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RESEARCH ARTICLE

A mutation in monoamine oxidase (MAO) affects the evolution of stress behavior in the blind cavefish *Astyanax mexicanus*

Constance Pierre[1], Naomie Pradère[1], Cynthia Froc[2], Patricia Ornelas-García[3], Jacques Callebert[4] and Sylvie Rétaux[1,*]

ABSTRACT

The neurotransmitter serotonin controls a variety of physiological and behavioral processes. In humans, mutations affecting monoamine oxidase (MAO), the serotonin-degrading enzyme, are highly deleterious. Yet, blind cavefish of the species *Astyanax mexicanus* carry a partial loss-of-function mutation in MAO (P106L) and thrive in their subterranean environment. Here, we established four fish lines, corresponding to the blind cave-dwelling and the sighted river-dwelling morphs of this species, with or without the mutation, in order to decipher the exact contribution of *mao* P106L in the evolution of cavefish neurobehavioral traits. Unexpectedly, although *mao* P106L appeared to be an excellent candidate for the genetic determinism of the loss of aggressive and schooling behaviors in cavefish, we demonstrated that it was not the case. Similarly, the anatomical variations in monoaminergic systems observed between cavefish and surface fish brains were independent from *mao* P106L, and rather due to other, morph-dependent developmental processes. However, we found that *mao* P106L strongly affected anxiety-like behaviors. Cortisol measurements showed lower basal levels and an increased amplitude of stress response after a change of environment in fish carrying the mutation. Finally, we studied the distribution of the P106L *mao* allele in wild populations of cave and river *A. mexicanus*, and discovered that the mutant allele was present – and sometimes fixed – in all populations inhabiting caves of the Sierra de El Abra. The possibility that this partial loss-of-function *mao* allele evolves under a selective or a neutral regime in the particular cave environment is discussed.

KEY WORDS: Cortisol, Dopamine, Environmental change, Serotonin

INTRODUCTION

Monoaminergic systems control a variety of physiological functions in vertebrates, ranging from stress response (Dinan, 1996; Winberg et al., 1997) to gut motility (Bülbring and Crema, 1958; Gershon, 2013; Mawe and Hoffman, 2013), metabolic homeostasis (El-Merahbi et al., 2015), immune function (Khan and Deschaux, 1997; Nicole and Randy, 2013) and reproduction (Prasad et al., 2015). They also play roles in brain, heart, ocular and craniofacial development (Baker and Quay, 1969; Moiseiwitsch, 2000; Ori et al., 2013; Sodhi and Sanders-Bush, 2004; Souza and Tropepe, 2011). Crucially, due to their central neuromodulatory functions, they control multiple aspects of animal behavior: aggressiveness (Edwards and Kravitz, 1997; Nelson and Trainor, 2007; Olivier, 2004; Popova, 2006), locomotion (Beninger, 1983; Brocco et al., 2002; Gabriel et al., 2009; Pearlstein, 2013; Perrier and Cotel, 2015), sleep and arousal (Jouvet, 1999; Oikonomou et al., 2019; Scammell et al., 2017), food intake (Pérez-Maceira et al., 2016; Voigt and Fink, 2015) and olfaction (Gaudry, 2018).

Plastic changes in monoaminergic systems can occur in all animal species during acclimation to variations in the environment such as temperature, altitude or season (e.g. Hernádi et al., 2008; Nakagawa et al., 2016; Stefano and Catapane, 1977; Vaccari et al., 1978). Evolutionary changes in monoaminergic systems are much less studied. However, inter-species and intra-species genetic variations in monoaminergic pathway genes associated with different behaviors have been reported (e.g. Bergey et al., 2016; Staes et al., 2019). This opens the possibility that genetically encoded evolutionary changes in monoaminergic pathways could contribute to adaptation of species to their environment.

The fish *Astyanax mexicanus* is an excellent model to study adaptation after an environmental shift. It comes in two inter-fertile forms: sighted and pigmented fish living in rivers of Northern Mexico, and blind depigmented morphs living in 31 caves in North-East Mexico (Elliott, 2018; Mitchell et al., 1977). Surface-like, common ancestors of the two extant morphs colonized caves about 20,000 years ago, and have since then adapted to the total and permanent darkness of the subterranean environment (Fumey et al., 2018; Policarpo et al., 2020 preprint).

Surface fish (SF) and cavefish (CF) display many morphological, behavioral and physiological differences. Cavefish eyes degenerate during development, and their brains show multiple differences when compared with river-dwelling conspecifics (Rétaux et al., 2016). They also have more teeth and modifications of their craniofacial bone structure (Atukorala et al., 2013; Gross et al., 2014; Yamamoto et al., 2003). Cavefish are albino as they do not synthetize melanin (McCauley et al., 2004), and they have a low metabolic rate (Hüppop, 1986; Moran et al., 2014; Salin et al., 2010) and an altered intestinal motility (Riddle et al., 2018). Behavioral differences are manifold: most CF are non-aggressive (Elipot et al., 2013; Espinasa et al., 2005), do not school (Kowalko et al., 2013), sleep very little (Duboué et al., 2011) and show intense exploratory behavior (Patton et al., 2010). They are attracted to vibrations (Yoshizawa et al., 2010) and have exceptional olfaction (Hinaux et al., 2016). This ensemble, called 'cavefish behavioral syndrome', is often considered as adaptive for cave life.

At the molecular level, cavefish originating from the Pachón cave carry a point mutation in the gene for monoamine oxidase (*mao*; P106L), the serotonin- and catecholamine-degrading enzyme

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(Elipot et al., 2014). P106L decreases MAO enzymatic activity, which results in enhanced monoamine levels in the CF brain – fish possess only one form of MAO, in contrast to mammals. Because of the many roles of monoaminergic systems in development, physiology and behavior, the P106L *mao* mutation could be responsible for some of the cavefish special traits.

Here, we have systematically analysed the phenotypic effects of the P106L *mao* mutation at the neuroanatomical, behavioral and physiological levels. We also studied the distribution and the evolutionary history of the P106L mutation, in order to discuss its adaptive nature and its impact on cavefish evolution.

MATERIALS AND METHODS

Fish husbandry

Laboratory stocks of *Astyanax mexicanus* (De Filippi 1853) surface fish (origin: Texas) and cavefish (Pachón cave, Tamaulipas, Mexico) were obtained in 2004–2006 from the Jeffery Laboratory at the University of Maryland, College Park, MD, USA and were then bred in our local facility (Gif-sur-Yvette, France). Fishes were maintained at 23–26°C on a 12 h:12 h light:dark cycle and they were fed twice a day with dry food. The fry were raised in Petri dishes and fed with an unlimited quantity of micro-worms (*Panagrellus* sp., obtained from the local amateur aquarist network), after opening of the mouth. Animals were treated according to the French and European regulations for handling of animals in research. S.R.'s authorization for use of *A. mexicanus* in research is 91-116 and the Paris Centre-Sud Ethic Committee protocol authorization number related to this work is 2017-05#8604. The animal facility of the Institute received authorization 91272105 from the Veterinary Services of Essonne, France, in 2015.

Wild fish samples

Our fin clips collection from wild cavefish and surface fish populations (exact locations available on request) were obtained during field sampling trips between 2013 and 2019, under field permits 02241/13, 02438/16, 05389/17 and 1893/19 from the Mexican Secretaría del Medio Ambiente y Recursos Naturales to P.O.-G. and S.R. To obtain a fin clip, a small part of the caudal fin (approximately 3–4 mm^2) was cut with a razor blade. The individual fin clips were stored in tubes in 100% ethanol.

Fish lines

To obtain the Pachón CF without the P106L mutation, we crossed heterozygote fish identified among our laboratory Pachón breeding colony (Fig. 1A), taking advantage of the fact that the P106L mutation is not fixed in the Pachón population. To obtain a SF line carrying the mutation, a cross between a SF (wild-type *mao*) and a CF carrying the P106L (homozygote mutant) was followed by four backcrosses with SF (Fig. 1B). At each generation, we selected fish carrying the P106L mutation (heterozygotes) and backcrossed them with SF to dilute the cave genome little by little. Then, to obtain SF homozygote mutants, we intercrossed the last generation together. Theoretically, after five generations, the percentage of cave genome is 3.13%. Note that the generation time between the spawn of the *n* generation and the spawn of the *n*+1 generation was about 8 months.

mao (P106L) and *mc4r* (G145S) allele genotyping

Wild fin clips preserved in 100% ethanol and laboratory fin clips were genotyped as follows. We performed a crude lysis with proteinase K in lysis buffer (100 mmol l^{-1} Tris, 2 mmol l^{-1} EDTA, 0.2% Triton, 0.01 µg $µl^{-1}$ PK), followed by PCR and purification of DNA (NucleoSpin Gel and PCR Clean-up). For the *mao* gene: primer F-GGGAAATCATATCCATTCAAGGGG; primer R-CT-CCATGCCCATCTTGTCCATAG (see Elipot et al., 2014). For the *mc4r* gene: F-ATGAACATATCGCAGCACCA; R-TCACACAC-AATCAGAAGAAAGC (see Aspiras et al., 2015). We used the sequencing service of Eurofins Genomics (https://www.eurofinsgenomics.eu/). Homozygotes and heterozygotes at position 106 were easily detected and identified on sequence chromatograms (see Fig. 2B; the reverse sequences are shown).

mao genomic fragment sequencing

A fragment spanning 3838 bp on the *mao* gene was sequenced in 110 wild-sampled individuals originating from different caves and surface rivers. The fragment includes the exons 4 (where the P106L

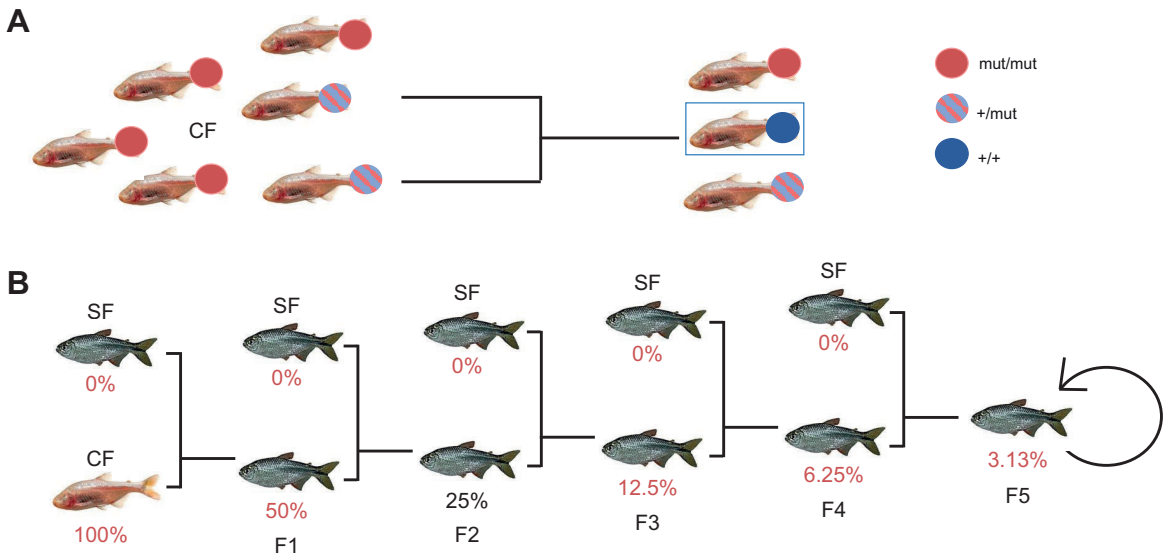

Fig. 1. Generation of genetic lines of *Astyanax mexicanus* by crosses. (A) Generation of cavefish (CF) without the P106L *mao* mutation by crosses between heterozygotes. The dots on fishes indicate their genotype: mutant (mut/mut; red), non-mutant (+/+; blue) or heterozygote (+/mut; striped). (B) Generation of surface fish (SF) carrying *mao* P106L by backcrosses. Percentage values in red denote the theoretical percentage of cavefish genome at each generation (F).

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mutation is located), 5 and 6, and occurs between positions NC_035915.1:32708790 and NC_035915.1:32713975. This fragment was subdivided into four smaller fragments, which were easier to sequence. The primers used were: Fw1-TTGTGCCTCTGTGGTGATGA, Rv1-AGTGCCGGAACCTAAAGGA; Fw2-AGCTCGCTAGCTAAATGTGTGA, Rv2-GAAGAATGCTTGCTGGAGCTG; Fw3-TCTCATCTGCTTGTTGATGGCT, Rv3-TCCCCTAGGAGCAACGAAAC; Fw4-TTTTATGGTGGCATGCAGAAGTG, Rv4-ATGATACTGCAAGCGAAGCC. Primers were designed with the primer-designing tool of NCBI (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). We used the sequencing service of Eurofins Genomics.

Immunofluorescence, immunohistochemistry and imaging

These experiments were performed on 6 days post-fertilization (dpf) wild-type SF, mutant SF and their non-mutant siblings, and CF (mutant and non-mutant). Larvae (6 dpf) were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), gradually dehydrated in ethanol, and permeabilized for 30 min in 1:1 ethanol:xylene at room temperature (RT), then 10 min in 1:2 ethanol:acetone at −20°C. The samples were rehydrated in PBS and 1% Tween (PBST). In addition, for immunohistochemistry, samples were incubated in PBST with 6% H_2O_2 for 30 min at RT. Next, the inferior jaws of the larvae were removed to make the ventral brain more accessible to antibodies. The samples were incubated in blocking buffer [10% sheep serum, 1% Triton, 1% Tween 20, 1% DMSO, 1% bovine serum albumin (BSA) in PBST] for 2 h, then incubated with the primary antibody (rat anti-serotonin, MAB352, 5/1000, Chemicon; rabbit anti-tyrosine hydroxylase, SAB2701683, 2/1000, Sigma Aldrich) in buffer (1% sheep serum, 1% Triton, 1% DMSO, 1% BSA in PBST) for 3 days, followed by the secondary antibodies (biotinylated goat anti-rat antibody, 112-066-072, 1/500, Jackson ImmunoResearch; goat anti-rabbit Alexa Fluor 635, A31577, 1/500, Life Technologies) and DAPI (4/1000) for immunofluorescence. For immunohistochemistry, revelation was performed using an avidin/biotin complex coupled with peroxidase (ABC kit, Universal Vectastain kit PK6200) and diaminobenzidine (Sigma Aldrich).

Brains were dissected by removing the palate and were mounted in Vectashield (Vector Laboratories) and glycerol for immunofluorescence and immunohistochemistry, respectively. Images were taken on a Leica confocal Sp8 and a Nikon AZ100 Macrozoom for immunofluorescence and immunohistochemistry, respectively. Images were corrected for brightness and contrast in ImageJ or Photoshop, but no other corrections were made (Fig. 3).

Behavioral analyses

Aggressiveness

These experiments were performed on 3 months post-fertilization (mpf) wild-type SF, mutant homozygotes SF, their non-mutant and heterozygotes siblings, and CF (mutant homozygotes, non-mutant and heterozygotes). Fish (3 mpf) were used in a resident-intruder test, as in Elipot et al. (2013), to allow comparison with previously obtained data. Fish were placed individually in tanks (12 cm×9 cm) with 200 ml of water for the night. In the morning, intruders were transferred into tanks of residents, and a video of 1 h was recorded. Attacks between the two fish were counted manually using ODREC software (Observational Data Recorder; https://sourceforge.net/projects/odrec/).

Shoaling

The experiments were performed on wild-type SF, mutant homozygotes SF, their non-mutant and heterozygotes siblings and CF (mutant homozygotes, non-mutant and heterozygotes, of several ages). Groups of six fish were put in tanks of 25×18 cm (1.2 liters) for 2-month-old fish, and 40×23 cm (3.6 liters) for 5-month-old fish. The results were identical at the two ages, and 5 mpf fish are shown in Fig. 4B. In Fig. 4B, N=1 corresponds to one group of the same morphotype and genotype. Tanks were placed on an infrared-emitting floor. A video of 10 min was recorded after 10 min of habituation, and the inter-individual distances (IID) and nearest-neighbor distances (NND) were calculated with ViewPoint software (http://www.viewpoint.fr/en/home). IID and NND were calculated from 10 min recordings. IID and NND were simulated for a random distribution of the fish with a program writer using Scilab v.5.5.2 (https://www.scilab.org/) (ran on graphs; black bars). Values of IID and NND for a random distribution of fish were calculated with 100,000 positioning simulations of six points (=fishes) in a square of the dimensions of the real tank. Note that we used the term ‘shoaling’ (as opposed to ‘schooling’) because we did not measure angles between fish.

Locomotion

Locomotion was studied as a developmental time course analysis, from 6 dpf to 5 mpf. For larval stages, the experiments were performed on wild-type SF and CF, both mutants and non-mutants. For 2- and 5-month-old fish, the experiments were performed on wild-type SF, mutant homozygote SF, their non-mutant and heterozygote siblings, and on CF (mutant homozygotes, non-mutants and heterozygotes). Larvae (6 dpf) were placed individually in 24-well plates, 2-month-old fish were placed in 12×9 cm tanks (200 ml) and 5-month-old fish in 19×10 cm tanks (600 ml). Tanks were placed on an infrared-emitting floor. Videos were recorded with an infrared detecting camera placed above the tanks, after 10 min, 30 min, 1 h or 24 h of habituation, depending on the experiment. For the measure of locomotion in groups, groups of six 5-month-old fish were placed in tanks of 40×23 cm (5 liters) on infrared-emitting floors, with a 12 h:12 h light:dark cycle. Videos of 10 min were recorded and locomotion was measured with ViewPoint software.

Food intake

One-year-old fish (CF: homozygote mutants, heterozygotes and non-mutants; older and bigger fish were used for this assay to increase the precision of weight measures) were placed individually in tanks (25×11×10 cm) for 5 days with no food available. On the fourth day, they were weighed. On the fifth day, food was added to the tanks, and the fish were weighed 1 h after feeding. The same protocol was used to measure food intake in groups, except that the fish were in groups of 20, and in their home tank at the fish facility.

Anxiety behaviors

The same protocol as for the recording of locomotion was used, on 5-month-old fish, alone in their tank. The different stress behaviors were analysed manually with ODREC (‘freezing’: the fish is immobile, paralysed and sometimes loses equilibrium and stays on the side; ‘thigmotaxis’: fast swimming, head against the wall of the tank; ‘erratic movements’: the fish swims very fast, and frequently changes direction, with angles of 90 deg; ‘attempts to dive’: the fish swims vertically with frenzy, head against the bottom of the tank, sometimes moving horizontally along the tank, sometimes not). Freezing, thigmotaxis and erratic movements are described and widely used to measure anxiety in other fish (Blaser and Gerlai, 2006; Blaser et al., 2010; Cachat et al., 2010; Champagne et al., 2010; Maximino et al., 2010; Schnörr et al., 2012). Although bottom dwelling was described as an anxiety behavior (Cachat et al.,

2010; Chin et al., 2018; Egan et al., 2009; Levin et al., 2007), to our knowledge attempts to dive were not, and could actually correspond to a form of thigmotaxis as they represent efforts to escape to the bottom of the tank. Freezing, thigmotaxis, erratic movements and attempts to dive were measured on wild-type SF, homozygote mutant SF and their non-mutant siblings. Only thigmotaxis was measured in CF (both mutants and non-mutants), as CF do not express other stress behaviors.

Cortisol and monoamine levels

Monoamines were measured in wild-type SF, and mutant and non-mutant CF. Cortisol was measured in homozygotes mutants, heterozygotes and non-mutant SF (siblings), as well as in mutant and non-mutant CF, and in different contexts. Measurements were made at various ages, systematically indicated in the text and figures.

Larvae (6 dpf) were immersed in water at 1°C. The heads were immediately cut and recovered to measure noradrenalin, and the bodies were recovered to measure noradrenalin and adrenalin. A sample (N=1) was formed with 15 heads or 15 bodies, in 400 µl of HCl (10^{-3} mol l^{-1}).

Groups of four to six adults (5 mpf) were placed in tanks (fish facility home tanks for several weeks, novel tank for 10 min or 24 h). They were quickly immersed in water at 1°C, and immediately frozen to avoid losing blood during dissection. The head was immediately cut and the brain dissected out and placed in 400 µl of HCl (10^{-3} mol l^{-1}) to measure monoamines, while the body (with gills) was placed in 1 ml of HCl (10^{-3} mol l^{-1}) to measure cortisol. Samples were stored at −80°C.

High-performance liquid chromatography assays were performed on fresh tissue, as previously described (Gamache et al., 1993; Kema et al., 1993). Before analysis, the brains were homogenized at 10,000 rpm in 500 µl of HCl 0.001 mol l^{-1} containing ascorbic acid and EDTA (10 µmol l^{-1}), using Ultra-Turrax T8 (IKA, Staufen, Germany). Homogenates were then centrifuged at 22,000 ***g*** for 20 min at 5°C. The supernatants were collected and filtered through a 10 kDa membrane (Nanosep, Pall, NY, USA) by centrifugation at 7000 ***g***. A 20 µl aliquot of sample was analysed for serotonin by fluorometric detection (Kema et al., 1993). The concentrations of dopamine, norepinephrine and metabolites [3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA) and 5-hydroxyindoleacetic acid (5-HIAA)] were measured by electrochemical detection on a serial array of coulometric flow-through graphite electrodes (CoulArray, Thermo Fisher Scientific Dionex, Les Ulis, France) (Gamache et al., 1993). Cortisol was analysed by immuno-chemiluminescence (kit Cortisol 8D15 840607/R4; Abbott Alinity, Abbott Park, Illinois, USA).

Cardiac rhythm

The experiments were performed on 8 dpf homozygote mutants, heterozygote and non-mutant SF (siblings), and mutant and non-mutant CF. Larvae (8 dpf) were anesthetized in a 140 ppm tricaine solution, and the cardiac beats were counted manually under a binocular microscope. We cannot rule out that tricaine had a bradycardic effect. However, if any, the effect was the same on all fish lines. Note that the concentration of tricaine used here was low and this method was preferred to low temperature for example, which had a clear and immediate effect on cardiac frequency.

Statistical analyses

No statistical method was used to pre-determine sample size. No data were excluded from analysis, and sample allocation was random after genotyping. Sex was not considered in the analyses as it impossible to determine sex in *A. mexicanus* before the age of 6–7 months without dissection/sacrifice. Analyses (except automated analyses by ViewPoint software) could not be blinded: for anatomical analyses, brains from SF or CF are easily recognizable by eye size, and for behavioral analyses, the investigator can also easily recognize the morphs on the videos. All experiments (except anatomy for mutant SF and their siblings in Fig. 3 and feeding assay in group in Fig. 6) were reproduced at least twice (=technical replicates), and the results were pooled (=biological replicates, corresponding to the N values indicated on graphs). All graphs show means±s.e.m., and the number of samples included is indicated on graph bars. In all graphs, bar outlines indicate morphotype (blue: SF; red: CF) and bar shading indicates genotype [blue: *mao* wild-type/non-mutant (+); red: *mao* mutant (m); hatched: heterozygote (+/−)].

Statistical analyses (except for two- and three-way ANOVA below) were performed with BiostaTGV, using R (https://biostatgv.sentiweb.fr/). All data were analysed in appropriate pair-wise comparisons using non-parametric Mann–Whitney tests (normal distribution was not tested). P-values are shown in the figures (***P<0.0001, **P<0.001 and *P<0.01). More detailed statistical analyses were carried out for Fig. 7 to account for the effect of time and its potential interactions with the genotype and morphotype of the fish.

For Fig. 7A, Mann–Whitney pairwise comparisons were completed by a Kruskal–Wallis analysis, accounting for all comparisons of independent means across morph and genotypes.

For Fig. 7B, in order to test for a variation of stress behavior over habituation time, Friedman tests were performed for each SF genotype (repeated measures: the same fish were tested at 10 min, 24 h and 96 h). In addition, a two-way ANOVA with repeated measures was performed to account for the time (i.e. 10 min, 24 h, 96 h) and the genotype (wild-type or mutant) of the three SF groups repeatedly assayed. Tukey's multiple comparisons tests were performed to highlight significant differences.

For Fig. 7C, a three-way ANOVA was performed to account for the three investigated factors, i.e. genotypes, morphotypes and contexts (time spent in a familiar or novel tank), prior to examining significant differences between each independent cortisol sample using a Holm–Sidak multiple comparisons test (GraphPad Prism). A one-way ANOVA was also performed to test for variations over time for each type of sample. Full summary statistics for Fig. 7 are given in Table S1.

RESULTS

Distribution of *mao* alleles in wild *A. mexicanus* populations

To estimate the spread of *mao* P106L in natural populations and its potential relevance to cave adaptation, we analysed fin clips sampled during field expeditions. We genotyped 232 cavefish from 11 caves, and 425 surface fish from several rivers in the region of Ciudad Valles, SLP, México (Fig. 2). Caves are subdivided into three geographical groups: Micos, Guatemala and El Abra, in which the different CF populations probably derive from independent colonization events (Bradic et al., 2012). The P106L *mao* mutant allele was found in high proportions in all sampled caves of the El Abra group (from north to south: Pachón, Tinaja, Los Sabinos, Curva, Toro, Chica), but not the Micos and Guatemala groups (Fig. 2A). The wild-type allele was recovered in Pachón, Tinaja, Toro and Chica, and all fish sampled in Curva (N=23) and Los Sabinos (N=33) were homozygous mutants. This suggested that the mutation has reached fixation in these two caves.

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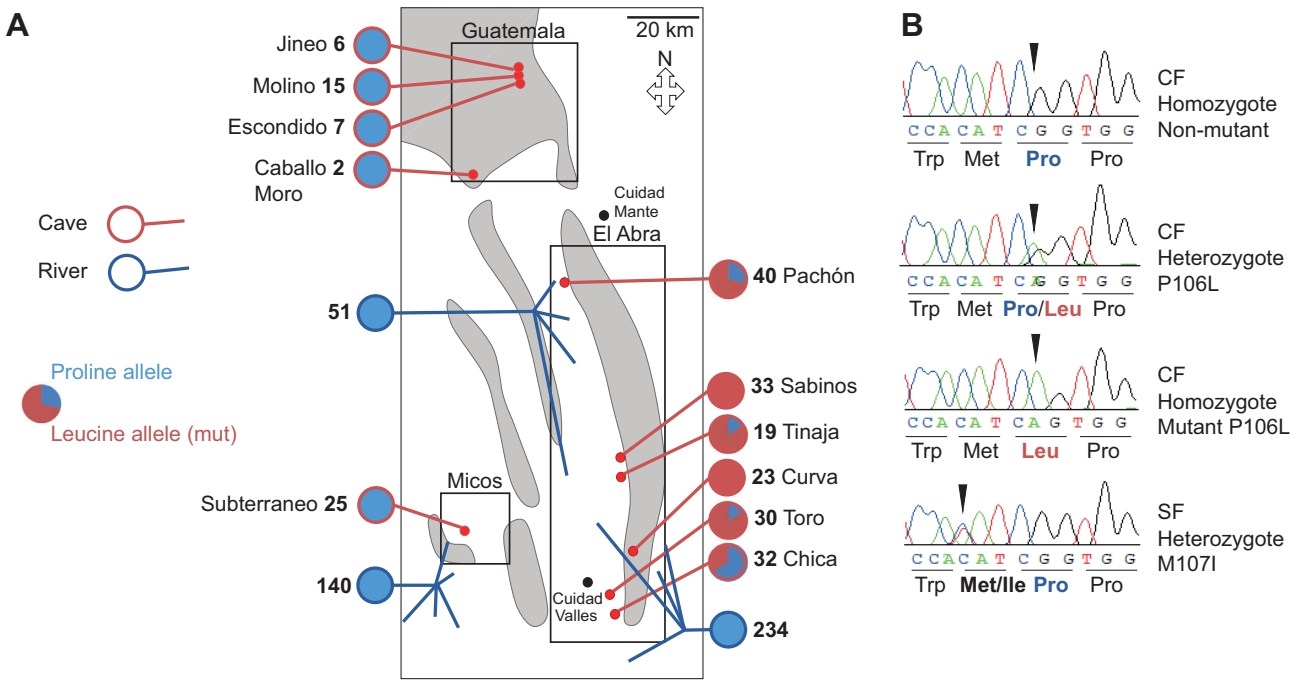

Fig. 2. Distribution of *mao* alleles in the wild. (A) Proportion of mutant alleles for 11 caves (red edges) and several zones of sampling at the surface (13 locations, blue edges). The number of fish sampled is indicated. (B) Sequence chromatograms of wild samples centered on MAO amino acid 106.

Conversely, the P106L mutation was never found in the 425 genotyped SF (=850 alleles). It is noteworthy that in *mao* exon 4 which contains the P106L mutation and in exons 5/6 which were also amplified, we identified a synonymous (not shown) and a non-synonymous mutation at position 107 (M107I, adjacent to P106L) (Fig. 2B). This indicated the presence of polymorphisms in *mao* in natural river populations of *A. mexicanus* (see also Fig. S5 and Discussion). Thus, the P106L mutation is either absent from the SF populations, or present at a very low frequency.

To study the phenotypic effects of *mao* P106L, we systematically compared four fish lines: SF and Pachón CF, wild-type or mutant/P106L. To generate these lines, we took advantage of the presence of heterozygous individuals in our Pachón CF laboratory colony: Pachón CF without the mutation were obtained by crosses between two heterozygotes (Fig. 1A), and SF carrying the mutation were obtained by successive backcrosses (Fig. 1B; Materials and Methods).

mao P106L does not affect the neuroanatomy of monoaminergic systems

The P106L mutation decreases MAO enzymatic activity, resulting in an increase of monoamine levels in the brain and body (Elipot et al., 2014; C.P., J.C. and S.R., unpublished observations). As serotonin can modulate neurogenesis and differentiation of the serotonergic system and its targets (Lauder, 1993; Pérez et al., 2013; Urtikova et al., 2009; Vitalis et al., 2013; Whitaker-Azmitia et al., 1996), we wondered if *mao* P106L could be responsible for anatomical variations reported in the serotonergic system between the two *Astyanax* morphs (Elipot et al., 2013). We also analysed catecholaminergic neurons, because links between the different monoaminergic systems are reported (Di Giovanni et al., 2008, 2010). Neurons were labeled using immunohistochemistry against serotonin (5-HT) and tyrosine hydroxylase (TH), respectively, on 6 dpf larvae of the four different lines.

The fish serotonergic system is composed of three clusters of neurons in the hypothalamus plus the rhombencephalic raphe. In agreement with Elipot et al. (2013), the size of the PVa (anterior paraventricular nucleus) was larger in CF than in SF (Fig. 3A–D,M). Here, we found that the PVi (intermediate nucleus) was also larger in CF. However, the size of these 5-HT clusters was similar in P106L mutants (SF or CF) and their non-mutant counterparts. Thus, the P106L mutation does not modify the size of 5-HT clusters (proxy of neuron numbers).

The fish dopaminergic system is more diffuse and organized in many discrete clusters, showing different cell shapes and labeling intensities (Rink and Wullimann, 2002) (Fig. 3E–K). We counted neuron numbers in some of them. For some clusters like the anterior pre-optic parvocellular group, the number of cells was the same for all four studied lines. For others like the posterior tuberal nucleus, CF possessed more cells than SF (Fig. 3N). However, similar to the 5-HT system, we could not detect any influence of the P106L mutation on the number of cells or the organization of the dopaminergic and noradrenergic systems (Fig. 3N; Fig. S1).

Overall, these data suggest that the differences observed between SF and Pachón CF in serotonin PVa and PVi sizes, or else in posterior tuberculum and telencephalic dopaminergic cell numbers, were not due to *mao* P106L but rather to other, morph-dependent developmental factor(s).

mao P106L and the cavefish 'behavioral syndrome'

A major difficulty inherent to studies comparing behaviors in the two *Astyanax* morphs is to disentangle the contribution of blindness and genetics for the expression and evolution of CF behavioral traits. Indeed, numerous behaviors are visually driven. Using the powerful approach allowed by the comparison of the effects of the P106L mutation in the four genetic lines, we try to provide definitive answers to some long-standing questions.

Previous studies have shown that deprenyl, a selective MAO inhibitor, decreases aggression and schooling in SF (Elipot et al., 2013; Kowalko et al., 2013). As these behaviors are typically lost in CF, we first tested whether *mao* P106L could be responsible for their evolution.

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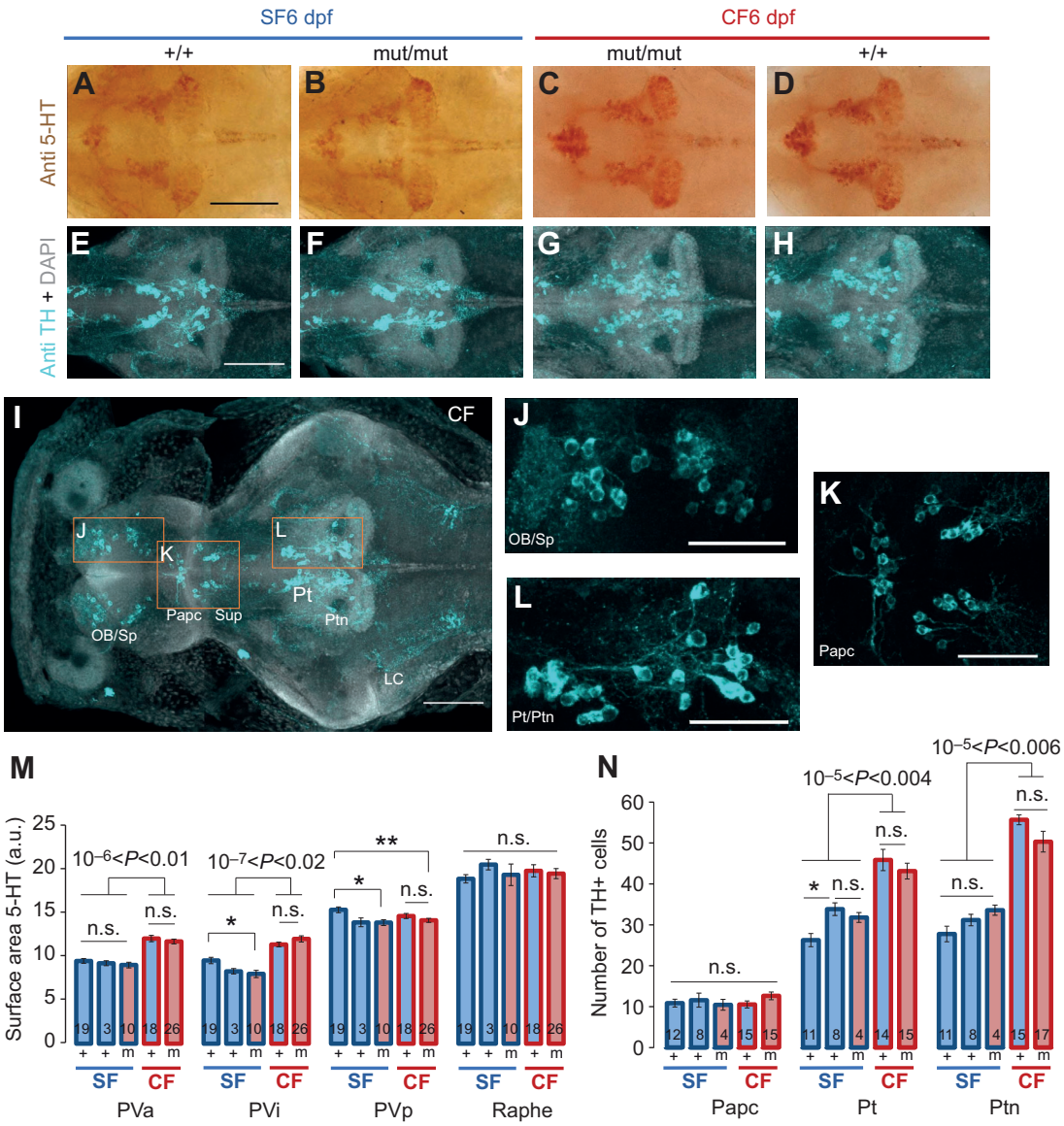

Fig. 3. 5-hydroxytryptamine and dopamine systems in 6 dpf larvae. (A–H) Immunohistochemistry for 5-hydroxytryptamine (5-HT) (A–D) and immunofluorescence for tyrosine hydroxylase (TH) (E–H) in brains of the four lines. (I) Image of a CF whole brain, showing a general view of TH-positive cells. Scale bars: 100 µm. (J–L) High magnification views of TH-positive clusters in olfactory bulb and subpallium (OB/Sp), pre-optic anterior parvocellular (Papc) and suprachiasmatic (Sup) clusters, posterior tuberculum (Pt) and posterior tuberal nucleus (Ptn). Scale bars: 50 µm. LC, locus coeruleus. (M) Size of 5-HT clusters; PVa/i/p, anterior/intermediate/posterior paraventricular nucleus. (N) Number of TH-positive cells in Papc, Pt and Ptn. Non-mutant SF (+) are wild-type (first bar of each series), or mutant (m) siblings (second bar). n.s., not significant. $^{**}P<0.001$ and $^{*}P<0.01$.

Aggressiveness was assessed using a resident-intruder test, by counting the number of attacks performed between two individuals in 1 h. As described, SF were much more aggressive than Pachón CF (Fig. 4A) (Elipot et al., 2013). However, there was no difference in aggressiveness between mutated and non-mutated SF, nor between mutated and non-mutated CF. We concluded that *mao* P106L has no influence on the evolution of aggressive behavior in *Astyanax*.

Similarly, we analysed shoaling by measuring inter-individual and nearest-neighbor distances (IID and NND) in groups of six fish (Fig. 4B). As reported (Kowalko et al., 2013), in SF IID and NND in the light were shorter than in the dark. In the dark, values were identical to those obtained by simulation of a random distribution of the fish (black bars, Fig. 4B). Thus, SF shoal in the light and light is required for shoaling. This behavioral pattern was markedly different when SF groups were habituated in their tanks for a few days (Fig. S2): there, SF decreased shoaling in the light and reached IID values similar to CF or random fish distribution; and their mean IID at night was even greater, suggesting that they voluntarily moved away from each other.

For CF in contrast, IID and NND were similar to those obtained with a random fish distribution, showing that they do not shoal. Finally, regarding *mao* genotypes, SF with or without P106L, and CF with or without P106L were indistinguishable, both in the light and the dark, both without or with habituation (Fig. 4B; Fig. S2). Therefore, *mao* P106L has no influence on the evolution of shoaling in *Astyanax*.

Next, we studied locomotion, a 'simple' trait. At 6 dpf, CF larvae were more active than SF larvae, but again there was no influence of the P106L mutation on the distance swum (Fig. 5A).

For older fish aged 2 or 5 months, CF were still more active than SF (Fig. 5B). The distance traveled by mutant SF was similar to wild-

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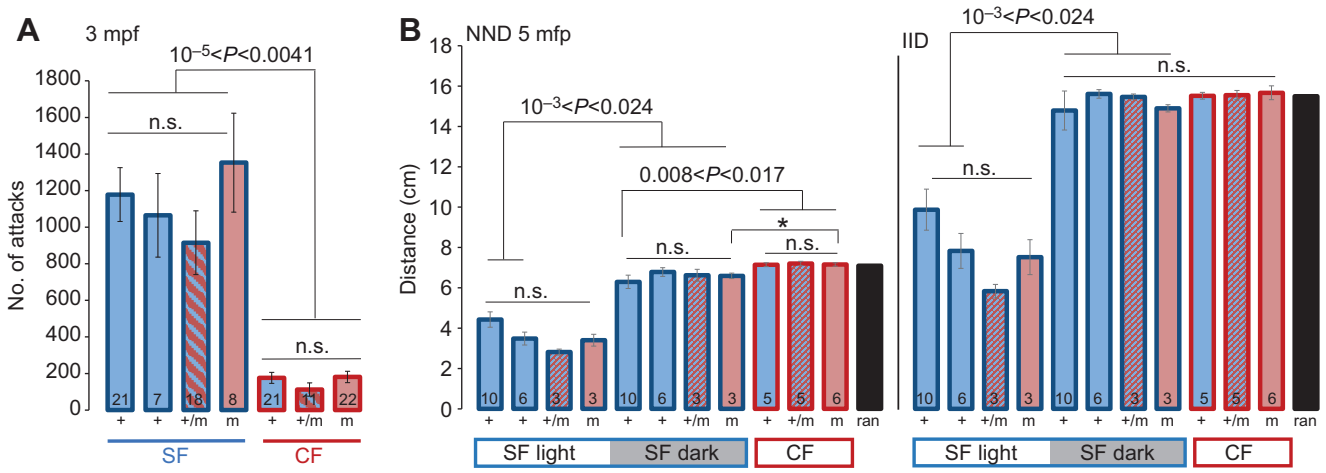

Fig. 4. Aggression and schooling. (A) Number of attacks over 1 h for fish of indicated genotypes. (B) Nearest-neighbor distance (NND) and inter-individual distances (IID) in groups of six fish, measured in the light or in the dark. Non-mutant SF (+) are wild-type (first bar of each series), or mutant (m) siblings (second bar). n.s., not significant. *P<0.01. **P<0.001 and *P<0.01).

type siblings. However, in CF at these older ages, an effect of the mutation progressively emerged, with mutant CF being more active than CF carrying the wild-type *mao* allele. Thus, the P106L mutation specifically enhanced locomotion in adult CF, but not in SF.

The locomotion recordings above were performed after 1 h of habituation, as commonly done in zebrafish assays. However, we noticed that CF placed in a novel tank for locomotion assay displayed strong thigmotaxis and frenetic swimming (Fig. 5C), i.e. behaviors described as anxiety-related behaviors in zebrafish (Blaser and Gerlai, 2006; Maximino et al., 2010; Schnörr et al., 2012). This occurred immediately after transfer into the test tank and continued after 1 h (Fig. 5C), suggesting that CF were still stressed

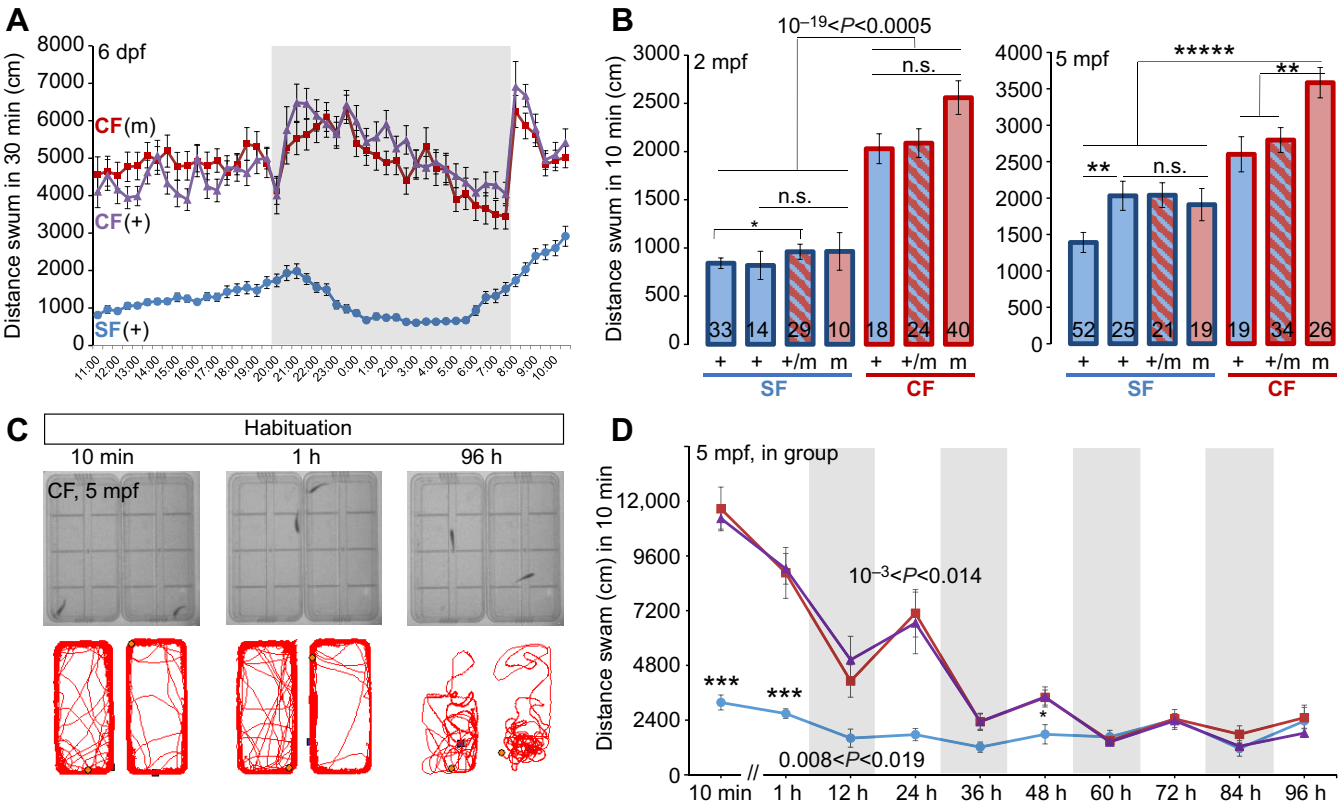

Fig. 5. Locomotion. (A) Distance traveled by 6 dpf larvae in SF (N=46), P106L mutant (m) CF (N=30) and non-mutant CF (N=26) during 30 min recordings. (B) Distance traveled during 10 min recordings by 2- or 5-month-old fish, 1 h after the beginning of the experiment. Non-mutant SF (+) are wild-type (first bar of each series), or mutant (m) siblings (second bar). (C) Screenshots of 5-month-old CF in test tanks and their trajectories over 10 min periods, at 10 min, 1 h and 96 h after the beginning of the experiment. (D) Distance traveled during 10 min recordings for groups of six fish, over 5 days. Fish tested are SF (blue; N=5), P106 L mutant CF (red; N=10) and non-mutant CF (purple; N=12). n.s., not significant. ***P<0.0001, **P<0.001 and *P<0.01.

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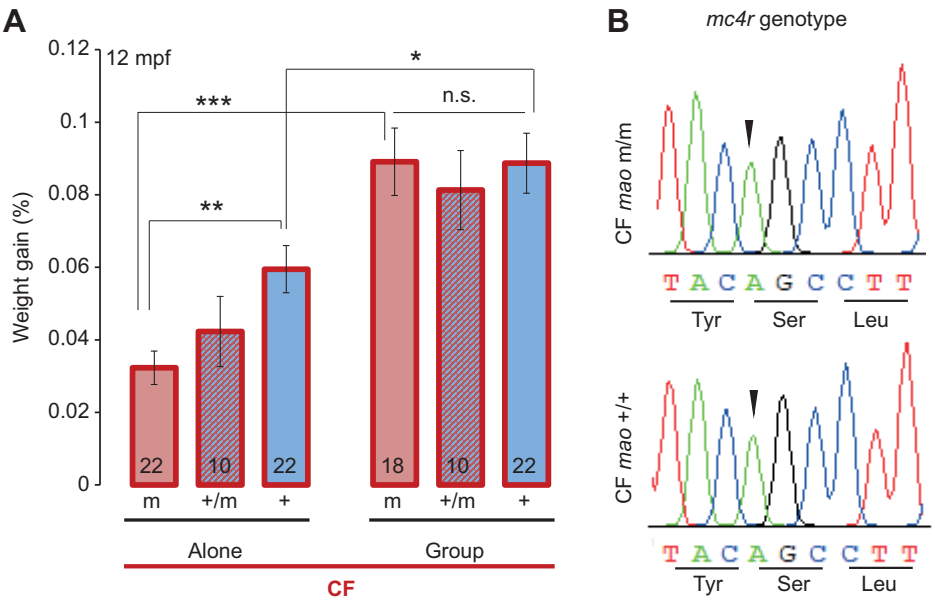

Fig. 6. Food intake in CF and *mc4r* genotype. (A) Weight gain in adult CF, either alone with 5 days of habituation, or in groups in their facility home tank. The same fish (genotypes indicated) were tested in the two conditions. (B) Sequence chromatograms centered on the Gly-to-Ser *mc4r* mutation of a P106L *mao* mutant CF (top), and a non-mutant CF (bottom). Both carry the homozygous *mc4r* mutation. n.s., not significant. ***P<0.0001, **P<0.001 and *P<0.01.

by the novel environment. Moreover, SF also still displayed anxiety behaviors such as freezing 1 h after transfer to a novel tank (not shown). We concluded that a 1 h habituation period was insufficient to accurately quantify locomotion, without being parasitized by stress behaviors. Moreover, as SF are social animals as shown by schooling, being alone in a tank certainly could be stressful.

To circumvent these biases, we studied locomotion in groups and analysed the time course of activity over 5 days (Fig. 5D). Strikingly, CF were initially more active than SF, but progressively decreased locomotion and reached SF levels after 60 h. Moreover, after 3 days of habituation there was no more influence of the morphotype or of the P106L mutation on the activity level. These data suggested that *mao* P106L increased locomotion only if the fish was alone and in a novel tank.

Finally, as serotonin plays a role in the control of food intake (Pérez-Maceira et al., 2016; Voigt and Fink, 2015) we quantified food intake in CF, alone or in groups (Fig. 6A). Alone, P106L mutant CF ate half as much food as non-mutants. Heterozygotes were intermediate. In groups, fish with all *mao* genotypes ate more than when they were alone, and there was no further influence of *mao* P106L on food intake. Thus, similar to locomotion, *mao* P106L affected food intake only when fish were alone.

A mutation in the *mc4r* gene, which causes hyperphagy in CF, has been described (Aspiras et al., 2015). This mutation is not fixed in the Pachón population, so we genotyped the individuals used in food intake assays. All the Pachón fish used carried the same *mc4r* allele, the mutant one (Fig. 6B). Thus, the differences in food intake reported above cannot be due to melanocortin signaling, and are specific to *mao* P106L mutation.

Taken together, these data suggest that *mao* P106L is not responsible for the evolution of aggressive or social behaviors it was previously suspected to control. Importantly, for other traits such as locomotion and feeding, the effects of P106L strongly depended on the context (habituation time, group size), suggesting that these behavioral phenotypes were rather read-outs of another parameter, such as stress or anxiety.

Effects of *mao* P106L on stress behaviors and stress hormones

We measured adrenalin and noradrenalin (NA) levels in 6 dpf larvae. Mutant CF had elevated levels of NA in heads (Fig. 7A) and bodies (not shown), and elevated levels of adrenalin in bodies (Fig. 7A). Importantly, the adrenalin and NA levels were identical in SF and non-mutant CF, demonstrating that *mao* P106L alone was fully responsible for increased levels of the two 'stress catecholamines' in mutant CF.

Together, these data suggest that *mao* P106L could increase fish anxiety. To test this hypothesis, we quantified stereotyped stress behaviors 10 min, 24 h and 96 h after fish were placed alone in a novel tank. For SF, we quantified four behaviors: freezing, thigmotaxis, erratic movements and attempts to dive (see Materials and Methods).

A key observation was that 5 mpf SF had individual preferences for the expression of stress behaviors: some fish almost exclusively displayed freezing, while others only showed thigmotaxis, and this preference could vary over time (Fig. S3B,C). Therefore, it was impossible to compare anxiety by quantifying specific stress behaviors independently. Instead, we considered the total time spent showing at least one of the four stress behaviors (Fig. 7B). Just after the beginning of the test, mutant and wild-type SF spent equivalent time exhibiting stress behaviors. After 24 and 96 h of habituation, mutant SF spent much more time exhibiting stress behavior than siblings and wild-type SF. The time course of response to stress was different as a function of the genotype (two-way ANOVA repeated measures: $F_{1.898,45.54}$=12.38, P<0.0001 for time; $F_{2,24}$=29.41, P<0.0001 for genotype). We concluded that the P106L *mao* mutation increased anxiety in SF.

However, measures of the time performing thigmotaxis in CF (the only stress behavior expressed by cave morphs among the four defined above) did not show any difference between mutant and non-mutant fish (Fig. S3A; Movies 1–3).

To bring support to the involvement of *mao* P106L in the evolution of anxiety behaviors in *Astyanax*, we measured cortisol levels, a reliable stress indicator, in the four fish lines, 10 min and 24 h after 5 mpf fish were placed in a novel tank, as well as in their facility home tanks (Fig. 7C). In their home tanks, P106L mutant CF had less cortisol than non-mutant CF (P=0.036, Mann–Whitney test). The same tendency was observed for SF (P=0.063). Thus, the P106L mutation seemed to reduce basal cortisol levels in a familiar tank. After 10 min in a novel tank, cortisol increased for all the lines, as expected. For both morphs, the *mao* mutants showed cortisol

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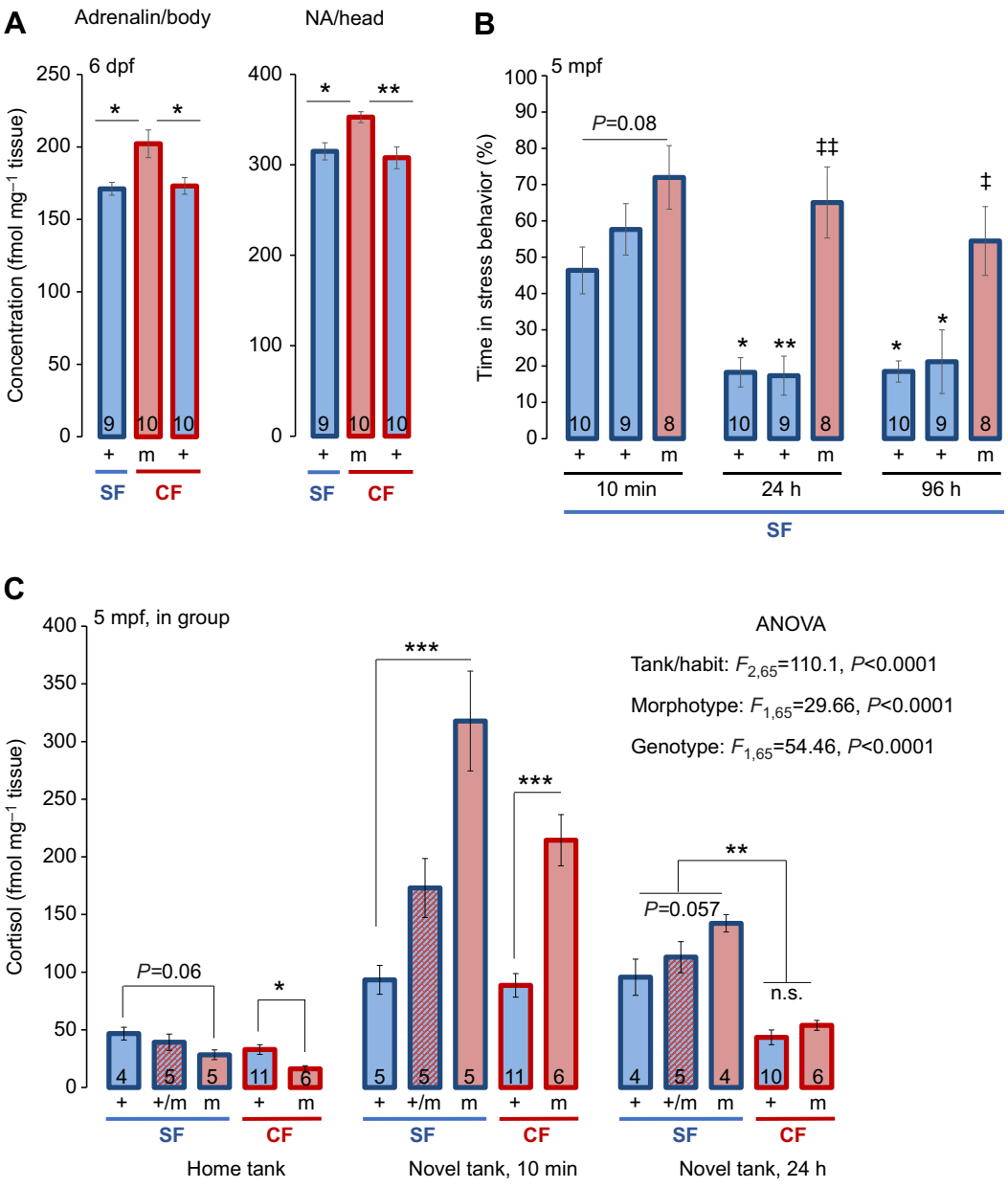

Fig. 7. Catecholamine, cortisol and stress behavior measurements. (A) Adrenalin and noradrenalin (NA) in bodies and heads, respectively, of 6 dpf larvae; Kruskal–Wallis test for adrenaline (P=0.032) and for noradrenalin (P=0.003). (B) Percentage of time spent exhibiting stress behavior (freezing/thigmotaxis/erratic movements/attempts to dive) in 5-month-old SF. Non-mutant SF (+) are wild-type (first bar of each series), or mutant (m) siblings (second bar). Recordings were performed 10 min, 24 h and 96 h after the fish was transferred alone to a novel tank. *Time comparisons, ‡genotype comparisons, two-way repeated measures ANOVA. Friedman test for wild-type (P=0.024), siblings (P=0.003) and mutants (P=0.32/n.s.). (C) Cortisol in 5-month-old fish held in groups of six individuals in their home tank, in a novel tank for 10 min, and in a novel tank for 24 h (+/m: heterozygotes). The most relevant statistical comparisons are indicated on the graph (Mann–Whitney) and inset (three-way ANOVA). Exhaustive comparisons are given in Table S1. n.s., not significant. ***P<0.0001, **,‡‡P<0.001 and *,‡P<0.01.

increases that were approximately twice as high as their non-mutant counterparts (Fig. 7C). SF heterozygotes were intermediate. This demonstrated that *mao* P106L potentiates the stress reaction in response to a change of environment. Finally, after 24 h in a novel tank, cortisol levels returned to initial levels for non-mutant CF, but not for mutant CF and for the three genotypes in SF. Moreover, mutant SF still tended to have slightly higher cortisol levels than wild-type SF (P=0.057, Mann–Whitney test). This suggests that the mutation still influenced cortisol/stress after 24 h in a novel tank: P106L mutants needed a longer time to habituate. Finally, regarding the SF/CF comparison, CF cortisol levels returned to baseline more rapidly than SF. A three-way ANOVA revealed that context/habituation represented the main source of variation (46%) and showed overall significant interactions between the three parameters, i.e. morphotype, genotype and habituation ($F_{2,65}$=3.145; P=0.0497) as well as between dual combinations of parameters (morph×genotype: $F_{1,65}$=6.972, P=0.0104; genotype×habituation: $F_{2,65}$=49.90, P<0.0001; morph×habituation: $F_{2,65}$=4.033, P=0.0223). Thus, we have uncovered an interaction between the P106L *mao* mutation, which confers stressability in *Astyanax*, and a morph-dependent variable, which increases the time of recovery to baseline in SF.

In parallel to cortisol, we also measured monoamine levels (Fig. S4). Ten minutes after stress a twofold variation of brain NA

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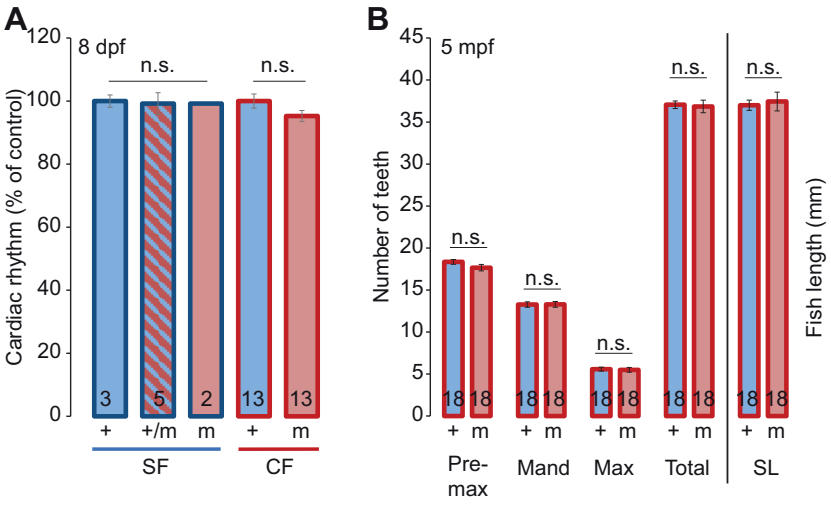

Fig. 8. Cardiac rhythm and number of teeth. (A) Cardiac rhythm in larvae. (B) Number of teeth on pre-maxilla (Pre-max), mandible (Mand) and maxilla (Max), total number of teeth (Total) and standard length (SL) in P106L mutant (m) and non-mutant (+) CF.

and 5-HT levels occurred for mutant fish but not for wild-type ones, and in both morphs, further supporting an involvement of these monoamines in stress response.

Together, these results show that the P106L *mao* mutation increases anxiety in a novel environment in *Astyanax*.

Effects of P106L mutation on other candidate phenotypes

In mice, serotonin and MAO-A modulate cardiac rhythm, development and function (Abzalov et al., 2015; Mialet-Perez et al., 2018; Nebigil and Maroteaux, 2001; Nebigil et al., 2000; Stoyek et al., 2017). However, CF have a low metabolic rate (Hüppop, 1986; Moran et al., 2014; Salin et al., 2010), so we checked if *mao* P106L modified *Astyanax* cardiac rhythm. In both morphs, mutant and non-mutant fish presented the same cardiac rhythm (Fig. 8A), suggesting no influence of *mao* P106L on this physiological parameter.

Serotonin affects craniofacial and tooth development (Moiseiwitsch, 2000; Moiseiwitsch et al., 1998; Reisoli et al., 2010). CF possess more teeth than SF (Atukorala et al., 2013), so we tested if *mao* P106L could be involved. Mutant CF had the same number of teeth as non-mutants, ruling out any influence of the P106L mutation on this anatomical trait, or on fish size (Fig. 8B).

DISCUSSION

mao alleles in the wild

We have built a phylogeographical map of *mao* alleles. The P106L mutation was present exclusively in El Abra caves, with the mutant allele found in large proportions and fixed in two caves. Either P106L has been selected, or the observed proportions have been reached by genetic drift, possibly helped by bottleneck effects during colonization or later.

Two hypotheses arise concerning the origin of the P106L mutation: (i) it appeared in a cave of the El Abra group and then spread to other caves through karstic subterranean water networks, or (ii) it appeared in the ancestral surface population, and individual(s) carrying P106L entered one or several El Abra caves. According to the latter hypothesis, the P106L allele may still be present in the surface population, in low proportions, as is the case for the *mc4R* mutation (Aspiras et al., 2015). As we did not find the mutated allele in river populations, we could not discriminate between the two hypotheses.

The fact that the mutation is not fixed in most El Abra caves suggests that it is recent. In an attempt to reconstruct the evolutionary history of the mutation, we screened polymorphisms in a genomic region of ∼4 kb around P106L, for SF and CF originating from nine different caves, in 110 wild-sampled individuals (Fig. S5). The goal was to find haplotypes corresponding to the mutant and wild-type *mao* alleles, respectively. The total number of polymorphisms encountered in the 4 kb *mao* fragment was consistent with current knowledge about the ecology of different populations (Fig. S5A). Polymorphism was high in the river population, which is large, as well as in the Chica cave, where introgression of surface alleles is frequent (Elliott, 2018; Mitchell et al., 1977; Torres-Paz et al., 2018). This also explains why the P106L allele is found at lower frequency in Chica compared with other El Abra caves. Conversely, polymorphism was lowest in Curva – where the P106L mutation is also fixed. The Curva cave is tiny, and although the population size is unknown, we can suppose it is small. Our findings regarding shared polymorphisms between cave populations are also consistent, with many polymorphisms shared within groups of caves, and few between groups (Fig. S5B). Clear haplotypes emerged (Fig. S5C), but unfortunately a simple scenario for evolutionary history of the P106L mutation in caves was impossible to draw, as too many recombination events took place. Nevertheless, haplotype distributions strongly suggest that the P106L mutation has traveled and colonized El Abra caves without being counter-selected (conclusions on positive selection cannot be drawn, see below).

Cavefish behavior

Many studies have demonstrated the involvement of serotonin in aggression in vertebrates (Edwards and Kravitz, 1997; Nelson and Trainor, 2007; Olivier, 2004; Popova, 2006). In most models, an increase in 5-HT neurotransmission causes a decrease in aggression (Carrillo et al., 2009; Summers and Winberg, 2006; Winberg and Nilsson, 1993). Accordingly, pharmacological treatments with serotonin re-uptake inhibitors or receptor agonists decrease aggression, whereas antagonists or synthesis inhibitors increase aggression (Buchanan et al., 1994; Deckel and Fuqua, 1998; Filby et al., 2010; Lopez-Mendoza et al., 1998; Lynn et al., 2007; Perreault et al., 2003; Sperry et al., 2003). Moreover, long-term supplementation of tryptophan (5-HT precursor, essential amino-acid) in food also activates the 5-HT system, and reduces aggression in trout (Winberg et al., 2001). Conversely, some studies found no change in aggression after manipulations of 5-HT neurotransmission (Filby et al., 2010; Winberg and Thörnqvist, 2016), suggesting a complex role for serotonin in the expression of agonistic behavior in fish.

In the species *A. mexicanus*, cavefish are less aggressive than surface fish (Elipot et al., 2013; Espinasa et al., 2005). Therefore, the P106L *mao* mutation was an ideal candidate to serve as the genetic basis of reduced aggression. However, here we demonstrate that even though *mao* P106L does increase 5-HT levels in 5 mpf fish (Fig. S4), it does not affect aggressive behavior, regardless of vision being present or not. This is even more surprising because Elipot et al. (2013) had shown that SF under acute fluoxetine treatment were less aggressive. An explanation is that an acute increase in 5-HT produced pharmacologically may differ from the chronic MAO inhibition produced by the mutation, which could induce plastic changes in neuronal networks and homeostatic compensations to elevated 5-HT levels. Elipot et al. (2013) also performed developmental manipulations in CF using cyclopamine (Shh signaling inhibitor), which resulted in a decrease in size of the PVa and raphe clusters and an increase in aggression. Here, we did not observe any change in 5-HT cluster size caused by *mao* P106L at 6 dpf – but note that we cannot exclude anatomical changes in the mature brain – only changes in 5-HT levels, which may not suffice to change aggression. Finally, as dopamine (DA) stimulates aggression in mammals (Nelson and Trainor, 2007; Rodríguiz et al., 2004) and maybe also in fish (Filby et al., 2010; Winberg and Nilsson, 1993), we can hypothesize that the increases in both 5-HT and DA caused by *mao* P106L could compensate for each other, resulting in no change in aggression.

Schooling or shoaling (as studied here) has advantages as it reduces the threat of predation and facilitates visual food search (Miller and Gerlai, 2011). This behavior is supported by vision and lateral line (Partridge and Pitcher, 1980). We found that light is necessary for SF to shoal, which is consistent with vision being required for this behavior (Kowalko et al., 2013). Moreover, SF shoaled less when they were habituated in their tank, consistent with other fish studies describing a reduction of shoaling due to habituation to the environment (Al-Imari and Gerlai, 2008; Delaney et al., 2002). Indeed, fear is one of the driving forces of shoaling, and if habituated fish are disturbed, they start schooling again (C.P. and S.R., personal observations).

Forming a school is important for predator detection and escape. However, it requires vision, and becomes dispensable in environments such as caves where predators are absent. Moreover, in the dark, anti-predator advantages of forming a school are lost, and schooling becomes disadvantageous as from the predator point of view, all prey is in the same area. Most social fish species do not school at night (Pavlov and Kasumyan, 2000). We found that SF shoals also disperse at night.

Little is known about neural bases of schooling, but the DA system seems involved as treatments with DA agonists and antagonists affect schooling (Echevarria et al., 2008; Scerbina et al., 2012). Even though *mao* P106L changes brain DA levels, we could not show any effect on schooling, again regardless of the fish having eyes or not. Finally, Kowalko et al. (2013) showed reduced schooling in SF after deprenyl treatment. Again, a possible explanation is that deprenyl causes an acute inhibition of MAO activity, whereas the mutation corresponds to a chronic inhibition. We can also predict that chronic MAO inhibition should induce modifications of expression of monoamine receptors, transporters and other signaling components that rescue a 'normal' neuromodulation despite high neurotransmitter levels. Such compensation phenomena have been described (Evrard et al., 2002).

Previous studies on *Astyanax* have shown that CF are more active than SF at adult (Carlson and Gross, 2018; Yoshizawa et al., 2015) and larval (Duboué et al., 2011) stages. In larvae this difference is due to both sleep reduction and increased waking velocity (Duboué et al., 2011). In adults, it is mostly due to sleep reduction, and hyperactivity has little contribution to the phenotype (Yoshizawa et al., 2015). Our results are consistent with that: larval CF are more active, but there is no difference in adults after 72 h of habituation. As we did not measure sleep, we do not know the contributions of sleep loss and waking velocity to these results. Also, Carlson et al. (2018) showed higher activity in adult CF than in SF. A major difference with our study is that they recorded single fish, whereas we recorded groups. SF are social animals, and a SF alone in a tank displays more freezing than in a group (authors' personal observations). It is therefore not surprising to observe reduced locomotion in SF compared with CF, in the solo condition.

Importantly, it appears that 1 h is not enough for habituation to a novel environment in our fish model, as both SF and CF still display stress behaviors. The difference of locomotion between CF and SF (alone or in groups) measured 1 h after transfer in the test tank could be explained by the fact that CF spent ∼100% of their time doing thigmotaxis (hence they moved), whereas SF also spent some time freezing (hence no movement). In these conditions, it is therefore doubtful that the activity recorded is 'true' locomotion. It rather corresponds to a biased read-out of stress behaviors. Accordingly, the only effect of the *mao* mutation we observed on 'locomotion' was for 5-month-old CF placed alone in a novel tank after 1 h (Fig. 5B). Both mutant and non-mutant CF spent almost 100% of their time doing thigmotaxis, so we can conclude that the difference in distance traveled is only due to increased velocity in mutants. The swimming of mutant CF was more frenetic, which is consistent with our measures of cortisol levels. We did not observe this difference in frenzy and velocity when fish were in groups. This suggests that the group has some anxiolytic effect, even in CF, usually considered as non-social animals.

Many studies have reported a decrease in locomotor activity when serotonin levels are increased (Fingerman, 1976; Gabriel et al., 2009; Perreault et al., 2003; Winberg et al., 1993). Conversely, an increase of DA activity usually results in increased locomotion in mammals (Beninger, 1983) and fishes (Boehmler et al., 2007; Bretaud et al., 2004; Godoy et al., 2015; Irons et al., 2013; Jay et al., 2015; Lambert et al., 2012; Tran et al., 2015). However, although the P106L mutation changes 5-HT and DA levels, it did not affect locomotor activity. Changes in 5-HT and DA levels may compensate.

Several lines of evidences converged towards P106L *mao* mutation increasing fish anxiety: higher catecholamine levels, lower food intake when fish are alone in their tank, and higher locomotion in a novel tank. The literature also supports a link between serotonin and anxiety (Chen et al., 2004; Herculano and Maximino, 2014; Lillesaar, 2011).

To validate this hypothesis, we quantified stress behaviors using a novel tank test, known to induce anxiety in fishes (Bencan et al., 2009; Blaser et al., 2010; Cachat et al., 2010; Egan et al., 2009; Kysil et al., 2017; Levin et al., 2007) and other species (Moriarty, 1995; Simon et al., 1994; Treit and Fundytus, 1988). Fish have many displays of stress behaviors, used to measure anxiety: freezing, thigmotaxis (wall-following), erratic movements, leaping and bottom dwelling (diving) (Bencan et al., 2009; Blaser and Gerlai, 2006; Blaser et al., 2010; Cachat et al., 2010; Egan et al., 2009; Gerlai et al., 2006; Levin et al., 2007; Maximino et al., 2010; Schnörr et al., 2012; Speedie and Gerlai, 2008). *Astyanax* SF display all of these behaviors in a novel tank (Chin et al., 2018; authors' personal observations), whereas CF only display thigmotaxis (Abdel-Latif et al., 1990; Patton et al., 2010; Riedel,

1998; Sharma et al., 2009; Teyke, 1989). Such differences in stress-elicited behaviors could be due to the absence of predators in natural cave environments. In rivers, stress behaviors like freezing, erratic movements and diving are necessary to escape from predators and hide. In caves, these behaviors are probably not selected or even counter-selected, as freezing reduces the time spent foraging, and erratic movements consume energy.

Consistent with our prediction, P106L mutant SF spent more time expressing anxiety behaviors than non-mutant SF. We also expected more thigmotaxis in mutant CF than in non-mutants, but it turned out not to be the case. A possible explanation could be that in CF, the robust thigmotaxis elicited by a novel environment may not entirely be an anxiety behavior and an attempt to escape, but may also be a form of exploratory behavior.

In zebrafish and rodents, thigmotaxis is considered an anxiety behavior, as an anxiolytic treatment like diazepam decreases it, and an anxiogenic treatment like caffeine promotes it (Schnörr et al., 2012; Treit and Fundytus, 1988). Such experiments were not conducted in *Astyanax* SF, but their behaviors in a novel tank are very similar to zebrafish. Cavefish may be different. Interestingly, in CF the duration of thigmotaxis is shorter in a simple environment than in a complex environment, or when the fish is placed in a familiar environment, except when memory consolidation has been impaired (Teyke, 1989). Moreover, CF can detect spatial changes in their environment (Burt De Perera, 2004), and fish can build a cognitive spatial map (Burt De Perera, 2004; Rodriguez et al., 1994). Finally, CF swim slightly more parallel to the wall than SF when performing thigmotaxis (Sharma et al., 2009), consistent with thigmotaxis possibly corresponding to a different expression in CF (exploratory) and SF (escape). Then again, spatial learning in fishes involves the telencephalon (Broglio et al., 2010; Rodríguez et al., 2002; Saito and Watanabe, 2006), and CF still display thigmotaxis and habituation after telencephalic ablation (Riedel, 1998). Moreover, as thigmotaxis is an anxiety behavior in SF and other species, including humans (Kallai et al., 2005, 2007), the most parsimonious hypothesis is that it is also anxiety related in CF. A simple way to confirm this idea (or not) will be to treat CF with anxiolytic/anxiogenic drugs. Finally, the two hypotheses (anxiety and exploration) are not mutually exclusive, and thigmotaxis elicited by stress may allow exploration of the environment. Anyway, there must be a stress component in thigmotaxis, as CF placed in a novel environment do show a marked increase in cortisol levels.

In fish (and mammals), an increase of plasmatic cortisol occurs after or during a stressor, like sight of a predator (Barcellos et al., 2007), crowded environment (Ramsay et al., 2006), social stress (Øverli et al., 1999; Tea et al., 2019), chasing (Gesto et al., 2008), confinement (Backström et al., 2011; Gallo and Jeffery, 2012; Kiilerich et al., 2018; Schjolden et al., 2006; Vijayan et al., 1997) or novel tank (Kysil et al., 2017). The hypothalamus releases corticotropin-releasing factor (CRF), which induces ACTH release in the blood stream, which in turn stimulates cortisol release by inter-renal cells of the head kidney. This is the hypothalamic–pituitary–inter-renal axis (HPI) axis, homolog to the hypothalamic–pituitary–adrenal (HPA) axis of mammals (Mommsen et al., 1999; Wendelaar Bonga, 1997). Cortisol has multiple effects on organs and tissues. It induces a re-allocation of energy to cope with stress, with an increase of gluconeogenesis, lipolysis and proteolysis, and an inhibition of growth, reproduction and immune responses (Aluru and Vijayan, 2009; Faught and Vijayan, 2016; Harris and Bird, 2000; Mommsen et al., 1999).

A previous study concluded there was a reduction of anxiety in CF compared with SF (Chin et al., 2018). Here we show that the difference between the two morphs is more subtle than 'more or less stressed'. In normal conditions (home tank), P106L mutants have lower cortisol levels than non-mutants. In a novel tank, P106L mutants have higher cortisol levels. In the wild, as most fish in the Pachón cave are mutants, we can assume that they are less stressed than SF in normal conditions, and more stressed after stressful stimulus. An explanation may lie in the link between monoamines and cortisol release during the stress response. Indeed, positive or negative modulations of cortisol release by catecholamines and serotonin are known (Höglund et al., 2002; Medeiros et al., 2010; Rotllant et al., 2006; Saphier et al., 1995; Winberg et al., 1997), and this would regulate the intensity of the stress response (Gesto et al., 2015). Here, 10 min after stress we detected a twofold variation of brain NA and 5-HT levels in mutant but not wild-type fish, in both morphs (Fig. S5), providing strong support for the involvement of monoamines in the regulation of the stress response.

We suggest that the change in basal monoamine levels due to the P106L mutation could alter the balance of 5-HT and DA neuromodulation on the HPI axis. It is also possible that chronic inhibition of MAO by the mutation could induce neuronal plasticity and changes in the monoamine network, and their effect on the HPI axis. Indeed, acute MAO inhibition by deprenyl has no effect on cortisol levels in humans (Koulu et al., 1989), while MAO-A knockout/inactivation induces a decrease in corticosterone levels (Popova et al., 2006). Why a chronic inhibition of MAO induces changes in the regulation of the HPI axis remains unclear and needs further investigation.

Finally, CF cortisol levels returned to basal levels more quickly than SF. Therefore, there is a morph-dependent parameter that accelerates cortisol decrease in CF. Strickler and Soares (2011) found that cannabinoid receptor *CB1* expression is up-regulated in CF, and the endocannabinoid system is known to modulate the mammalian HPA stress axis, specifically enhancing recovery to baseline following stress (Hillard et al., 2017; Micale and Drago, 2018). The up-regulation of *CB1* in CF is a good candidate for explaining faster recovery of basal cortisol levels in CF.

mao P106L: selected or not?

The high proportion of mutant alleles in El Abra caves raises the question whether the mutation could be advantageous for cave life, and could be selected. Conversely, it would be counter-selected in rivers.

Rivers are stressful environments, with both biotic (predators, parasites) and abiotic stressors (changes in temperature/salinity/turbidity/water levels). Caves are more stable, buffered environments, with no predators, probably less parasitism (intermediate hosts are not always present), and little variation in water parameters (Mitchell et al., 1977). Because of the deleterious effects of cortisol on growth, immune response, reproduction and metabolism, chronically high levels of cortisol can impair all these physiological processes (Madison et al., 2015; Pickering and Pottinger, 1989).

For CF, having very low basal cortisol levels could be adaptive as growth, reproduction and the immune system would be spared. This could also facilitate energy storage and contribute to the lower CF metabolic rate (Hüppop, 1986; Moran et al., 2014; Salin et al., 2010). As caves are a 'quiet' environment, at least during the dry season, we can suppose that the large increase in cortisol observed after stress in our laboratory study would not occur frequently in the wild – and in any case, it would rapidly return to basal levels.

For SF instead, lower basal cortisol levels could decrease alertness, and impair energy mobilization and predator escape. Moreover, high cortisol increases induced by moderate stress, i.e.

over-reaction to stress, could be deleterious in the long term. Accordingly, *Brachyrhaphis episcopi* from high-predation environments show a milder stress response than individuals from low-predation environments (Brown et al., 2005). This could prevent energy expenditure for small stressors, and save energy for important ones like predators.

Thus, the mutation may actually be counter-selected in rivers, and positively selected in caves. A less 'adaptationist' hypothesis would be that the mutation is essentially neutral in caves, and evolves under a genetic drift regime. Future studies will need to address this question. As monoaminergic systems are involved in a variety of developmental, physiological and behavioral processes, it is also possible that *mao* P106L has other unexplored phenotypic effect(s); if so, we cannot exclude that these effect(s) may contribute to all or part of the selective process, if any.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: C.P., S.R.; Methodology: C.P., C.F., J.C., S.R.; Software: C.F.; Validation: C.P., S.R.; Formal analysis: C.P., S.R.; Investigation: C.P., N.P., J.C., S.R.; Resources: P.O.-G.; Data curation: C.P., S.R.; Writing - original draft: C.P., S.R.; Writing - review & editing: S.R.; Visualization: C.P., S.R.; Supervision: S.R.; Project administration: S.R.; Funding acquisition: S.R.

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Supplementary information

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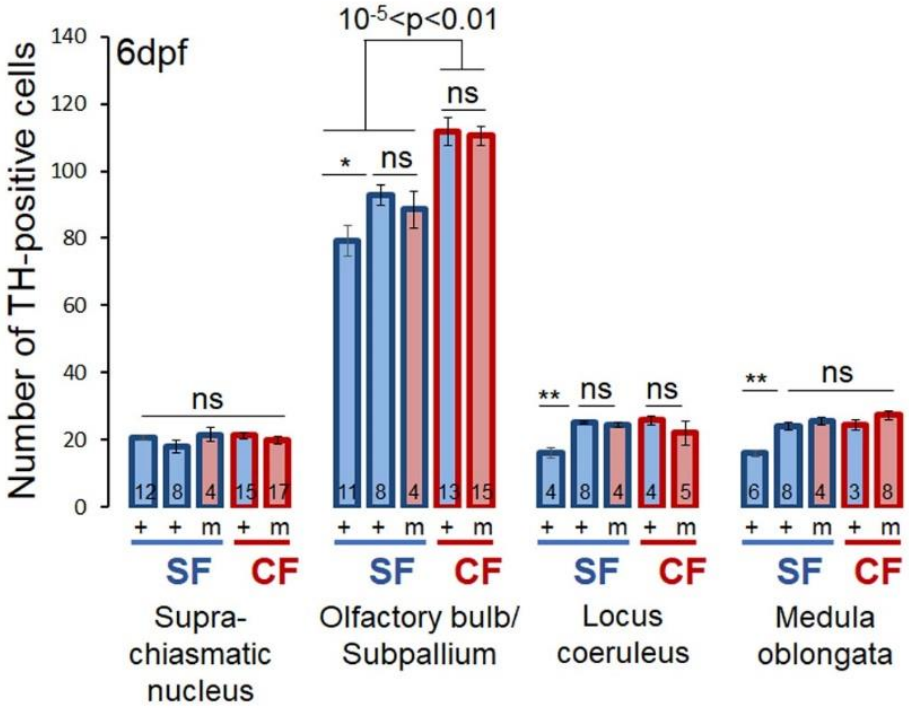


Figure S1. TH positive cells, additional clusters.

Number of TH-positive cells in suprachasmatic nucleus, olfactory bulb and subpallium, locus coeruleus and medulla oblongata, for SF and CF, both mutant homozygotes (m) or non-mutant (+). Non-mutant SF (+) tested are either wildtype (first bar), or mutants siblings (second bar).

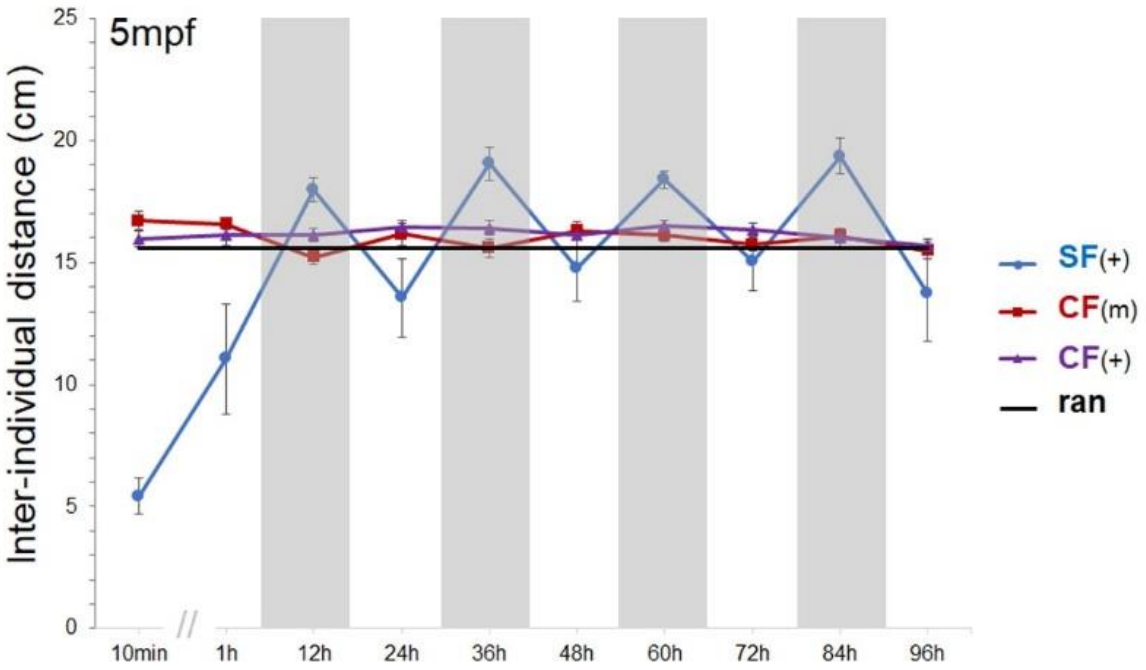


Figure S2. Shoaling in SF and CF, measured over a one week period.

Mean of inter-individual distance (IID) measured during the day or during the night, over 5 days in groups of 6 fish: n=10 groups for P106L mutant CF (red line), n=12 groups for non-mutant CF (purple line), and n=5 groups for non-mutant SF (blue line). The black line is the mean value of inter-individual distance obtained with 100.000 simulations of random distribution of fish (ran).

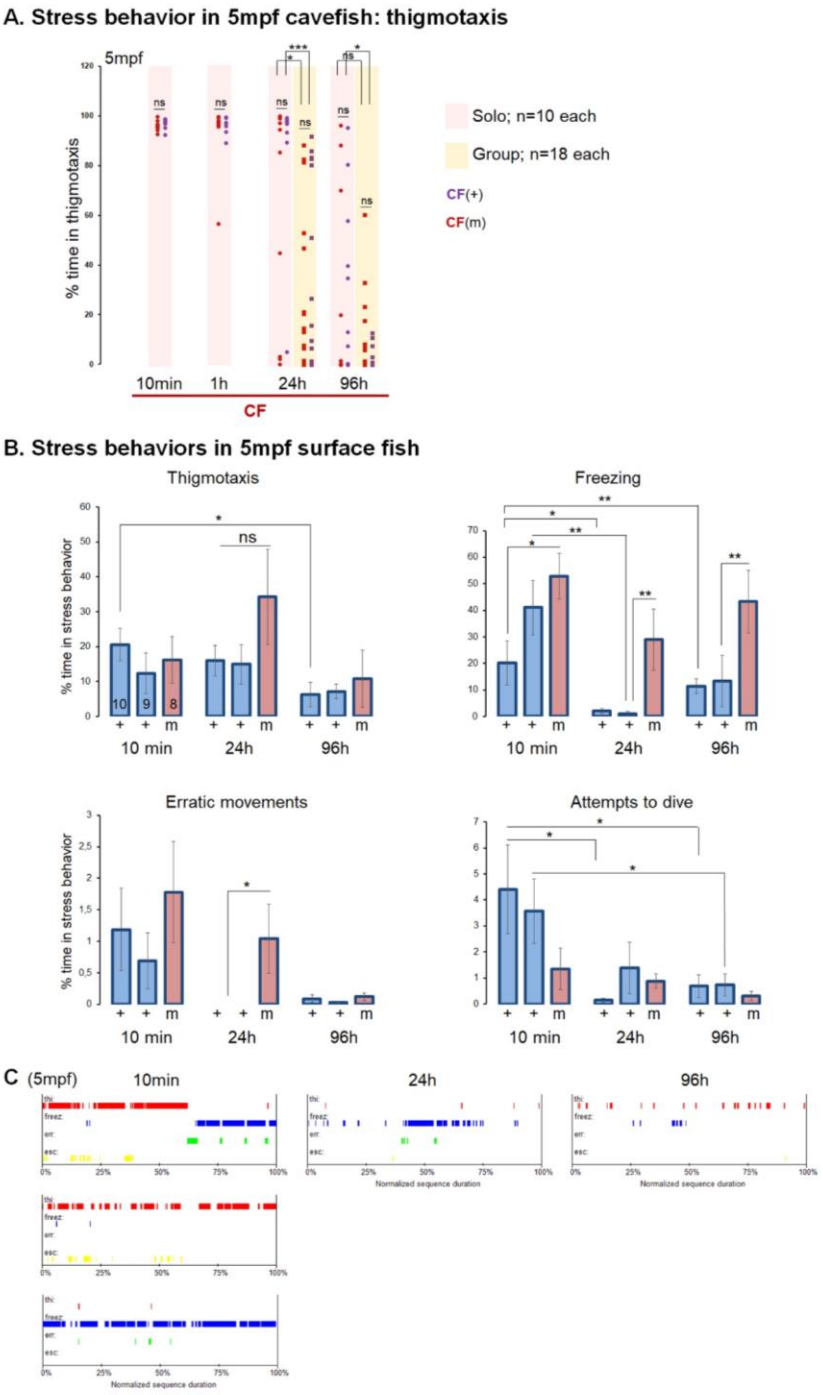


Figure S3. Stress behaviors in 5mpf CF and SF.

(A) Thigmotaxis in CF. Measurements of the percentage of time doing thigmotaxis 10min, 1h, 24h or 96h after the fish was/were put alone (pink shade) or in group (yellow shade) in a novel environment. The measurements were performed on P106L mutant CF (red) and non-mutant CF (purple), aged 5 months. Data are presented as scattered plots to represent the inter-individual variability of this phenotype at the two later time-points.

At 10min and 1h after transfer in a new tank, CF spent ~100% of their time doing thigmotaxis, regardless of their *mao* genotype. It was impossible to score groups of fish at these times (i.e., no

yellow bars for 10min and 1h) because frenetic swimming led to loss of the track of individual fish (manual scoring).

At 24h and 96h after transfer in a new tank, data could be obtained for the 4 conditions: mutant and non-mutant, solo or groups. As inter-individual variability was high, there was no detectable significant effect of the *mao* mutation on thigmotaxis behavior, either alone or in group. However and interestingly, there was a significant reduction of thigmotaxis, for both the mutant and the non-mutant CF, when the fish were in groups as compared to solo. This may suggest that the group has an anxiolytic effect on CF, although the CF morphotype of *A. mexicanus* is often described as a "non-social" animal because it does not show collective behavior such as schooling or shoaling. Of note, we cannot rule out the possibility that the "anxiolytic effect" observed here was not due to the size of the tank. Indeed, groups were tested in larger tanks (40x23cm; 5l) than single fish (19x10cm; 600ml), hence the possibility of a confinement stress in the later.

(B) Stress behavior repertoire in SF. Measurements of the percentage of time doing thigmotaxis, freezing, erratic movements and attempts to dive. The measurements were performed on P106L mutant SF (m) and non-mutant SF (+), and 10min, 24h or 96h after the fish was put alone in a novel environment. Non-mutant SF tested were either wt (first bar of each group), or siblings of mutants SF (second bar).

(C) Representative ethograms for SF, showing the alternation of periods where the fish display thigmotaxis ('thi' in red), freezing ('freez' in blue), erratic movements ('err' in green) and attempts to dive ('esc' in yellow). The 3 ethograms of the top line belong to the same fish, recorded 10min, 24h and 96h after being put in a novel environment. The 3 ethograms of the first column belong to 3 different fish recorded 10min after being put in a novel environment. Note the individual preferences for a given behavior, which can also vary along time for a single individual. Hence, the large error bars on graphs in A, and the necessity to combine all stress behaviors for analysis (as shown in Fig. 7B).

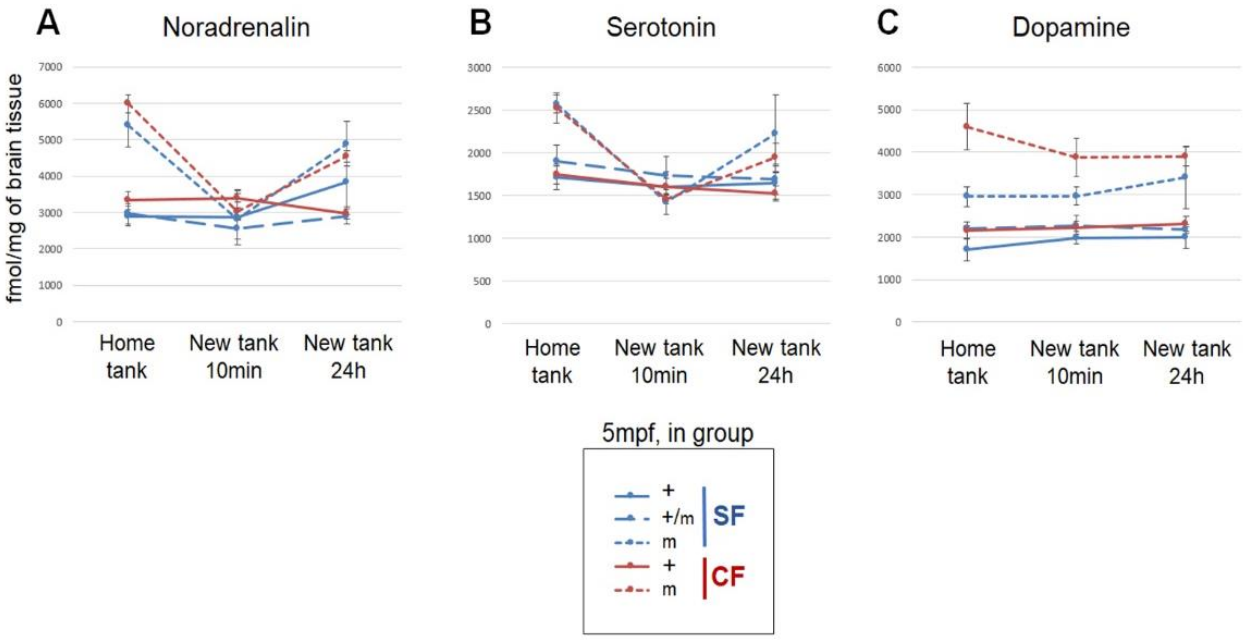


Figure S4. Levels of serotonin, noradrenaline and dopamine in the brain before, during and after a stressor (novel environment), in 5mpf fish in groups.

Noradrenaline (A), serotonin (B) and dopamine (C) measurements in the brains SF and CF, both P106L mutant homozygotes (m, dotted lines) and non-mutant (+, continuous lines), and heterozygotes SF (+/m, long dotted lines). Measurements were performed on fish held in groups of 6 in their home tank (HT) at the fish facility, in a novel tank for 10 min (NT10), and in a novel tank for 24h (NT24). Only mutants show strong variations of brain NA and 5-HT after stress.

Statistics for 5-HT: HT mCF versus HT +CF p=0.0011 ; HT mSF versus HT +SF p=0.0159 ; HT mCF versus NT10 mCF p=0.0022 ; HT mSF versus NT10 mSF p=0.0079 ; NT24 mCF versus NT24 +CF p =0.0312.

Statistics for NA: HT mSF versus HT +SF p=0.0159 ; HT mSF versus HT m/+SF p=0.0079 ; HT mCF versus HT +CF p=0.0002 ; HT mCF versus NT10 mCF p=0.0022 ; HT mCF versus NT24 mCF p=0.0022 ; NT10 mCF versus NT24 mCF p=0.0022 ; NT24 mSF versus NT24 m/+SF p=0.0159 ; NT24 mCF versus NT24 +CF p=0.0002.

Statistics for DA: HT mSF versus HT +SF p=0.0317 ; HT mSF versus HT m/+SF p=0.0079 ; HT mCF versus HT +CF p=0.0031 ; NT10 mSF versus NT10 +SF p=0.0159 ; NT10 mCF versus NT10 +CF p=0.0002 ; NT24 mCF versus NT24 +CF p=0.0010.

A

Location	Number of individuals	Number of polymophisms intra-location
Surface	20	57
Pachon	20	23
Los sabinos	10	3
Tinaja	12	18
Curva	10	1
Chica	10	32
Molino	9	5
Escondido	7	1
Caballo moro	2	1
Subterraneo	10	13

B

C

	NC_035915.1:32713783	NC_035915.1:32713768	NC_035915.1:32713768	NC_035915.1:32713733	NC_035915.1:32713703	NC_035915.1:32713347	NC_035915.1:32713238	NC_035915.1:32712805	NC_035915.1:32712378	NC_035915.1:32711901	NC_035915.1:32711590
SF	TGACTAG	A	A	T	delA and WT	C and T	WT	G and T	C and T	C	A
Pa	AGCTA	T	C	T	delA	C	WT	T	C	C	A
Tin	TGACTAG	A	A and C	T and C	delA and WT	T	WT	G	T and C	T	A and C
Sab	TGACTAG	A	A	C	WT	T	WT	G	T	T	A and C
Cur	AGCTA	T	C	T	delA	C	deletion 7nt	G	T	T	
Chi	AGCTA	T	C	T	delA	C	deletion 7nt	T	C	T	A

Figure S5. Polymorphism along a ~4 kb fragment of the *mao* gene, and *mao* haplotypes.

(A) Number of wild-sampled individuals tested and number of polymorphisms in the ~4kb fragment around the P106L position in the *mao* gene, detected at each sampling site. For instance, we sequenced 20 fish from the river population, and we found 57 polymorphic positions distributed along the sequenced fragment. (B) Number of shared polymorphisms between surface populations and cave populations from El Abra, Guatemala and Micos groups. Darker colors indicate a high number of shared polymorphisms. Note that they are mostly shared within groups of caves. (C) Haplotypes reconstitution

for the P106L mutant allele. Each column corresponds to a polymorphic position. The first line depicts the haplotypes encountered in SF (all Proline at position 106 – homozygote wildtype), and the next lines are haplotypes associated with the Leucine106 (P106L) mutant allele in cave populations of the El Abra group. For instance, all P106L mutant individuals sequenced from Pachón cave had the sequence AGCTA (first column in the table) at the position 32713783, whereas all P106L mutants from the Tinaja cave had the sequence TGACTAG instead at that same position. The P106L mutation is in position NC_035915.1:32711472.

Table S1. Summary statistics for figure 7.

Three-way ANOVA	**Ordinary**				
Alpha	0,05				
Source of Variation	% of total variation	P value	P value summary	Significant?	
Habituation	**46,51**	<0.0001	****	Yes	
Morphotype	6,266	<0.0001	****	Yes	
Genotype	11,51	<0.0001	****	Yes	
Habituation x Morphotype	1,704	0,0223	*	Yes	
Habituation x Genotype	21,09	<0.0001	****	Yes	
Morphotype x Genotype	1,473	0,0104	*	Yes	
Habituation x Morphotype x Genotype	1,329	0,0497	*	Yes	
ANOVA table	SS (Type III)	DF	MS	F (DFn, DFd)	P value
Habituation	263092	2	131546	F (2, 65) = 110.1	P<0.0001
Morphotype	35448	1	35448	F (1, 65) = 29.66	P<0.0001
Genotype	65090	1	65090	F (1, 65) = 54.46	P<0.0001
Habituation x Morphotype	9639	2	4820	**F (2, 65) = 4.033**	**P=0.0223**
Habituation x Genotype	119278	2	59639	**F (2, 65) = 49.90**	**P<0.0001**
Morphotype x Genotype	8333	1	8333	**F (1, 65) = 6.972**	**P=0.0104**
Habituation x Morphotype x Genotype	7516	2	3758	**F (2, 65) = 3.145**	**P=0.0497**
Residual	77682	65	1195		
Posthoc					
Holm-Sidak's multiple comparisons test	Mean Diff.	Significant?	Summary	Adjusted P Value	
HT:SF+ vs. HT:SFm	18,34	No	ns	0,9988	
HT:SF+ vs. HT:CF+	13,79	No	ns	0,9988	
HT:SF+ vs. HT:CFm	30,56	No	ns	0,9544	
HT:SF+ vs. NT 10min:SF+	-46,66	No	ns	0,6957	
HT:SF+ vs. NT 10min:SFm	-271,1	Yes	****	<0.0001	
HT:SF+ vs. NT 10min:CF+	-41,82	No	ns	0,6601	
HT:SF+ vs. NT 10min:CFm	-167,7	Yes	****	<0.0001	
HT:SF+ vs. NT 24h:SF+	-48,92	No	ns	0,6957	
HT:SF+ vs. NT 24h:SFm	-95,7	Yes	**	0,0087	
HT:SF+ vs. NT 24h:CF+	3,265	No	ns	0,9996	
HT:SF+ vs. NT 24h:CFm	-7,198	No	ns	0,9996	
HT:SFm vs. HT:CF+	-4,555	No	ns	0,9996	
HT:SFm vs. HT:CFm	12,22	No	ns	0,9988	
HT:SFm vs. NT 10min:SF+	-65	No	ns	0,1205	
HT:SFm vs. NT 10min:SFm	-289,5	Yes	****	<0.0001	
HT:SFm vs. NT 10min:CF+	-60,16	No	ns	0,0661	
HT:SFm vs. NT 10min:CFm	-186,1	Yes	****	<0.0001	
HT:SFm vs. NT 24h:SF+	-67,26	No	ns	0,1417	
HT:SFm vs. NT 24h:SFm	-114	Yes	***	0,0003	
HT:SFm vs. NT 24h:CF+	-15,08	No	ns	0,9988	
HT:SFm vs. NT 24h:CFm	-25,54	No	ns	0,9789	
HT:CF+ vs. HT:CFm	16,77	No	ns	0,9957	
HT:CF+ vs. NT 10min:SF+	-60,45	No	ns	0,0653	
HT:CF+ vs. NT 10min:SFm	-284,9	Yes	****	<0.0001	
HT:CF+ vs. NT 10min:CF+	-55,61	Yes	*	0,0136	
HT:CF+ vs. NT 10min:CFm	-181,5	Yes	****	<0.0001	
HT:CF+ vs. NT 24h:SF+	-62,7	No	ns	0,0885	
HT:CF+ vs. NT 24h:SFm	-109,5	Yes	****	<0.0001	
HT:CF+ vs. NT 24h:CF+	-10,53	No	ns	0,9988	
HT:CF+ vs. NT 24h:CFm	-20,99	No	ns	0,9789	
HT:CFm vs. NT 10min:SF+	-77,22	Yes	*	0,0174	
HT:CFm vs. NT 10min:SFm	-301,7	Yes	****	<0.0001	
HT:CFm vs. NT 10min:CF+	-72,38	Yes	**	0,0045	
HT:CFm vs. NT 10min:CFm	-198,3	Yes	****	<0.0001	
HT:CFm vs. NT 24h:SF+	-79,48	Yes	*	0,0254	
HT:CFm vs. NT 24h:SFm	-126,3	Yes	****	<0.0001	
HT:CFm vs. NT 24h:CF+	-27,3	No	ns	0,9082	
HT:CFm vs. NT 24h:CFm	-37,76	No	ns	0,7277	
NT 10min:SF+ vs. NT 10min:SFm	-224,5	Yes	****	<0.0001	
NT 10min:SF+ vs. NT 10min:CF+	4,839	No	ns	0,9996	
NT 10min:SF+ vs. NT 10min:CFm	-121,1	Yes	****	<0.0001	
NT 10min:SF+ vs. NT 24h:SF+	-2,256	No	ns	0,9996	
NT 10min:SF+ vs. NT 24h:SFm	-49,04	No	ns	0,6376	
NT 10min:SF+ vs. NT 24h:CF+	49,92	No	ns	0,2551	
NT 10min:SF+ vs. NT 24h:CFm	39,46	No	ns	0,7277	
NT 10min:SFm vs. NT 10min:CF+	229,3	Yes	****	<0.0001	
NT 10min:SFm vs. NT 10min:CFm	103,4	Yes	***	0,0003	
NT 10min:SFm vs. NT 24h:SF+	222,2	Yes	****	<0.0001	
NT 10min:SFm vs. NT 24h:SFm	175,4	Yes	****	<0.0001	
NT 10min:SFm vs. NT 24h:CF+	274,4	Yes	****	<0.0001	
NT 10min:SFm vs. NT 24h:CFm	263,9	Yes	****	<0.0001	
NT 10min:CF+ vs. NT 10min:CFm	-125,9	Yes	****	<0.0001	
NT 10min:CF+ vs. NT 24h:SF+	-7,095	No	ns	0,9996	
NT 10min:CF+ vs. NT 24h:SFm	-53,88	No	ns	0,2437	
NT 10min:CF+ vs. NT 24h:CF+	45,08	No	ns	0,1203	
NT 10min:CF+ vs. NT 24h:CFm	34,62	No	ns	0,6962	
NT 10min:CFm vs. NT 24h:SF+	118,8	Yes	****	<0.0001	
NT 10min:CFm vs. NT 24h:SFm	72,04	No	ns	0,0661	
NT 10min:CFm vs. NT 24h:CF+	171	Yes	****	<0.0001	
NT 10min:CFm vs. NT 24h:CFm	160,5	Yes	****	<0.0001	
NT 24h:SF+ vs. NT 24h:SFm	-46,79	No	ns	0,7275	
NT 24h:SF+ vs. NT 24h:CF+	52,18	No	ns	0,2994	
NT 24h:SF+ vs. NT 24h:CFm	41,72	No	ns	0,7277	
NT 24h:SFm vs. NT 24h:CF+	98,97	Yes	***	0,0004	
NT 24h:SFm vs. NT 24h:CFm	88,51	Yes	**	0,0075	
NT 24h:CF+ vs. NT 24h:CFm	-10,46	No	ns	0,9988	

Mann-Whitney pairwise comparisons

	SF + NT10	SF + NT24	SF +/- anim	SF +/- NT10	SF +/- NT24	SF m HT	SF m NT10	SF m NT24	CF m HT	CF m NT10	CF m NT24	CF + HT	CF + NT10	CF + NT24
SF + HT	0.111	0.057	0.412			0.063			0.009			0.137		
SF + NT10				0.007			0.007			0.004			0.661	
SF + NT24					0.555			0.057			0.019			0.007
SF +/- HT				0.007	0.007	0.222			0.008			0.742		
SF +/- NT10					0.095		0.0555			0.428			0.008	
SF +/- NT24								0.190			0.004			0.0006
SF m HT							0.007	0.015	0.051			0.319		
SF m NT10								0.015		0.082			0.0004	
SF m NT24											0.009			0.0019
CF m HT										0.002	0.002	0.036		
CF m NT10											0.002		0.0001	
CF m NT24														0.219
CF + HT													0.000034	0.173
CF + NT10														0.002

	SF + NT10	SF + NT24	SF +/- anim	SF +/- NT10	SF +/- NT24	SF m HT	SF m NT10	SF m NT24	CF m HT	CF m NT10	CF m NT24	CF + HT	CF + NT10	CF + NT24
SF + HT	NS	NS	NS			NS			**			NS		
SF + NT10		NS		**			**			**			NS	
SF + NT24					NS			NS			*			**
SF +/- HT				**	**	NS			**			NS		
SF +/- NT10					NS		NS			NS			**	
SF +/- NT24								NS			**			***
SF m HT							**	*	NS			NS		
SF m NT10								*		NS			***	
SF m NT24											**			**
CF m HT										**	**	*		
CF m NT10											**		***	
CF m NT24														NS
CF + HT													*****	NS
CF + NT10														**

one-way ANOVA for each group, along time

SF + Statistique observée Qobs : 4.8660986357807

p-value : 0.033428951624457 *

Test d'homogénéité de variance de Bartlett

Méthode : Bartlett test of homogeneity of variances
Statistique observée Qobs : 2.5648540679301
p-value : 0.27736331270786
Paramètre du test : 2

SF +/- Statistique observée Qobs : 15.190245363751

p-value : 0.00051533731390284 ***

Test d'homogénéité de variance de Bartlett

Méthode : Bartlett test of homogeneity of variances
Statistique observée Qobs : 5.3132918848822
p-value : 0.07018322599511
Paramètre du test : 2

SF m Statistique observée Qobs : 30.287214416875

p-value : 3.3611768813376E-5 ***

Test d'homogénéité de variance de Bartlett

Méthode : Bartlett test of homogeneity of variances
Statistique observée Qobs : 16.998091696251
p-value : 0.00020366260138521
Paramètre du test : 2

CF + Statistique observée Qobs : 16.140270446008

p-value : 1.9435396964723E-5 ***

Test d'homogénéité de variance de Bartlett

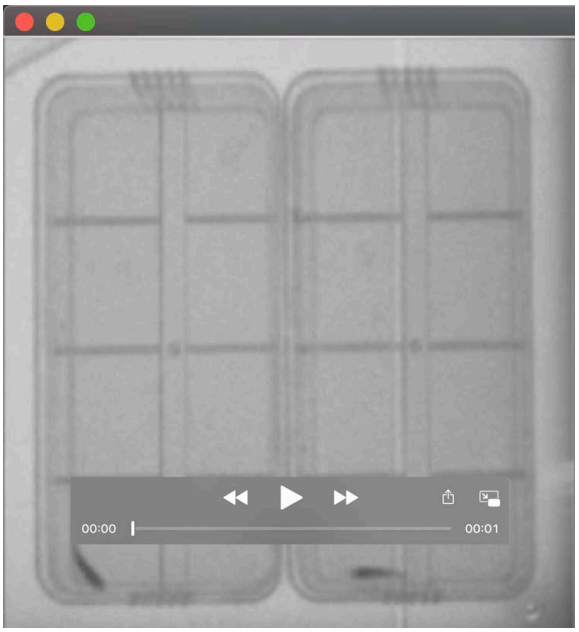
Méthode : Bartlett test of homogeneity of variances
Statistique observée Qobs : 7.3937770507278
p-value : 0.02480057289771
Paramètre du test : 2

CF m Statistique observée Qobs : 64.137389491156

p-value : 4.4607854863108E-8 ***

Test d'homogénéité de variance de Bartlett

Méthode : Bartlett test of homogeneity of variances
Statistique observée Qobs : 19.814791926837
p-value : 4.9804960850732E-5
Paramètre du test : 2



Movie 1: thigmotaxis in CF, 10min after being placed in a novel tank.

Movie 2: thigmotaxis in CF, 1hour after being placed in a novel tank.

Movie 3: thigmotaxis in CF, 96hours after being placed in a novel tank.