zone was lifted and fish behaviors were video recorded during 15 min.

The day following the last behavioral observation (173 dph), all adults were sacrificed and their sex (hermaphrodite) was confirmed by testing for the presence of ovaries in their reproductive system.

### Statistical analysis

Statistical data analyses were performed with the software R (version 4.2.1), using  $\alpha=0.05$ .

We used parametric analyses in which the assumed distribution of residuals was matched to the data (Wilson & Hardy 2002) and, when possible, continuous data were transformed to meet standard assumptions. However, non-parametric tests were chosen when parametric conditions were not satisfied or when transforming data was inconclusive. To find the best transformation to apply, we used the 'bestNormalize' function (R package 'bestNormalize') on the explanatory variable. Here, only the cortisol data were transformed (log transformation). When significant effects were found, post hoc multiple comparison tests were run for parametric tests with the 'emmeans' function (package 'emmeans') to establish which modalities significantly differed from each other. For all analyses, we started with the maximal model and removed non-significant interactions to test the significance of main effects. Chi-square and F statistics were calculated using the 'Anova' function (package car), which performs type 2 analysis of variance for Linear and Generalized Linear Mixed Models (LMMs and GLMMs), that is, invariant to the order in which effects are entered into the model (Fox & Weisberg 2024).

First, an LMM was run to investigate how cortisol levels (log-transformed) varied with age (factor with three levels: 137, 154 and 171 dph), lineage (factor with three levels: DC4, DC11, EPP) and interaction between age and lineage, taking individuals as random factor ('lmer' function, 'lme4' package). Fish standard body length (log-transformed) was also included as fixed factor because this trait was significantly influenced by an interaction between age and lineage in this study (Appendix). Then, cortisol levels (log-transformed) repeatability [R] was calculated separately for each lineage using the 'rpt' function from the 'rptR' package. The conditional repeatability was computed, and bootstrapped 95% confidence intervals [IC] were calculated from 1000 bootstraps to assess the significance of [R] from 0. The conditional repeatability estimates the proportion of trait variation that is attributed to among-individual differences after adjusting for fixed effects within each lineage. A significant repeatability would imply that, over time, among-individual differences explain a significant proportion of trait variation (Biro & Stamps 2015). Note that we were not able to compute conditional repeatability for behaviors because of many zeros in our dataset (i.e., fish did not exit shelter during the test), which caused the estimated [R] to collapse to zero.

GLMM assuming binomial distribution of errors (link function='logit') was then performed to test the effect of cortisol levels (log-transformed), age, lineage and two-way interactions on the probability of fish exiting from shelter (0=fish did not exit from shelter, 1=fish exited from shelter), taking individuals as random factor ('glmer' function, 'lme4' package). Fish body length (log-transformed) was included as fixed factor in the model.

Finally, a GLMM assuming zero/one inflated beta distribution of errors (link function='logit') was used to investigate the effect of cortisol levels (log-transformed), age, lineage, and two-way interactions on the proportion of time spent before leaving shelter, taking individuals as random factor ('glmmTMB' function, 'glmmTMB' package). The proportion of time before leaving shelter was calculated as 'latency before exiting from shelter (s) / video duration (s)'. Fish body length (log-transformed) was also added as fixed factor in the model.

## Results

Analyses showed a main effect of both fish age (LMM:  $\chi^2_2=18.005$ ,  $P<0.001$ ) and lineage (LMM:  $\chi^2_2=20.03$ ,  $P<0.001$ ; Table 1) on cortisol levels, but no effect of interaction between age and lineages (LMM:  $\chi^2_4=5.422$ ,  $P=0.247$ ).

Cortisol levels were significantly higher at 171 dph (mean±SE: 832.9±68.00 pg.ind<sup>-1</sup>) than at 154 dph (586.5±55.8 pg. ind<sup>-1</sup>; post hoc multiple comparison tests:  $P < 0.001$ ) and 137 dph (371.31±42.50 pg.ind<sup>-1</sup>; post hoc multiple comparison tests:  $P = 0.009$ ), but no difference was found between 154 and 137 dph (post hoc multiple comparison tests:  $P = 0.773$ ; Fig. 4A). Moreover, cortisol levels in EPP individuals were significantly lower (437.4±33.28 pg.ind<sup>-1</sup>) than fish from both DC4 (982.7±102.3 pg. ind<sup>-1</sup>; post hoc multiple comparison tests:  $P < 0.001$ ) and DC11 (859.0±70.56 pg.ind<sup>-1</sup>; post hoc multiple comparison tests:  $P = 0.001$ ), whereas no difference was found between DC4 and DC11 (post hoc multiple comparison tests:  $P = 0.891$ ; Fig. 4B).

In addition, there was significant among-individual variation in cortisol levels (i.e., significant effect of ‘individual’), either in DC4 (repeatability [ $R$ ]=0.433; [CI]=[0.138, 0.660];  $P < 0.001$ ), DC11 (repeatability [ $R$ ]=0.423; [CI]=[0.146, 0.631];  $P < 0.001$ ) or in EPP lineage (repeatability [ $R$ ]=0.335; [CI]=[0.122, 0.508];  $P < 0.001$ ). However, no significant among-individual variation was found regarding probability of exiting the shelter in the DC4 (repeatability [ $R$ ]=0.03; [CI]=[0, 0.342];  $P = 0.391$ ), DC11 (repeatability [ $R$ ]=0.035; [CI]=[0, 0.301];  $P = 0.384$ ) or in EPP lineage (repeatability [ $R$ ]=0.001; [CI]=[0, 0.181];  $P = 0.494$ ). No significant among-individual variation was found in the proportion of time spent before leaving the shelter in the DC4 (repeatability [ $R$ ]=0.03; [CI]=[0, 0.342];  $P = 0.391$ ), DC11 (repeatability [ $R$ ]=0.035; [CI]=[0, 0.301];  $P = 0.384$ ) or in EPP lineage (repeatability [ $R$ ]=0.001; [CI]=[0, 0.181];  $P = 0.494$ ).

The probability of exiting from shelter (GLMM with binomial distribution of errors:  $\chi^2_2 = 8.621$ ,  $P = 0.013$ ) and the proportion of time before exiting from shelter (GLMM with zero/one inflated beta distribution of errors:  $\chi^2_2 = 6.405$ ,  $P = 0.041$ ) were significantly influenced by interactions between fish cortisol levels (log-transformed) and lineage (Table 2). When they had low cortisol levels, fish from the EPP lineage had a lower probability of exiting from shelter and spent more time before exiting from shelter compared to DC4 and DC11 individuals (Fig. 5A–B). In addition, probability of exiting from shelter increased with higher cortisol levels in EPP mangrove rivulus while it decreased for DC4 and DC11 individuals (Fig. 5A). The proportion of time before exiting from shelter decreased with higher cortisol levels in EPP fish while it increased for the DC4 and DC11 lineages (Fig. 5B).

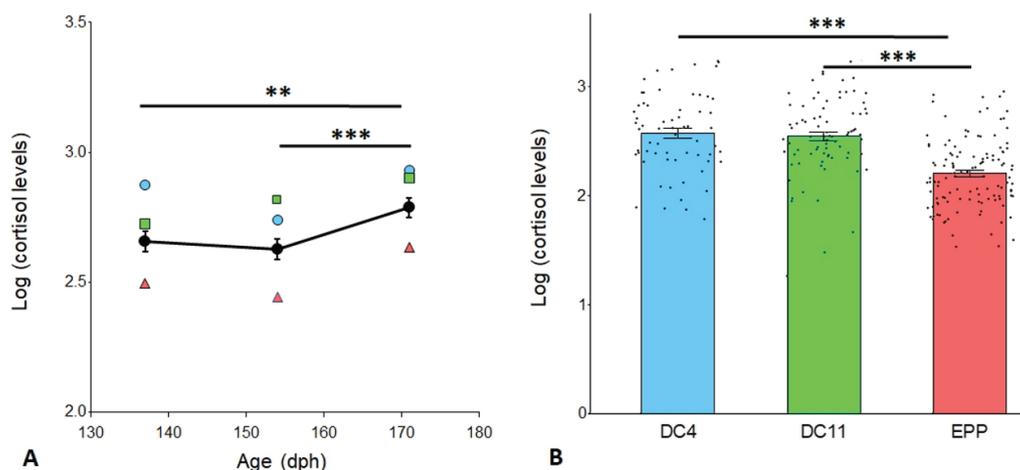


Figure 4 – **A.** Mean (± SE) cortisol levels (Log-transformed) in fish according to their age (N = 88 for 137, 154 and 171 dph). blue circles: mean Log (cortisol levels) for DC4 lineage; green rectangles: mean Log (cortisol levels) for DC11 lineage; red triangles: mean Log (cortisol levels) for EPP lineage. **B.** Mean (± SE) cortisol levels (Log-transformed) in fish according to their lineage (N = 21, 27 and 40 respectively for DC4, DC11 and EPP lineage). \*\* =  $P < 0.01$ ; \*\*\* =  $P < 0.001$ .

TABLE 1

Effect of fish age, body length (log-transformed) and lineage on cortisol levels (log-transformed; Linear Mixed Model). P values of significant explanatory variables are highlighted in bold font.

Factors affecting cortisol levels	Estimates	SE	df	$\chi^2$	P
Intercept	2.824	0.186			
Body Length	-0.037	0.686	1	0.003	0.956
Age			2	18.01	< <b>0.001</b>
155 dph	-0.028	0.041			
172 dph	0.132	0.044			
Lineage			2	20.34	< <b>0.001</b>
DC11	-0.035	0.077			
EPP	-0.323	0.079			
Residuals			259		
Total			264		

TABLE 2

Effect of fish age, lineage, cortisol levels (log-transformed) and fish body length (log-transformed) on bold/shy behaviors (Generalized Mixed Model). P values of significant explanatory variables are highlighted in bold font.

Factors affecting probability of exiting from shelter	Estimates	SE	df	$\chi^2$	P
Intercept	1.421	2.282			
Body length	4.025	3.862	1	1.086	0.297
Age			2	4.324	0.115
155 dph	-0.680	0.335			
172 dph	-0.514	0.347			
Lineage			2	0.382	0.826
DC11	-4.172	2.953			
EPP	3.346	2.739			
Cortisol levels	-0.805	0.750	1	2.621	0.105
Lineage * cortisol levels			2	8.621	<b>0.013</b>
DC11	1.533	1.038			
EPP	-1.434	1.003			
Residuals			256		
Total			264		
Factors affecting proportion of time before exiting from shelter	Estimates	SE	df	$\chi^2$	P
Intercept	0.466	1.384			
Body length			1	0.524	0.469
Age			2	2.550	0.279
155 dph	0.276	0.186			
172 dph	0.246	0.193			
Lineage			2	0.209	0.901
DC11	1.953	1.737			
EPP	-1.576	1.601			
Cortisol levels	0.414	0.457	1	3.122	0.078
Lineage * cortisol levels			2	6.405	<b>0.041</b>
DC11	-0.725	0.610			
EPP	0.647	0.579			
Residuals			256		
Total			264		

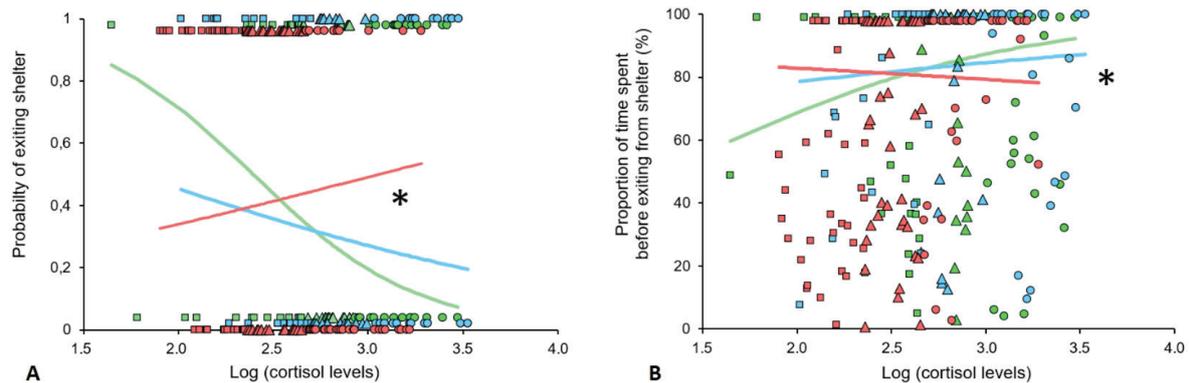


Figure 5 – Effect of interaction between fish cortisol levels (log-transformed) and lineage (green = DC4; blue = DC11; red = EPP) on A) probability of exiting from the shelter, and B) proportion of time before exiting from the shelter (%). Rectangles = 138 dph; circles = 155 dph; triangles = 172 dph; \* = significant interaction with  $P < 0.05$ .

## Discussion

In fish, little is known about how repeatable, among-individual variance endocrine traits may predict inter-individual variability in behavioral expression. By studying intra- and inter-individual variation in water-borne cortisol levels in three isogenic lineages of the self-fertilizing mangrove rivulus *Kryptolebias marmoratus*, our work aimed to investigate in a fish species whether this hormone shows individuality and predicts expression of behavior associated with bold/shy personality. However, even if the correlation between water-borne and blood/plasma cortisol levels has been demonstrated in different fish species (e.g., Earley *et al.* 2008; Wong *et al.* 2008; Friesen *et al.* 2012; Gabor & Contreras 2012), such a correlation still remains unknown in the mangrove rivulus. Interpretations should therefore be taken with caution.

Repeatability of stress response has been demonstrated in vertebrates (Schoenemann & Bonier 2018) and has been observed in both captive and wild populations in fish (Schjolden *et al.* 2005; Cook *et al.* 2011, 2012; Samaras *et al.* 2016; Houslay *et al.* 2019, 2022). However, to our knowledge, only two studies to date have directly tested for repeatable, among-individual variance in basal cortisol levels (i.e., pre-stress) in fish, and results are inconclusive. Pre-stress cortisol levels were not consistent either in wild population of the Largemouth bass *Micropterus salmoides* (Cook *et al.* 2011) or in the three-spined sticklebacks *Gasterosteus aculeatus* (Fürtbauer *et al.* 2015). In the present experiment, we found that water-borne cortisol levels collected before behavioral tests (i.e., under relatively low-stress conditions) were significantly higher at the third time point of hormone collection (i.e., at 171 dph) compared with the two previous ones (i.e., at 137 and 154 dph). This contrasts with the study of Wong *et al.* (2008) who demonstrated that water-borne cortisol levels decreased after 3–4 handling repetitions in the convict cichlid fish *Amatitlania nigrofasciata*. In the African cichlid fish *Pseudocrenilabrus multicolor victoriae*, water-borne cortisol levels did not differ between fish being handled one or more times (Friesen *et al.* 2012). Our results may suggest that handling during hormone collection caused stress to mangrove rivulus. However, one would then expect to observe a significant increase in water-borne cortisol levels at the second time point of hormone collection. We rather propose that the difference we observed in cortisol levels in each individual was due to external factors we did not test during this study. Despite this within-individual temporal variation, we observed that inter-individual variability in water-borne cortisol levels was repeatable over time in each of the three lineages, that is, cortisol levels showed individuality in a fish species. In most mammals and fishes, cortisol is involved in mediating behavior

expression in a stressful context (Mesquita *et al.* 2009; Crespi *et al.* 2012), and consistent individual differences in personality are related to differences in the responsiveness of individuals to environmental stimuli (Wolf *et al.* 2008). Thus, consistent differences in cortisol levels over time between individuals may lie beneath inter-individual variability in expression of behaviors associated with animal personality in the mangrove rivulus.

There are some indications of association between endocrine and behavioral traits in a stressful context in a range of taxa (Cockrem 2013), but few studies have investigated repeated observations on both traits in fish (Martins *et al.* 2011; Fürtbauer *et al.* 2015; Samaras *et al.* 2016). The relationship between personality and cortisol levels varies between species and might be more complex than expected (Boulton *et al.* 2015). For example, cortisol levels before behavioral tests and shyness were positively related within individuals of the three-spined sticklebacks *Gasterosteus aculeatus* (Fürtbauer *et al.* 2015), whereas a meta-analysis by Niemelä & Dingemanse (2018) showed that bold individuals tended to be characterized by high cortisol levels. Here, we found that water-borne cortisol levels interacted with lineage to predict two behavioral patterns that are associated with bold/shy personality, suggesting that the relationship between cortisol levels and behavior differed according to genetic background. The studies that have demonstrated genetic variation for plasma cortisol levels in fish (e.g., Barton 2002; Pottinger 2010) usually focused on post-stress response (Pottinger & Carrick 1999; Massault *et al.* 2010; Boulton *et al.* 2011). Here, cortisol levels also differed between three lineages of the mangrove rivulus, which is consistent with a previous study in the same species (Garcia *et al.* 2015). Overall, our data support a genetic basis of cortisol levels in fish and indicates that cortisol levels predict behaviors associated with bold/shy personality in the mangrove rivulus.

As expected, the correlation between cortisol levels and behavior in fish from the DC4 and DC11 lineages both differed from fish belonging to the EPP lineage. DC4 and DC11 individuals originally came from the same population at Dove Creek (South Florida) whereas EPP rivulus originated from the Emerson Point Preserve (West Florida). Environmental variables that may have led to evolutionary endocrine levels differing between DC and EPP populations in the wild are unknown, but different abiotic and biotic factors are known to influence inter-population variability in cortisol levels, such as predation (Fürtbauer *et al.* 2015), temperature (Alfonso *et al.* 2023) or environmental contaminants (Rohonczy *et al.* 2021). In the present study, we investigated the variability of both intra- and inter-individual cortisol levels in fish whose parent has been collected in these three different populations and then raised in same controlled conditions in the lab (i.e., common-garden experiment). This suggests that cortisol level variability as we observed between lineages and its link with rivulus behavior is inherited rather than environmentally triggered (de Villemereuil *et al.* 2016). However, disentangling whether such observed differences in heritability are genetic or non-genetic requires further investigations. For example, epigenetic mechanisms (i.e., DNA methylation, histone modifications, and non-coding RNAs; Jensen 2013) are good candidates because they have the potential to affect phenotypic traits such as hormones and behaviors by causing alterations in gene expression (Jablonka & Raz 2009; Petitjean *et al.* 2019). Thanks to its fascinating characteristic to naturally produce very low inter-individual genetic variability in the same isogenic lineage, exploring epigenetic modifications associated with hormonal variability in *K. marmoratus* could help to unravel the underlying (epi)genetic mechanisms that may explain differences between lineages in cortisol levels and their relationship with bold/shy behavior.

## Conclusion

By investigating both intra- and inter-individual variation in water-borne cortisol levels in three isogenic lineages of the mangrove rivulus *Kryptolebias marmoratus*, we demonstrated for the first time that levels of this hormone show individual variation and support the hypothesis that cortisol levels have a genetic basis. We also found that water-borne cortisol levels interacted with genetic components to

affect bold/shy behaviors expression, suggesting that cortisol levels can predict behaviors associated with fish personality traits. We suggested future research to focus on potential molecular mechanisms underlying consistent endocrine-behavioral relationships.

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### Data availability

Data will be made available on request.

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## Appendix

### S1. Difference in standard body length between isogenic lineages of adult *Kryptolebias marmoratus*

TABLE S1

Effect of lineage identity and fish age on standard body length. P values of significant explanatory variables are highlighted in bold font.

Factors affecting body length	Estimates	SE	df	$\chi^2$	P
Intercept	1.816	0.03			
Age			2	221.4	< <b>0.001</b>
154 dph	0.098	0.020			
173 dph	0.110	0.020			
Lineage			2	65.75	< <b>0.001</b>
DC11	-0.062	0.046			
EPP	0.238	0.042			
Age * Lineage			4	11.72	<b>0.020</b>
154 dph * DC11	0.027	0.027			
154 dph * EPP	-0.025	0.025			
173 dph * DC11	0.074	0.027			
173 dph * EPP	0.030	0.025			
Residuals			256		
Total			264		

Our analysis demonstrated that Standard Body Length of adult *K. marmoratus* was significantly influenced by an interaction between fish age and lineage identity (Linear Mixed Model:  $\chi^2_2=11.72$ ,  $P=0.020$ ). Fish from the EPP lineage were significantly longer at 137 (mean $\pm$ SE: 2.05 $\pm$ 0.02 cm), 154 (2.13 $\pm$ 0.03 cm) and 173 dph (2.19 $\pm$ 0.02 cm) than both DC4 (1.82 $\pm$ 0.03 cm, 1.91 $\pm$ 0.02 cm, and 1.93 $\pm$ 0.04 cm respectively for 137, 154 and 173 dph; post hoc multiple comparison tests: all  $P < 0.001$ ) and DC11 lineages (1.75 $\pm$ 0.04 cm, 1.88 $\pm$ 0.04 cm, 1.94 $\pm$ 0.03 cm respectively for 137, 154 and 173 dph; post hoc multiple comparison tests: all  $P < 0.001$ ). However, no significant difference was found between DC4 and DC11 rivulus ( $P=0.37$ , 0.72 and 0.97, respectively for 137, 154 and 173 dph). Fish from the DC4, DC11 and EPP lineages were significantly longer at 173 dph than at 137 dph (post hoc multiple comparison tests: all  $P < 0.001$ ). However, DC11 and EPP fish were longer at 173 dph than at 154 dph (post hoc multiple comparison tests:  $P < 0.01$  and  $P < 0.001$ , respectively for DC4 and EPP fish), whereas DC4 and DC11 fish had the same body length at 154 and 173 dph (post hoc multiple comparison tests:  $P=0.83$ ).