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Early impact of domestication on aggressiveness, activity, and stress behaviors in zebrafish (*Danio rerio*) using mirror test and automated videotracking

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Fish domestication progresses through five levels: from the initial acclimatization to captivity (Level 1), to the life cycle completion in captivity (Level 4), and even to the implementation of selective breeding programs (Level 5). Domestication leads to phenotypic changes over generations, sometimes from the very first generation. Behavioral traits are among the first to change. However, in fish, potential behavioral changes during early domestication have been little studied. Therefore, we studied potential behavioral changes among early and advanced levels of domestication in a model species, the zebrafish (*Danio rerio*), using a mirror test experiment, commonly used to assess traits involved in activity, aggressiveness, and stress in this species. We compared these traits between wild zebrafish in captivity (F0; Level 1), the first generation of their captive-born offspring (F1; Level 4), and three laboratory strains (AB, TU, and WIK; Level 5). Each fish was individually filmed and tracked using an automated procedure for 5 min. Nine behavioral traits and one activity-related trait were characterized for each individual based on the movements and positioning of the fish. We applied a principal component analysis (PCA) and tested the significance of potential differences between groups using an analysis of similarities (ANOSIM). We applied an indicator value analysis (IndVal) to determine which traits were most expressed by each group. We detected differences between groups and across domestication levels. More specifically, we highlighted differentiations between different levels of domestication (e.g. between F1, AB, TU, and WIK) as early as the beginning of the domestication process (i.e. F0 *vs.* F1), but also within the same level of domestication (i.e. AB *vs.* TU). Based on PCA and IndVal, (i) F0 and F1 tended to show stronger expression of stress-related traits than the other groups, (ii) F0 was more active than others, and (iii) TU was more aggressive than AB. Our results confirmed that domestication can change fish behavior, even in the first generation born in captivity, although these modifications remain limited. In contrast, we did not observe any general trends correlated with domestication levels, given that AB and TU diverged in their aggressiveness levels, and WIK differed only from F1. This result needs to be generalized to other species but also considered for domestication for aquaculture. If future studies confirm that the changes observed at the beginning of the domestication process remain limited and that there is no consistent evolutionary trend across generations in fish, this would highlight the crucial importance of selecting the right species from the outset of domestication. It would also emphasize the need to design selective breeding programs that shape fish stocks with the most desirable characteristics. To our knowledge, this study is one of the few to examine the behavior of wild zebrafish alongside laboratory strains, offering a unique insight into the early stages of domestication.

Keywords Activity, Aggressiveness, Domestication, Stress, Zebrafish

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Domestication is a mutualistic relationship in which one species (the "domesticator"; e.g. humans) provides lasting, multigenerational support to another (the "domesticate") in exchange for a predictable supply of a resource or services in a captive environment more or less controlled by the domesticator (adapted from[1,2]). This relationship between humans and plants or animals led to the development of agriculture[3], including aquaculture[4]. Domestication is a gradual process which, in fish, has been broken down into five levels[5]. Level 1 corresponds to the acclimatization of wild individuals to a human-controlled environment. Level 2 is reached when part of the life cycle is completed in captivity. Levels 3 and 4 correspond to the completion of the life cycle, first with the regular introduction of wild individuals to reinforce fish stocks (level 3), then without the introduction of wild individuals (level 4). Finally, level 5 corresponds to the implementation of selective breeding programs (i.e. artificial selection consciously driven by humans) to improve productivity and other key characteristics for aquaculture purpose. About 250 fish species have been the subject of domestication programs for aquaculture, but only 75 have reached at least level 4 and only 30 level 5[6].

During domestication, a population of a species (the domesticate) adapts through phenotypic modifications, over generations, to the captive environment and to human control of its resources and life cycle[7,8]. In fish, the phenotypic modifications concern behavioral, morphological, phenological, and physiological traits[7,9]. Previous studies, in economically important (e.g. European seabass, *Dicentrarchus labrax;* brown trout*, Salmo trutta*) and model species (e.g. zebrafish, *Danio rerio*)[10], showed that behavioral traits in fish can be modified very early by domestication[9,11]. First, compared with their wild counterparts, domesticates tend to have a greater tameness, leading to a reduction in fear in the face of a potential threat (e.g. less predators avoidance[12] and bolder behavior facing a new object[13] in brown trout). Second, domestication tends towards a reduction in the intensity of response to stress linked to the rearing environment: domesticates appear to be less stressed than wild fish[9], as observed in percids, where the domesticates have a better tolerance to chronic confinement[14]. Third, changes in foraging strategy and exploratory behavior are frequently observed[9]. For instance, selected Atlantic salmon (*Salmo salar*) have a greater feed consumption than captive-born wild offspring[15] and domesticated masu salmon (*Oncorhynchus masou*) swim more at the surface than their wild counterparts[16]. Fourth, a change in risk-taking behavior is also associated with domestication (i.e. wild fish exhibit a more rapid response to predators and generally avoid risky areas with high predation level in comparison to domesticated fish[9]). Finally, a change (decrease or increase) in aggressiveness towards conspecifics also occurs. For instance, selection for fast growth in medaka (*Oryzias latipes*) favors less aggressive fish when food is highly localized[17], while Atlantic salmon domesticated for many generations are more aggressive[9]. Overall, these changes can result from (i) direct, conscious or unconscious selection by humans and the captive environment, and (ii) indirect selection pressures that arise as by-products of breeding programs targeting traits, such as growth rate or flesh quality[7]. Although there is an abundance of literature available, those consequences have mainly been established by comparing (i) strains at different advanced levels of domestication (e.g. level 4 vs. level 5) and (ii) 'farmed' or 'domesticated' individuals with the captive-born descendants of wild fish (i.e. level 3 and above)[10,18,19]. In contrast, few studies have investigated the behavioral differences between wild fish (i.e. levels 1 and 2) and captive-born fish (levels 3 and above). Yet, as behavioral changes can appear very early in the domestication process, such a comparison is crucial to the understanding of the evolution of behavioral traits during fish domestication.

Understanding the consequences of domestication is important for fish production in aquaculture. First, resistance to stressors commonly encountered in aquaculture (e.g. transport, confinement, handling) is crucial to develop a successful production of a fish species[7,20]. Second, tolerance to conspecifics in a limited space is essential for production[21]. Otherwise, intensive rearing conditions can lead to the emergence of aggressive behavior (e.g. attacks or biting), causing injuries, as well as triggering stress, immune suppression, mortality, or unequal competition for food, with negative consequences for individual welfare, fish rearing, and production[11,22]. Third, animal activity is also important for aquaculture, as it contributes to the total energy budget (e.g.[23]). Therefore, changes in activity, aggressiveness, or stress response during domestication can be useful or detrimental to production goals, as these traits are involved in the fish welfare, and the allocation of energy resources to key biological functions (including growth, which is important for animal production)[9,22].

Zebrafish (*Danio rerio*) is a model species for aquaculture research thanks to its ease of breeding (i.e. small size, short and simple life cycle, easy rearing), its basic biological traits and physiological responses similar to the most important cultivated species, and its many resources (e.g. strains, genomic resources, transgenics) which facilitate research[24]**.** This makes it possible to rapidly carry out intergenerational studies on a wide range of biological functions, and to infer conclusions to species of aquaculture interest. For the same reasons, it has also been proposed as a model for the study of domestication in fish[7]. Overall, zebrafish behavior is already widely studied for various types of research (e.g. ecotoxicology, biomedical, animal welfare[25–28]) and many specific behavioral tests are available (e.g.[29]). Previous studies have investigated the effects of domestication on zebrafish behavior, but they have primarily focused on comparing laboratory strains with wild-derived strains (i.e. those born and raised in captivity for at least one generation)[30–32]. No study has yet been able to assess the consequences of domestication in zebrafish from the very beginning of the process, specifically by considering individuals born in the wild as baseline. Therefore, we here investigated behavioral changes between wild and captive-born zebrafish. This offers a unique perspective by examining behavioral changes across different levels of domestication, from the wild populations to domesticated laboratory strains. By including wild populations, we aim to provide a deeper understanding of how domestication influences behavior, especially in its early stages. More specifically, we compared nine behavioral traits related to stress or aggressiveness and one activity-related trait (Table 1) between wild zebrafish, sampled in Bangladesh, acclimatized to captivity (F0, n = 30), their captive-born offspring (F1, n = 28), and three laboratory strains (AB, TU, and WIK, n = 59, 28, and 29, respectively). These five fish groups were at different levels of domestication: level 1 for F0, level 4 for F1, and level 5 for AB, TU, and WIK. As variations in environmental factors (e.g. water quality, temperature, feeding regimes) can impact fish behavior[33], we placed all fish under the same rearing conditions prior to behavioral testing and under the same

Trait	Description	Interpretation
Immobility	Number of *Frames* during which the fish is immobile without being in the *Contact Zone*. The fish is considered as immobile when it moves less than 0.5 cm of *Distance* for at least 5 s (i.e. 125 successive frames).	Stress-induced freezing[53]; characterized by a motionless state
Immobility Periods	Number of periods of successive *Frames* at which the fish is immobile.	Stress[53]
Total distance	Sum of *Distance* during the total number of *Frames*.	Activity[35]
Contact Zone %	Percentage of *Frames* (%) spent in the *Contract zone* compared to the *Total Number of Analyzed Frames* in proportion to the percentage of volume (%) occupied by this zone.	Aggressiveness[36]
Distant zone %	Percentage of *Frames* (%) spent in the *Distant zone* compared to the *Total Number of Analyzed Frames* in proportion to the percentage of volume (%) occupied by this zone.	Stress; as spending time in an area away from the mirror due to an aversive stimulus (adapted from[34,53])
Rapid movements toward the mirror	Number of rapid movement (i.e. *Velocity* upper to 20 cm/s) of the fish from the *Proximate Zone* to the *Contact Zone* without reversing *Direction*.	Aggressiveness[26,53]
Active stay at the mirror	Number of *Frames* during which the fish is in the *Contact Zone* and remains there for at least 1.2 s (i.e. 30 successive frames) without being immobile.	Aggressiveness[37]
U-turn	Number of U-turns detected during tracking. The fish is regarded as making a U-turn when it successively moves towards the mirror, reverse *Direction*, and moves rapidly (i.e. *Velocity* upper to 20 cm/s) away from the mirror.	Stress; expressed as quick escape from the opponent (adapted from[34])
Thigmotaxis	Number of *Frames* during which the fish remains close to the tank sides whether it moves or not. A fish is considered close to a tank side if its distance from it is less than the average body length observed in the fish population in question. There is one exception: a fish is not considered thigmotactic if it is in the *Contact Zone*.	Stress[34,53]
Thigmmobility	Number of *Frames* during which the fish remains close to the tank sides (thigmotaxis) and it is immobile (immobility).	Stress (adapted from[53])

Table 1. The behavioral and activity-related traits studied in the mirror test based on the tracking results to characterize activity, aggressiveness, and stress of each fish individual. Behavior descriptions and interpretations were modified from[26,34–36,53] or developed by the authors. "Trait" presents the studied traits. "Description" provides the description of each trait. Words in italics are parameters defined in Table 2. "Interpretation" describes whether the trait is related to the individual's activity, aggressiveness, or stress level; the higher the trait expression, the higher the activity, aggressiveness or stress level.

experimental conditions during testing. We compared fish groups through their behavioral response during a mirror-test experiment, commonly used to evaluate behavior in zebrafish[29,34–37], through an experimental workflow (Fig. 1A). We also compared the sexes to account for any divergence between them in the domestication process. Each fish was filmed individually in a mirror-test experiment for 5 min. To minimize observer bias, an automated tracking procedure was applied to each video obtained and the features studied were characterized for each individual, based on the movements and positioning of the fish in relation to the mirror and the edge of the experimental tank. We hypothesized that the higher is the level of domestication the lower are the aggressiveness, stress, and activity behaviors. Confirming this would align with general trends observed in fish domestication, particularly in the early stages, and support the idea that domestication favor behavioral traits beneficial for aquaculture production.

Material and methods

Model species, biological material, and pre-test rearing conditions

The zebrafish is a social freshwater species with a dominance hierarchy. Both sexes can exhibit aggressive behavior when interacting with their conspecifics, including when a solitary zebrafish encounters another individual[35,38]. Native to South Asia[39], several populations have been used in independent domestication programs for research purposes or pet production for decades, leading to the many current zebrafish strains[40,41].

Here, we used five fish groups ranging from level 1 to 5 of domestication: one wild population (F0), its captive-born offspring (F1, first generation born in captivity), and three laboratory strains: AB (A and B crossbreed), TU (Tübingen), and WIK (Wild India Karyotype). F0 was at level 1 as they were wild individuals acclimatized to a human-controlled environment. F1 was at level 4 as their life cycle is carried out in captivity without gene flow from wild organisms (i.e. no wild specimen where added to F1 fish stock). AB, TU, and WIK were more advanced in the domestication process (level 5 as they underwent selective breeding program previously) and domesticated several decades ago[42]. AB is a strain established in the 1970s. We considered AB to be at level 5 because individual females of the AB line had been screened for healthy, good looking embryos, and those females had been used to make next generations[43]. TU originates from Tübingen in the 1990s[44]. We regarded TU to be at level 5 as it had been cleaned up to remove embryonic lethal mutations[45]. WIK derives from a single pair mating (unlike the other studied strains) of second-generation wild-caught Indian zebrafish in the late 1990s[46]. We regarded WIK to be at level 5 as the strain had been established by selection of a subline free of embryonic and larval lethals with a probability of over 90%[46].

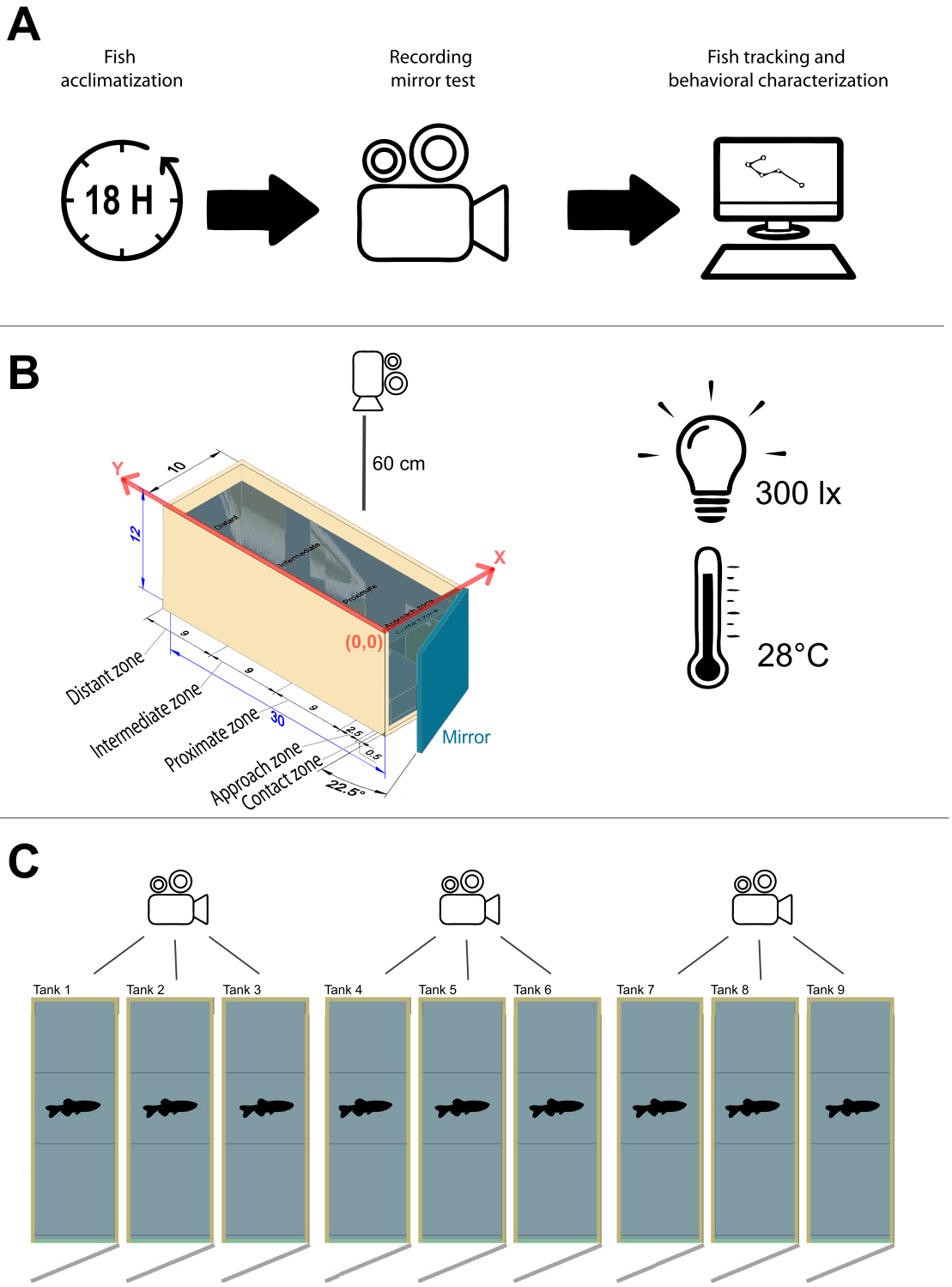

Fig. 1. Graphical overview of the mirror test experiment. (**A**) Experimental flow, which follows this order: (i) acclimatization of individual fish for 18 h in the experimental tank with all sides covered, (ii) uncovering of the mirror and video recording for 10 min, and finally (iii) analysis of video by automated tracking and behavioral characterization, considering 5 min of the recording. (**B**) The individual experimental tank used for mirror test with its dimensions (in cm), the five zones defined in relation to the mirror, and the standardized abiotic conditions for temperature (°C) and luminous flux (lx). Transparent red arrows indicate the axes (X, Y) and origin (0,0) of the Cartesian plane used to calculate fish coordinates during tracking. (**C**) An example of one of the series of nine independent experimental tanks filmed in parallel in blocks of three, each block being filmed by one camera.

We studied these three levels of domestication (i.e. Levels 1, 4, and 5) as they are the most important steps in domestication programs: the acclimatization to captivity, the breaking of links (i.e. introduction of individuals) between wild and farmed populations, and the implementation of selective breeding programs. We chose AB, TU, and WIK because they are three strains commonly used in laboratories and have not undergone genetic manipulation. Therefore, they correspond to an advanced level of domestication observed for fish in aquaculture. We used three strains to highlight if the behavioral characteristics were similar for the same level of domestication.

F0 had been sampled from June 17 to 28 2022 in a radius of 10 km around the Bangladesh Agricultural University (WGS84: 24°43′21.20″ N, 90°26′1.45″ E, Mymensingh, Mymensingh Division, Bangladesh). They had been acclimatized to captive conditions at the *Platforme Expérimentale en Aquaculture* (PEA, University of Lorraine, Vandoeuvre-lès-Nancy, France). F1 are the offspring of five F0 couples produced at the PEA in January 2023. AB, TU, and WIK had been purchased at the embryonic stage from the European Zebrafish Resource Center (Karlsruher Institute of Technology, Eggenstein-Leopoldshafen, Germany) in March 2022.

Fish individuals were housed in 3.5L tanks in an automatized rack system (ZebTEC Active Blue Stand Alone Tecniplast®) with recirculating water-controlled conditions: water temperature at 28 ± 0.5 °C; pH 7.5 ± 0.5; conductivity of 500 ± 50 µS/cm. The room photoperiod was set at 13 h light and 11 h dark with 30 min of dawn (starting at 8:00 a.m.) and dusk (starting at 9:00 p.m.). The light intensity was 300 lx at the water surface. The rearing conditions were defined by a consensus between the recommendations in the literature[47] and the abiotic conditions observed in the sampling zone, the latter obtained by AquaDesign[48]. The fish were fed four times daily at apparent satiation including three times with dry food (GEMMA Micro, Skretting) and one time with *Artemia* nauplii (EG > 225)[49]. Except for the F0, all other fish groups (F1, AB, TU, and WIK) have been raised under the same standardized conditions from 0 dpf to the age which they were tested. F0 was reared under these standardized conditions for 247 days before testing.

Mirror test

The mirror test is commonly used in zebrafish, initially to study aggressiveness-related behaviors[34,35,37,50,31]. Zebrafish do not recognize themselves in the mirror and treat their reflection as another fish[52]. When a solitary zebrafish encounters its reflection in the mirror, it may display aggressive behaviors such as approaching, bumping into and biting the image. The mirror test is also used to assess stress-related behaviors such as thigmotaxis (i.e. "wall-hugging"), swimming away from the mirror stimulus (i.e. escape) and spending time away from the mirror[34,53]. As the fish move during the experiment, we extend this test to measure activity as the total distance traveled by the fish during the behavioral assay (as already performed by[35]).

A set of 174 fish was tested with the mirror test: 15 females and 15 males for F0; 14 females and 14 males for F1; 30 females and 29 males for AB; 13 females and 15 males for TU; 14 females and 15 males for WIK. These sample sizes were chosen because around 15 individuals per group are commonly used in behavioral studies on zebrafish[31,54,55]. These individuals were sampled randomly from the rearing facilities. AB, TU, and WIK fish were tested between 332- and 360-days post-fertilization (dpf). F1 fish were tested at 120 dpf. The exact age of F0 wild individuals is unknown, but it is more than 247 dpf (i.e. time between the field capture and the behavioral experiment).

The experimental set-up for the mirror test consisted of a glass tank coupled to an inclined mirror (Fig. 1B). The experimental tank measured 30 × 10 × 12 cm (L × W × H) and was filled with 3L of system water (water height = 10 cm). The 12 × 12 cm mirror was placed at one end of the tank at a 22.5° angle[35]. Placing the mirror at a 22.5° angle behind the tank, creates the illusion where the reflection appears closer on one side and farther away on the other. This setup provides a lateral view of the "opponent," which is most effective in eliciting aggressive behavior[56]. Except for the side of the mirror, the vertical sides of the tank were covered with beige cardboard to visually isolate the fish from external disturbances. Lines were drawn at the bottom of the tank[36], to delimit five zones in the tank, named: "Contact", "Approach", "Proximate", "Intermediate", and "Distant" zones from closest to farthest from the mirror (Fig. 1B). The temperature of the experimental room was maintained to ensure the water temperature at 28 ± 0.5 °C, similar to the rearing conditions. The light intensity of the room at the surface of the water of each tank was 300 lx.

The fish were individually evaluated in sets of nine simultaneously (Fig. 1C). Thus, in each series, nine completely independent (i.e. thanks to beige cardboard on the adjoining sides between the tanks) experimental tanks were used (Fig. 1C). The day before the experimental phase, at 4:00 p.m., the fish were placed individually overnight in the experimental tank for 18 h (similarly to[31]) (Fig. 1A). During this time, (i) fish were not fed, (ii) a beige cardboard was placed between the mirror and the uncovered edge of the experimental tank, and (iii) a transparent plastic panel was placed over the aquarium to prevent the fish from escaping. After these 18 h, at 10:00 a.m., the cardboard paper on the mirror side was removed and the video recording began. We used three cameras (Sony Handycam DCR-SR72E, DCR-SR190E, and FDR-AX33; frequency of 25 frames/s) placed at 60 cm vertically above three experimental tanks each (Fig. 1C). Therefore, the experiment was (i) recorded in 2D and (ii) carried out in series with three blocks of three different fish recorded simultaneously (Fig. 1C).

Once a series was recorded, the fish were transferred back to the ZebTec Active Blue standalone rack. The experimental tanks used for the experiment were emptied, rinsed with 99.9% ethanol and then with water to eliminate any olfactory cues for future experimental tests.

Fish tracking

For each video recording, fish were individually tracked using the R-package *trackR*[58] in R version 4.0.3[59] to monitor their behavior and activity. The tracking began as soon as the cardboard in front of the last mirror of the block was removed and lasted 10 min, generating 15,000 frames.

Parameter	Definition
Direction	Direction of movement of the fish relative to the mirror. It (i) approaches the mirror as long as a decrease in the Y coordinate (see Fig. 1B) is observed between successive coordinates, and (ii) moves away from the mirror as long as an increase in the Y coordinate is observed between successive coordinates. The direction is considered to reverse when the fish moves more than 0.05 cm in the opposite direction between successive coordinates.
Distance	Euclidean distance traveled by the fish between two successive images, according to the fish's coordinates on the X and Y axes. It is expressed in cm.
Frame	Still image forming the video in which the fish was filmed. Here, the camera recorded 25 frames per second.
Time	Number of frames for a given period divided by 25. It is expressed in s.
Velocity	Distance multiplied by 25. It is expressed in cm/s.
Zone	Zone in which the head of the fish is located at each frame.
Total number of analyzed frames	Total number of frames successfully tracked (i.e. when the fish is detected after discarding frames with tracking errors).
Contact Zone	Zone closest to the mirror. Here, the width of the zone is 0.5 cm.
Proximate zone	Zone after the approach zone (between the approach zone and the intermediate zone). Here, the width of the zone is 9 cm.
Distant zone	Zone after the intermediate zone. It is also the zone furthest from the mirror. Here, the width of the zone is 9 cm.

Table 2. Parameters calculated from the data table obtained by tracking each fish to establish behavioral and activity-related traits for each fish. "Parameter" presents the parameters calculated. "Definition" shows how each parameter is calculated from the tracking results.

In line with several mirror-test studies, we aim at considering 5 min for videotracking[34,31,60]. However, we recorded 10 min, as we wanted to obtain a period of 5 min to characterize the behaviors after deducting one minute of acclimatization (i.e. 1 min after removing the cardboard in front of the last mirror of the block) and to leave time at the end of the experiment before the experimenter came to turn off the cameras.

The tracking produced a data table that includes the fish's coordinates on a Cartesian plane, with the origin (0, 0) positioned near the mirror (i.e., in the top right-hand corner of the tank) (Fig. 1B). Additionally, the table records the angle, length, and width of the fish for each frame of the video. These variables were converted into centimeters using the 9 cm proximate zone as a reference scale (Fig. 1B).

Behavioral characterization from tracking datasets

We characterized nine behavioral traits and one activity-related trait for each fish (Table 1) based on 18 parameters calculated from the data table obtained by tracking each fish (Table 2). The definition of behavioral traits and their interpretation in terms of activity, aggressiveness, and stress are based on previous studies (i.e.[34,53], see details in Table 1). Overall, the majority of these interpretations have already been proposed in previous studies of the mirror test in the literature (e.g.[26,34,53]: high levels of immobility, thigmotaxis, and time spent away from the mirror indicate increased stress, while spending more time close to the mirror suggests higher levels of aggression). We have added to these interpretations that the total distance covered can be used to measure the fish's overall activity during the test.

Parameter calculations and characterization of the behavioral and activity-related traits were performed in R.

First, we removed potential tracking errors, such as aberrant values and duplicated frames. These errors resulted from residues in tanks detected by *trackR*, leading to duplicate objects (i.e. artefacts and fish) being detected in the same image, or the residues and the fish merging to form a single object larger than the fish, generating outliers.

Second, as already performed in other zebrafish aggression assays[34,36,37], we (i) excluded the first minute of tracking as an acclimatization period after the cardboard on the side of the mirror was removed by the experimenter and (ii) considered only the following five minutes for the analysis. Consequently, a maximum of 7,500 frames were considered for behavior characterization. The number of frames considered for some individuals may be less than 7,500 because of (i) the exclusion of images with tracking errors (see above) and (ii) the non-detection of fish in some frames.

Third, we excluded specimens with insufficient tracking time (i.e. few hundred frames rather than several thousand frames) as such disparate observation times compromise comparability and may introduce bias into the conclusions. After this exclusion, the number of specimens considered for behavioral analyses was 169 fish: 15 females and 15 males for F0; 12 females and 12 males for F1; 30 females and 29 males for AB; 13 females and 15 males for TU; 13 females and 15 males for WIK. Therefore, five fish were removed from the further analyses.

Fourth, the parameters were calculated from the coordinates, angle, and length of the fish for each frame as well as the position of the fish in the zone over the experiment (Table 2).

Fifth, behavioral and activity-related traits were assessed based on parameters (Table 2). For traits corresponding to the proportion of time spent in each zone of the experimental tank (Table 1), we adjusted this proportion according to the volume occupied by the zone in the tank. As all fish were not tracked for 7,500 frames, we divided, for each fish, the results of the traits corresponding to a number of frames or a count over

the experiment (Table 1) by the total number of analyzed frames. This allowed considering the different usable observation times of different individuals.

The resulting dataset is available as Supplementary Table S1.

Statistical analyses

To take account of the complex interactions that can exist between the traits studied, we performed multivariate analyses[61]. All statistical analyses were carried out using R.

First, we assessed Pearson's correlations between traits using the R-package *ape 5.0*[62]. When traits highly correlated were detected (i.e. correlation coefficient higher that 0.7 and p-value < 0.05), one of the two was randomly chosen and the other was discarded. Therefore, we removed Thigmmobility that was highly correlated to Immobility ($r = 0.88$, $p = 0.00005$).

Second, we normalized all traits between 0 and 1 to give them the same weight in the multivariate analyses performed below using the R-package *heatmaply*[63].

Third, we performed a principal component analysis (PCA) using R-packages *FactoMineR*[64] and *factoextra*[65] to take account of correlations between the remaining variables and reduce the number of variables while retaining the essential information. We determined the number of PCA dimensions to consider, using the Kaiser-Guttman criterion[66].

Fourth, we tested the significance of potential differences between sexes and between fish groups on the basis of the principal component score using multivariate statistical tests. We checked the normality of the data and the multivariate homogeneity of group dispersions (variances) to identify the appropriate multivariate statistical tests. We highlighted that the normality of our data was not met, using a Mardia test using R-package *MVN*[67] (Mardia Skewness 'statistic = 300.833, p-value < $1e^{-05}$; Mardia Kurtosis' statistic = 9.207, p-value < $1e^{-05}$). With R-package *vegan*[50], we tested the multivariate homogeneity of group dispersions between sexes and between fish groups by using betadisper (i.e. analysis of multivariate homogeneity of variances of group dispersions) on a Mahalanobis distance matrix to calculate group dispersions, and then did an ANOVA to check the homogeneity of dispersions between groups. The results of these ANOVAs show that the multivariate dispersions between sexes are homogeneous ($F = 0.323$, p-value = 0.5705), but not between strains. ($F = 2.5954$, p-value = 0.03834). Consequently, we applied a perMANOVA (permutational multivariate analysis of variance) based on a Mahalanobis distance matrix to check the significance of potential differences between sexes using R-package *vegan*[68] and 10,000 permutations. PerMANOVA is a non-parametric statistical method used to test differences between groups on multivariate bases, which requires data homoscedasticity. To assess significance of potential differences between strains, we used an ANOSIM (Analysis of Similarities) based on a Mahalanobis distance matrix using R-package *vegan*[68] and 10,000 permutations. ANOSIM is a non-parametric method used to test differences between groups on multivariate databases which does not require data homoscedasticity. If ANOSIM detected a significant difference between fish groups, a pairwise ANOSIM with a Benjamini & Hochberg correction was applied to determine precisely which groups differed from each other. The function to perform the ANOSIM pairwise was created by the authors from the anosim function of the R-package *vegan*[68].

Fifth, we developed two approaches to detect trait expressions specific to certain fish groups. On the one hand, we developed a linear mixed-effects model fitted to the data for each trait individually (i.e. log + 1 transformation) via a restricted maximum likelihood approach using the R-package *lmer*[69] to account for individual variability and the potential random effect due to experimental setup (i.e. age, series, tanks, Fig. 1C). Then, we applied an ANOVA based on each linear mixed effect model to detect differences in traits between groups. If the ANOVA detected significant results, we then developed a Tukey-type post-hoc with the R-package *emmeans*[70]. On the other hand, we performed an indicator value analysis (IndVal)[71] using the R-package *labdsv*[72]. This analysis determines which behavioral and activity-related traits are most expressed by each of the five groups of fish. IndVal was used in conjunction with PCA to define traits whose expression is specific to one or more of the fish groups.

Results

One hundred and seventy-four fish were tracked and five were excluded as the tracking could only follow them over a much shorter period of time. The remaining 169 tracked fish were analyzed first by PCA and then potential differences between sexes and between fish groups on the basis of the principal component score using perMANOVA (i.e. between sexes) or ANOSIM (i.e. between F0, F1, AB, TU, and WIK). Finally, ANOVA based on linear mixed-effects models and an IndVal were applied to determine which behavioral and activity-related traits are most expressed by each of the five fish groups.

Differentiation between sexes and fish groups

Following the Kaiser-Guttman criterion, we considered the first four axes of the PCA as their eigenvalue was, in dimensions order, 2.9, 1.3, 1.2, and 1. These dimensions accounted for, in dimensions order, 32.3%, 15%, 13.1%, and 10.8% of the total variance, i.e. a total of 71.2% of the total variance (Fig. 2). The variables with the highest absolute contributions for each dimension were: Contact Zone % (27%), Distant Zone % (24%), Active stay at the mirror (20%), and Immobility (18%) for dimension 1; Immobility Periods (42%), U-turn (24%), Thigmotaxis (12%), Rapid movements toward the mirror (12%), and Immobility (9%) for dimension 2 ; Total Distance (57%), U-turn (21%) and Immobility Periods (14%) for dimension 3; Rapid movements toward the mirror (71%) for dimension 4 (for detailed results see Supplementary Table S2). Based on the distribution of fish in the space of these four first PCA dimensions (Fig. 2), (i) perMANOVA detected no difference between the sexes ($F = 0.5118$, p-value = 0.7346), (ii) while ANOSIM detected a significant difference between strains ($R = 0.1287$, p-value = $9.999e^{-05}$). The pairwise ANOSIM showed that all the fish groups are different from each other, except

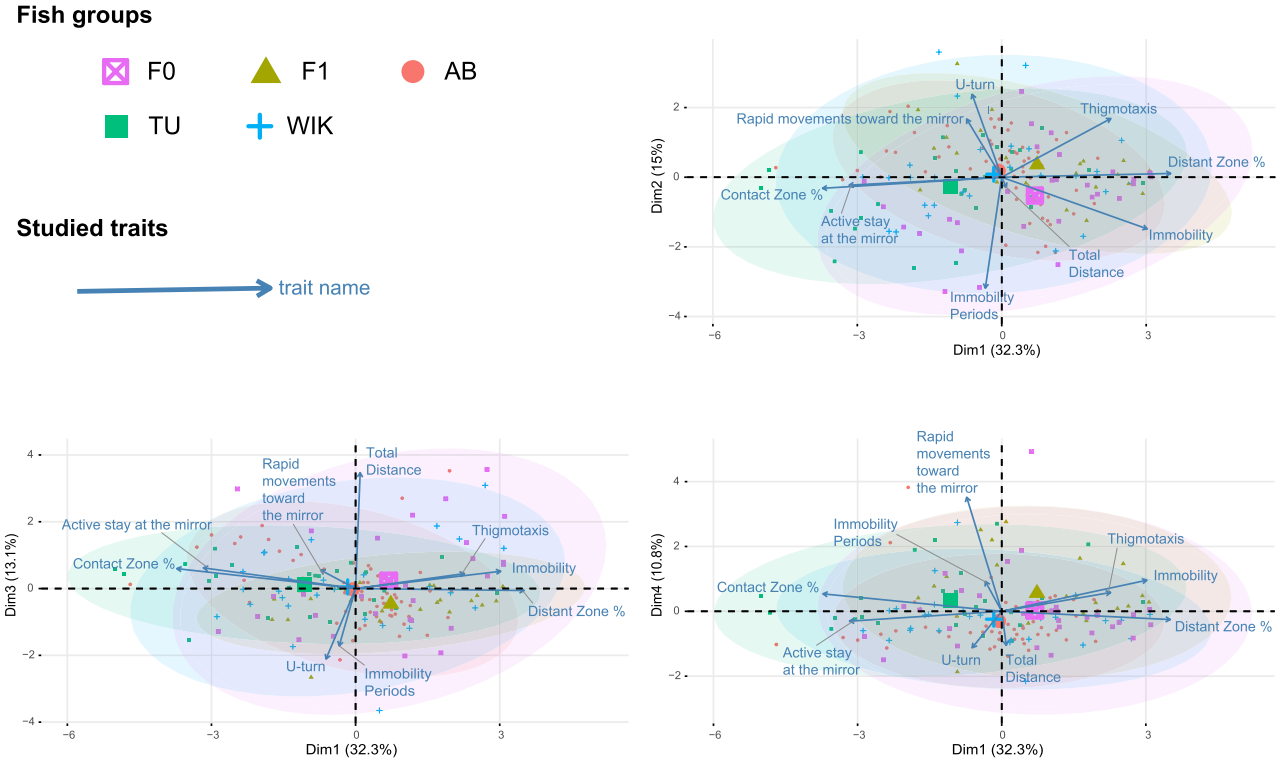

Fig. 2. The principal component analysis of the mirror test of five zebrafish groups. The analysis is based on nine behavioral and activity-related traits. 71.2% of the information (variances) contained in the data are retained by the first four principal components (Dim1, Dim2, Dim3, Dim4). The contributions of each trait on each dimension are indicated by blue arrows. Ellipses are confidence ellipses (0.95) around the mean of each fish group. Large crossed-out squares, triangles, circles, squares and crosses represent the mean of the different zebrafish groups. Small crossed-out squares, triangles, circles, squares and crosses represent zebrafish individuals of each fish group. F0 are wild organisms acclimatized to captivity (Level 1 of domestication). F1 are the offspring of F0 born in captivity (Level 4 of domestication). AB, TU, and WIK are laboratory zebrafish strains (Level 5 of domestication).

WIK which is not different from F0, AB, and TU (Table 3). ANOVA based on each linear mixed effect model of each trait individually also detected differences between fish groups (Supplementary Table S3).

Specific expression of behavioral and activity-related traits of differentiated fish groups

The contribution of the behavioral and activity-related traits to the PCA dimensions showed that (i) F0 and F1 were characterized by stress-related traits (F0 more by Immobility and F1 by Thigmotaxis), (ii) TU was characterized by aggressiveness-related traits (i.e. Contact Zone % and Active stay at the Mirror), (iii) F0 was characterized by an activity-related trait (i.e. Total Distance), and (iv) other strains were not characterized by any particular expression of the traits studied during the experiment (Fig. 2). These characteristic behaviors of each fish group were confirmed by IndVal and ANOVA based on each linear mixed effect model of each trait individually (Tables 4, S3).

Groups	F0	AB	TU	WIK
AB	**R = 0.290; p value = 0.0020**			
TU	**R = 0.1026; p value = 0.0042**	**R = 0.1409; p value = 0.0108**		
WIK	R = 0.0217; p value = 0.1503	R = 0.092; p value = 0.0519	R = 0.0099; p value = 0.2448	
F1	**R = 0.0844; p value = 0.0150**	**R = 0.166; p value = 0.015**	**R = 0.1413; p value = 0.0030**	**R = 0.1235; p value = 0.0042**

Table 3. Pairwise results of analysis of similarities between the five groups of zebrafish. F0 are wild organisms acclimatized to captivity. F1 are offspring of F0 born in captive condition. AB, TU, and WIK are zebrafish laboratory strains. R is chance-corrected estimate of the proportion of the distances explained by group identity; a value analogous to a coefficient of determination in a linear model. Statistically significant results are in bold (i.e. using a p-value threshold < 0.05 corrected by the Benjamini & Hochberg method).

Trait	Interpretation	F0	F1	AB	TU	WIK	p-value
Rapid movements toward the mirror	Aggressiveness	0.0152	0.1225	0.0086	0.1082	0.0125	0.07061
Active stay at the mirror	Aggressiveness	0.0266	0.0517	0.0521	**0.3942**	0.1200	0.00001
Contact Zone %	Aggressiveness	0.1270	0.1025	0.0894	**0.3047**	0.1584	0.00003
Distant Zone %	Stress	**0.2793**	0.2251	0.1577	0.1179	0.1832	0.00155
Immobility	Stress	**0.2788**	0.2761	0.0704	0.1096	0.1460	0.00057
Immobility Periods	Stress	**0.2850**	0.1332	0.0971	0.1950	0.1428	0.00084
Thigmotaxis	Stress	0.1872	**0.2382**	0.1988	0.1765	0.1860	0.00018
U-turn	Stress	0.1140	0.1520	0.1456	0.0829	0.1697	0.08549
Total Distance	Activity	**0.3511**	0.0002	0.2153	0.1819	0.2516	0.00025

Table 4. Results of analysis based on indicator value (IndVal) method to detect behavioral and activity-related trait expression that are specific and regular to studied zebrafish groups. Trait shows the studied traits. Interpretation shows whether the trait provides information on the individual's activity, aggressiveness, or stress level; the higher the trait expression, the higher the activity, aggressiveness or stress level. F0 are wild organisms acclimatized to captivity. F1 are offspring of F0 born in captive condition. AB, TU, and WIK are zebrafish laboratory strains. The table reports indicator value (i.e. the closer the value is to 1, the more it corresponds to a specific and regular trait for a group of fish) and p-value of the IndVal analysis. Bold indicator values indicate when group is characterized by this behavior when the p-value is lower than a threshold of 0.05 corrected by the Holm-Bonferroni method.

Overview of results

Overall, our analyses showed no differences between females and males of the zebrafish studied, but they did highlight differences between F0, F1, AB, TU, and WIK (Fig. 2; Table 3). The results detected differences between F0 and their F1 offspring (i.e. between domestication levels 1 and 4), and between AB and TU (i.e. between fish of the same domestication level). It should be noted that WIK (i.e. domestication level 5) was no different from F0 (i.e. level 1 of domestication). The differences observed concerned behaviors related to activity, aggressiveness and stress (Fig. 2; Table 4). Although F1 differed from F0 in Thigmotaxis and F0 was more active, both F0 and F1 were still characterized by greater stress than AB, TU and WIK. It should be noted that we did not detect a convergent trend for AB, TU, and WIK, which were at the same level of domestication.

Discussion

Potential biases

As biotic or abiotic variation can influence behavioral response in animals, it is important to standardize and control conditions before and during experiments to ensure reproducibility and quality of the results[33]. In this context, our mirror tests were carried out with standardized conditions on fish reared under standardized conditions (i.e. strictly the same) from hatching to mirror test for F1, AB, TU, and WIK. For fish born in the wild (F0), they experienced the same controlled and standardized conditions from their sampling to mirror test. As early-life experiences, interactions with conspecifics, predator, and prey interactions, as well as spatially complex habitats can impact fish behavior development through plasticity[11], results for F0 may be thus biased by differences in living conditions prior to sampling. Nevertheless, we argue that we minimized this potential bias by rearing F0 fish for 247 days in the same standardized conditions as F1, AB, TU, and WIK prior to the mirror tests. Indeed, shorter durations of acclimatization of wild specimens before behavioral experiments have already been used by other studies[73,74]. Moreover, studying the phenotypes of wild individuals intrinsically involves considering individuals with experience of specific, uncontrolled environmental conditions in the wild. This potential bias cannot thus be overcome.

Another potential bias was the age difference between fish. Fish from F1, AB, TU, and WIK were tested at a known age, with F1 being younger than the other three groups (i.e. 120 dpf *vs* 332 to 360 dpf). The age of the F0 was not precisely known, but they were at least 247 dpf. Although behavioral differences may appear with age in zebrafish, many behaviors exhibited by adult and larval zebrafish are quite similar[53]. More specifically, it has been found that aggression levels do not differ between juvenile and adult zebrafish[75]. Moreover, in the groups studied here, all the fish were adults (i.e. effective reproductions observed in the rearing tanks)[76] and far from the life expectancy age of zebrafish[77]. We argue thus that the age difference between our groups is not the main reason for the differences observed. Furthermore, the inclusion of age as a random effect in our linear mixed effect models does not change the observed differentiation (Table S3).

Domestication consequences on behavioral traits

Our results confirmed that domestication can modify fish behavior, even in the first generation born in captivity. However, the three categories of traits studied (i.e. activity, aggressiveness, and stress-related) do not seem to be affected in the same way by domestication.

For stress, there is a trend towards a decrease in the traits' expression from F0 and F1 towards groups that are more advanced in the domestication process (i.e. level 5) and have been produced in captivity for many generations (i.e. AB, TU, and WIK) (Fig. 2; Table 4). The same trend has already been observed for stress-related traits or startle response in other fish species (e.g.[7,78,79]) and in zebrafish[81,82]. The reduction in stress response during

domestication is potentially linked to lower post-stress levels of stress hormones in domesticated fish compared to their wild counterparts as already observed in many species (review in[7]). Stressors associated with captive rearing (e.g. handling, high fish density, presence of human operators) cause considerable selection pressures[83], affecting the fitness of individuals and favoring those with a lesser or shorter response to these stressors[7]. As inter-populational behavioral differentiation in zebrafish has most likely a genetic component (review in[30]) and the physiological stress response is highly heritable in fishes (e.g.[84]), the frequency of phenotypes linked to a lower or shorter stress response should thus increase over generations, leading to behavioral divergence between wild animals and groups that have reached advanced stages of domestication[7]. Our results are in line with this, but we suggest that the modification of stress-related traits is more correlated to the time elapsed since the beginning of domestication (i.e. the number of generations) than to the level of domestication (i.e. here, level 1 and level 4 are still characterized by higher stress than level 5; Fig. 2, Table 4). In the specific context of the mirror test, individuals experience stressors similar to those commonly encountered in the rearing environment, but also others specific to the experiment (e.g. isolation, absence of water flow). This suggests that domestication also modifies the response to stressors not commonly encountered in the rearing system as already observed in other fish species[80].

For aggressiveness, the studied aggressiveness-related traits are statistically different only for one of the laboratory strains (TU, Fig. 2; Table 4). This absence of a general trend is in line with an overview of domestication consequences on behavior of fish species that shows that aggressiveness or agonistic behaviors may be more[85–87] or less[17,88] expressed in domesticated than wild, or expressed similarly in both fish types[89]. At the intraspecific level, previous studies showed (i) laboratory strains (i.e. TM1 and SH) may present higher or lower levels of aggressiveness than recently derived wild-caught strains (i.e. fifth generation born in captivity)[31], and (ii) laboratory strains (i.e. AB, WIK, and TL, a derived strain from TU) may display different level of aggressiveness[60]. Therefore, the evolution of aggressive behavior does not seem to follow a general trend during the domestication process, at least in the species studied. We suggest that the differences in levels of aggressiveness observed between F1, AB, TU, and WIK could be explained by the specificities of their independent domestication history. Indeed, (i) different degrees of intraspecific competition over several generations in the rearing system[9], leading to a selection of aggressiveness (i.e. genetic heritability of aggressiveness in mirror test is known for zebrafish[29]), (ii) different objectives of artificial selection programs and correlated expressions between targeted socio-economical traits of interest and behavioral traits[90], or (iii) different geographical origins of founder populations[91] can lead to different change of aggressive or agonistic behaviors during the domestication. Currently, the unknowns and imprecisions regarding the domestication history of AB, TU, and WIK make it impossible to assess whether the different levels of aggressiveness could be explained by one or more of these points.

In our results, the expression of the studied activity-related trait is statistically higher for F0 than for other groups (Fig. 2, Table 4). Conversely to a previous study[55], we do not detect similar activity divergences between AB, TU, and WIK (Fig. 2, Table 4). It appears that the expression of activity-related traits in zebrafish does not consistently follow a pattern across different domestication levels. Further, when compared with the literature, this difference may be related to the short duration of our observation (e.g. only 5 min in our study *vs.* 48 h in[55]). Moreover, the fact that both AB and TU have been characterized as active strains and WIK as highly anxious[82], could explain a behavioral overlap between activity and anxiety-related behaviors linked to activity (e.g. hyperactivity), often described in zebrafish behavioral research[53]. This overlap highlights the complexity of interpreting activity measures in behavioral research. For F0, as this group is also characterized by higher expression of stress-related traits, we argue that higher expression of activity-related trait could be an artifact linked to hyperactivity after removing the mirror cover[36]. Therefore, we consider that our analyses of activity were not conclusive in assessing the impact of domestication. Although differences in activity levels were observed between groups, these findings cannot be definitively attributed to domestication based on the current analysis. Overall, our results reveal statistically significant divergences between F0 and F1 (Fig. 2, Table 3), suggesting that behavioral differentiation can appear very early in the domestication process. Behavioral differentiation within a single generation during domestication is already known in several fish species[9,11,92]. However, we note that the differentiation observed here between F0 and F1 is limited. Specifically, divergence between F0 and F1 is less pronounced than those observed in certain laboratory strains. This suggests that domestication has not resulted in a substantial significant gap between F0 and F1. Furthermore, our comparisons indicate that differentiation along the domestication process is primarily evident in stress-related traits (i.e. as F0 and F1 are characterized by stress indicators that distinguish them from the other groups), with no clear trend for traits related to activity or aggressiveness. This finding aligns with the specific behavioral responses observed in various zebrafish strains at level 5 of domestication[34,41,93,94]. Overall, our results confirm that domestication rapidly and commonly affects stress responses in fish[78,79].

Implications for aquaculture

For aquaculture purpose, fish species must be able to survive, grow, and ideally reproduce in captivity. This ability depends on the initial characteristics of the stock's founding individuals, their subsequent adaptations, or the adaptations of their offspring to rearing conditions[5,7,20,95]. The completion of the life cycle in captivity as well as the establishment of new fruitful fish production are facilitated by the expression of a set of key traits for fish farming that shape the aquaculture potential of a stock of organisms (i.e. quantification of the degree of expression of all key traits/functions that is favorable to domestication and production)[20]. Several of these key traits are behavioral (e.g. activity, aggressive behaviors, antipredator behaviors, group structure) or related to stress resistance[20]. Beyond the importance of considering the expression of these traits in fish candidates for a new domestication program[20], it is important to know whether the evolution of these traits during domestication tend to occur in a way that further facilitate the production and life cycle control of the organisms.

As already highlighted in the literature (review in[7]), our results suggest domestication tends to be accompanied by a decrease in the response to aquaculture-related stressors over generations, facilitating the continued production of organisms and the maintenance of animal welfare. Reduced stress levels can lead to better health, reproduction, and growth rates, as chronic stress is known to negatively impact immune function and development[22]. It should be noted, however, that our results show no significant change in this stress response from the first generation born in captivity (Fig. 2). This underlines the importance of minimizing potential stress to fish stocks early in the domestication process[22,96]. This includes adjusting factors such as tank design, stocking densities, and handling procedures to align with the reduced stress thresholds[9].

For aggressiveness, our results, congruent with literature (review in[7,9]), show a lack of consistent trends during domestication that universally benefit aquaculture production. We observed that one strain at the level 5 of domestication is more aggressive than other strains at the same level (i.e. AB and WIK) as already observed in other studies[97]. Therefore, it is conceivable that the type of conscious or unconscious selection applied in the past to certain strains may lead to more aggressive behavior.

Limitations and perspectives

Although this study offers new insights into the consequences of domestication on fish behavior, it should be seen as a first step towards a deeper understanding of the phenomenon due to its limitations.

First, this study used a single behavioral test (i.e. mirror test). Although the test used is regarded as appropriate (see materials and methods for details), a combination of several tests would capture a wider range of behavioral traits. For instance, a more comprehensive assessment of the consequences of domestication on zebrafish behavior should add (i) open-field tests to assess general activity and anxious behavior[54], (ii) novel object tests to assess exploratory behavior and boldness[54], and (iii) social interaction tests to examine social behavior and aggression more generally[36].

Second, although our study considered three important levels of domestication, it only studied populations with few or many generations spent in captivity (i.e. F0 and F1 *vs.* AB, TU, and WIK). This limits the study of the dynamics of behavioral changes over time. A more complete analysis would need to include more generations of follow-up, if possible from the wild population through to the implementation of selective breeding programs.

Third, the model species, rearing system, and mirror test design can provide insight for some species phylogenetically related (e.g. Cyprinid, widely used in aquaculture[98]), production system (e.g. recirculating aquaculture system, similar to here used rearing facilities), or aquaculture practices (e.g. containment and isolation). However, our results must be extrapolated with caution, as the experimental set-up and model species are phylogenetically distant from other aquaculture species and from other common aquaculture practices (e.g. ponds). Considering several phylogenetically distant model species and breeding systems to initiate their domestication would enable us to validate extrapolations from the present study.

Fourth, this study was conducted with a relatively small number of individuals from a single wild population for the F0 and F1 strains, which limits the scope of our findings. Drawing broad conclusions from this single wild population may not accurately reflect what occurs in other populations. However, the challenges involved in sampling, acclimating, and maintaining wild zebrafish in captivity make this limitation difficult to overcome. These challenges have led previous studies to avoid using wild zebrafish. Therefore, the results of this study, though constrained by the focus on a single population, provide valuable and rare insights into the early stages of domestication in zebrafish, an area that remains largely unexplored.

Beyond the above suggested perspectives to overcome the limitations of this study, future research should investigate the genetic basis and heritability of these behavioral traits (i.e. already known in some species[94]) to assess the potential benefits of selective breeding programs[19,99,100] and their relevance to influence the trajectory of change during domestication.

Finally, the present study did not directly investigate the consequences of behavioral changes during domestication on fish welfare. Although the reduction of stress over generations is undoubtedly beneficial for welfare, changes in aggressiveness raise questions about the consequences for fish when placed in intensive farming conditions. Therefore, the impact on welfare should be a central focus of future studies on the consequences of domestication[7,22,101].

Conclusion

We studied changes in expression of traits related to activity, aggressiveness, and stress between wild zebrafish and the first generation of their captive-born offspring, as well as with populations more advanced in the domestication process. As already observed in animals, reduction in the expression of stress-related characteristics was observed between groups at different levels of domestication (i.e. decrease with level 5), suggesting potential selection for stress tolerance during the process. However, no general trend was observed for traits related to activity and aggressiveness among groups, potentially highlighting the complex interplay of genetic background and environmental factors in shaping these behaviors under domestication, even within a species in fish. These specificities observed between the studied fish groups question the potential evolutionary convergences or parallels for behavioral traits previously suggested[7]. From an aquaculture viewpoint, our results suggest that the evolution of behavioral traits, impacting production, does not necessarily follow a constant general trend towards trait expressions more favorable to fish farming. However, it should be noted that our study is limited by the use of a single type of behavioral assay and inevitable potential bias in the F0 study (i.e. adult sampling in the wild, with no possible age control, unlike the other fish groups).

Future studies on the consequences of domestication on fish behavior should (i) consider a combination of behavioral assays to capture a wider range of behavioral traits, (ii) limit age divergences between groups or demonstrate that these divergences have little impact on the changes observed between generations, (iii) track

the dynamics of changes over time in greater detail by studying a larger number of successive generations, and (iv) investigate the underlying mechanisms driving behavioral changes under domestication.

Overall, our study contributes to advancing knowledge in fish behavior and domestication, offering insights that could enhance fish welfare and productivity in aquaculture. By refining our understanding of stress, aggressiveness, and activity responses in zebrafish under domestication, we lay the groundwork for sustainable aquaculture practices that promote resilience and efficiency in global food production systems.

Data availability

Dataset used for the analyses are available in Supplementary Table S1.

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Author contributions

E.D., C.C., A.P., and T.L. conceived and designed the experiment. E.D., C.C., A.P., S.L., A.H., and T.L. performed the experiment and analyzed data. E.D., C.C., A.P., and T.L. wrote the manuscript and all authors contributed to the final version. E.D., C.C., and T.L. equally contribute to this study.

Competing interests

The authors declare no competing interests.

Ethical approval

No specific permits were required for wild zebrafish sampling as collection did not occur in privately-owned areas, protected locations, or protected species. All the procedures used complied with national and international guidelines for the protection of animal welfare (Directive 2010/63/ EU). This study was conducted with the approval of the Animal Care Committee of Lorraine (CELMA n°66) and the French Ministry of Higher Education, Research, and Innovation (APAFIS #38648-202209221545630 v5), at the *Plateforme Expérimentale en Aquaculture* (PEA, University of Lorraine, Vandœuvre-lès-Nancy, France; registration number for animal experimentation D54-547–18).

Additional information

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