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Zebrafish *Danio rerio* shows behavioural cross-context consistency at larval and juvenile stages but no consistency between stages

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Abstract

Coping style is defined as a set of individual physiological and behavioural characteristics that are consistent across time and context. In the zebrafish *Danio rerio*, as well as in many other animals, several covariations have been established among behavioural, physiological and molecular responses. Nonetheless, not many studies have addressed the consistency in behavioural responses over time starting at the larval stage. Therefore, this study aimed to improve the understanding of behavioural consistency across contexts and over time in zebrafish from the larval to juvenile stages. Two distinct experiments were conducted: a larval stage experiment (from 8 to 21 days post fertilization, dpf) and a juvenile stage experiment (from 21 to 60 dpf). On one hand, the larval experiment allows to focus on the transition between 8 and 21 dpf, marked by significant morphological changes related to the end of larval stage and initiation of metamorphosis. On the other hand, the juvenile experiment allows to properly cover the period extending from the end of larval stage to the juvenile stage (60 dpf), including metamorphosis which is itself completed around 45 dpf. Within each experiment, boldness was determined using a group risk-taking test to identify bold and shy individuals. A novel environment test was then performed at the same age to evaluate consistency across contexts. Groups of fish (either bold or shy) were bathed in an alizarin red S solution for later identification of their initially determined coping style to evaluate behavioural consistency over time. Fish were then reared under common garden conditions and challenged again with the same behavioural tests at a later age (21 and 60 dpf in the larval and juvenile experiments, respectively). Behavioural consistency was observed across contexts, with bold fish being more active and expressing higher thigmotaxis regardless of age. There was, however, little behavioural consistency over age, suggesting behavioural plasticity during development. Moreover, the use of alizarin red S to conduct this experiment provides new perspectives for the further study of the longitudinal evolution of various traits, including behaviour, over life stages in fish.

KEYWORDS

coping style, fish, personality, vital staining

1 | INTRODUCTION

Coping style is defined as a coherent set of individual physiological and behavioural traits that are consistent across context and over time, *i.e.*, in different situations and at different times (Koolhaas *et al.*, 1999). The existence of coping styles has been demonstrated in many animal species, including fish, and is described as a *continuum* between two extreme phenotypes, which are often called proactive and reactive (Castanheira *et al.*, 2017; Ferrari *et al.*, 2015; Øverli *et al.*, 2007; Tudorache *et al.*, 2013; Wong *et al.*, 2019). Temperament (Reale *et al.*, 2007), personality (Gostling, 2001; Reale *et al.*, 2010) and behavioural syndrome (Sih *et al.*, 2004) are terms related to coping style, as defined by Koolhaas *et al.* (1999). In fish, proactive individuals are generally bolder (Alfonso *et al.*, 2019; Huntingford *et al.*, 2010; Øverli *et al.*, 2006), more aggressive (Øverli *et al.*, 2004) and more active (Øverli *et al.*, 2002), and explore their environment faster than reactive fish (Ferrari *et al.*, 2015; Millot *et al.*, 2009).

Behavioural consistency across contexts and over time has been shown in adult fish, including the zebrafish *Danio rerio* (Rey *et al.*, 2013; Toms and Echevarria, 2014). For example, boldness, aggression, fear and exploration, measured in five distinct contexts, were shown to be consistent over a period of 7 days (Toms and Echevarria, 2014). Rey *et al.* (2013) also showed that boldness, measured using a risk-taking test, was positively correlated with aggressive behaviour and highly consistent over 10 months in adults. In most cases, including those mentioned earlier, coping styles were, however, studied in juveniles or adults, and consistency over time was generally evaluated through repeated assays within a single developmental stage. Coping styles have been, however, occasionally determined at early stages, *i.e.*, larval stages in fish. Tudorache *et al.* (2015) showed that swimming behaviour differed in the larvae of zebrafish 8 days *post* fertilization (dpf), depending on the order of emergence from a sheltered compartment. Early emerging larvae displayed lower velocity and travelled less distance than later emerging ones. Moreover, early emerging larvae expressed higher cortisol levels in response to netting stress but reached baseline level faster than late emerging larvae. Pasquet *et al.* (2016) showed that northern pike *Esox lucius* larvae exhibited consistency in behavioural responses across contexts in maze and novel object tests. More active larvae visited a higher number of zones in the maze and spent more time near the novel object than others, indicating that coping styles were consistent across contexts at the larval stage. Such cross-context consistency at the early stage is contradictory to observations in the mangrove killifish *Kryptolebias marmoratus*, in which it was absent before the juvenile stage at 61 days *post* hatching (Edenbrow and Croft, 2011).

Ontogenic studies evaluating the consistency of coping style across life stages in fish are scarce. The consistency of coping style between early (likely larval) and later stages has been evaluated only in two studies using two species, mangrove killifish and nine-spine sticklebacks *Pungitius pungitius*, and the authors concluded that there was no behavioural consistency between these life stages (Edenbrow and Croft, 2011; Herczeg *et al.*, 2013). Nonetheless, in both studies, individuals were kept isolated to allow for later individual

identification. Such a rearing method can introduce several biases, such as divergence in physico-chemical variables, unintentional differences in feeding (especially with such small individuals) or stress induced by social isolation [*e.g.*, in zebrafish, Pagnussat *et al.* (2013)].

An option to circumvent these limitations is batch marking of larvae. Indeed, this method allows the rearing of fish, from different coping styles, under common garden conditions. Alizarin red S (C₁₄H₇NaO₇S) is a fluorescent dye that labels calcium-containing tissue and has thus been used to monitor skeletal mineralization *in vivo* in zebrafish (Bensimon-Brito *et al.*, 2016; DeLaurier *et al.*, 2010; Tu and Johnson, 2011). Recently, alizarin red S was successfully used for mass marking of several fish species at an early stage, showing no particular mortality or growth delay (Caraguel *et al.*, 2015; Lü *et al.*, 2015; Stanczak *et al.*, 2015). The mass marking of larvae can be performed by ingestion of *Artemia salina* previously bathed in an alizarin red S solution (Stanczak *et al.*, 2015) or by directly bathing the larvae in the solution (Caraguel *et al.*, 2015; Lü *et al.*, 2015). Thus, alizarin red S appears to be a promising non-invasive tool to mark larvae and monitor behavioural consistency in zebrafish across life stages. Such information is indeed essential for understanding how development and/or environment may shape behaviour and coping styles.

Thus, the objectives of this study were to evaluate behavioural consistency across contexts and over time in zebrafish from the larval to juvenile stages. Zebrafish generally hatch at 2–3 dpf and reach the juvenile stage at 45 dpf for finally being considered as adults around 90 dpf when reared at 28°C (Nüsslein-Volhard and Dahm, 2002; Singleman and Holtzman, 2014; Westerfield, 2000). Metamorphosis is the continuous process leading from larvae to juvenile. It has no absolute borders because it depends on the analysed trait and also because metamorphosis course depends on external factors such as temperature or food availability (Parichy 2009). As an example, skin pigmentation pattern changes from around 14 until around 30 dpf, with 22 dpf being defined as the middle of metamorphosis (Parichy and Turner 2003). The age of 21–22 dpf appeared to be pivotal for other physiological or morphological changes such as the end of the transition from skin to gill respiration (Hale, 2014), and the beginning of gonadal transformation from the “juvenile ovary” stage (Orban *et al.*, 2009). This age is also marked by changes in neurobehavioral abilities (Valente *et al.*, 2012). These different developmental steps shaped the rationale of the study for conducting two distinct experiments, a so-called larval experiment from 8 to 21 dpf and a juvenile experiment from 21 to 60 dpf. On one hand, the larval experiment allows to focus on the transition between 8 and 21 dpf, marked by significant morphological changes related to the end of larval stage and the initiation of metamorphosis. On the other hand, the juvenile experiment allows to properly cover the period extending from the end of larval stage to the juvenile stage (60 dpf), including metamorphosis.

Within each experiment, fish were screened to determine their boldness or shyness using a group risk-taking test and thereafter challenged in an individual novel environment test at the same age to first evaluate consistency across contexts. Second, behavioural consistency over time was monitored by comparing fish behavioural

responses between the two successive ages (*i.e.*, between 8 and 21 dpf and between 21 and 60 dpf) using alizarin red S marking for the identification of coping style.

2 | MATERIALS AND METHODS

This study was conducted with the approval of the Animal Care Committee of Poitou-138 Charentes # 84 COMETHEA (France) under project authorization number CE2012-23 and followed the recommendations of Directive 2010/63/EU.

2.1 | Fish rearing

The study was conducted on the zebrafish TU strain (ZFIN ID: ZDBGENO-990623-3), maintained at the Fish Ecophysiology Platform (PEP - http://wwz.ifremer.fr/pep_eng) and originating from the European Zebrafish Resources Center (EZRC, Karlsruhe, Germany).

Eggs were obtained by random pair-wise mating using adults from the stock at approximately 180 dpf to provide larvae for the behavioural experiments. Briefly, one male and one female were placed together in spawning boxes on the evening before the eggs were required (AquaSchwarz, Germany). Eggs were collected the following morning, and the fertilization rate assessed within 2 h of collection. Spawns with a fertilization rate > 80% were kept for experiments and placed in Petri dishes filled with 30-ml isotonic medium E3 (1 l: 17.2 g NaCl, 0.76 g HCl, 2.9 g CaCl₂ · 2 H₂O, 4.9 g MgSO₄ · 7H₂O) and methylene blue (250 µl.L⁻¹) and incubated at 28°C under a 14-h/10-h dark/light cycle as described in Alfonso *et al.* (2019). The next day, 10 eggs from six different spawns were mixed to avoid spawn effects to obtain 60 embryos for one Petri dish. Multiple Petri dishes were prepared, depending on the number of replicates needed. At 72 h post fertilization (hpf), after hatching, the chorions were removed from the Petri dishes, and the larvae returned to the incubator until 5 dpf. They were then transferred from Petri dishes to 1 l tanks. Initial behavioural tests and alizarin red S marking were performed at 8 and 21 dpf. After these first characterization and marking steps, larvae with both coping styles were maintained in a common garden, as explained later. Larvae were reared in 1 l tanks from 8 to 21 dpf (24 larvae per tank, *n* = 12 per coping style), and at 21 dpf, they were transferred to 10 l tanks in the rearing system (20 larvae per tank, *n* = 10 per coping style). They were reared under a 14 h/10 h dark/light cycle in both cases. Physical water conditions were maintained constant during the experiment: temperature of 27 ± 1°C, conductivity of 300 ± 50 µS.cm⁻¹ and pH 7.5 ± 0.5, using a mixture of reverse osmosis water and tap water, both initially treated with charcoal and sediment filters. Ammonia, nitrites and nitrates were monitored weekly and remained within recommended ranges (Lawrence, 2007). Fish were fed three times a day, *i.e.*, morning and evening with pellets and at midday with freshly hatched *A. salina*. The size of the food pellets was adapted to the age of the fish and size of the mouth. From 5 dpf to the end of experiment, fish were fed sequentially with 100, 200 and 300 µm SDS

(Special Diet Service; Dietex France, Argenteuil, France), according to the size of the fish.

2.2 | Experimental procedure to monitor behavioural consistency across contexts and over age

Two experiments, using different fish, covering the larval stage (larval experiment; from 8 to 21 dpf) and part of the juvenile stage (juvenile experiment; from 21 to 60 dpf) were performed; the steps are summarized in Figure 1. In both cases, the first step consisted of a risk-taking test performed in groups at 8 dpf (larval experiment) and 21 dpf (juvenile experiment) to measure boldness and thus assess coping style. Individuals were then kept isolated overnight. The following day, a novel environmental test was performed to evaluate behavioural consistency across contexts (see details in the “Behavioural experiments” section). After the second test, fish exhibiting one or the other coping style were bathed in vital stain using alizarin red S (C₁₄H₇NaO₇S; see details in the “Marking procedure” section) and fish of both coping styles were further reared under common garden conditions to avoid possible rearing bias or stress induced by isolation. Later, at 21 dpf for the larval experiment and 60 dpf for the juvenile experiment, the fish were again challenged to evaluate behavioural consistency over life stages and alizarin red S marking was read to determine the initial coping style.

2.3 | Behavioural experiments

All behavioural experiments were performed in the experimental room at 27°C, with a photoperiod synchronized to that of the rearing room, *i.e.*, 14/10 h (light/dark). After each experiment, the water was changed. The fish were fed once on the day of the experiment at the end of the day.

Videos for the larval stage behavioural experiments (8 or 21 dpf) were recorded using DanioVision (Noldus, The Netherlands). Videos for

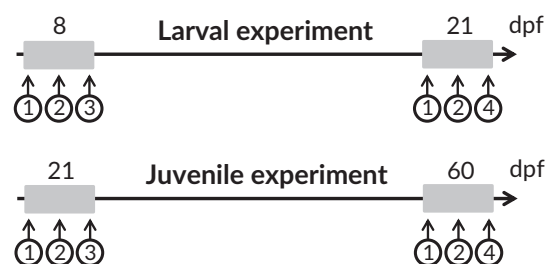


FIGURE 1 Graphical protocol used to monitor behavioural consistency across contexts and over time from larval to juvenile stages using the two different experiments. ⊙ Group risk-taking test for boldness screening; ⊕ individual behavioural characterization using either open-field test [at 8 and 21 days post fertilization (dpf)] or novel tank diving test (at 60 dpf); ⊗ marking using alizarin red S; ⊖ reading of alizarin red S staining. (■) experimental procedure period

the juvenile stage behavioural experiments (60 dpf) were recorded using an ICD-48E analogue camera (Ikegami, Japan) and a 2.1–13.5 lens (Fujinon, Japan) connected to a computer with an acquisition card and EthoVision XT 10.1 software (Noldus, The Netherlands). Data extraction and analyses were performed using EthoVision XT 10.1 software.

2.3.1 | Boldness screening using group risk-taking tests

The day before conducting the test, groups of fish were gently caught from their home tank and groups of 15 individuals were assembled. The groups of fish were placed in a Petri dish (90 mm in diameter × 14.2 mm in height) at both 8 and 21 dpf or in a 3 l tank at 60 dpf and then transferred to the experimental room for acclimation. The next day, the group risk-taking test was performed to assess boldness. Risk-taking tests were performed on 22 groups ($n = 330$ larvae) at 8 dpf and 4 groups ($n = 60$ larvae) at 21 dpf for the larval experiment. For the juvenile experiment, the risk-taking tests were performed on seven groups ($n = 105$ larvae) at 21 dpf and five groups ($n = 50$ fish) at 60 dpf.

Three different risk-taking test devices were used depending on the age of the fish. At 8 dpf, the device consisted of two Petri dishes (3.3 cm in diameter × 1 cm in height) connected to each other by a passage tube [0.5 cm in diameter × 0.6 cm in height, Tudorache *et al.* (2015)]. One Petri dish was covered with black tape, whereas the other one was not. At 21 dpf, the device consisted of a dark, covered compartment (4 cm in width × 7 in length cm × 2.5 cm in height) connected by an opening situated at the centre of the connecting face of the compartment to another one that was lit (6.5 cm in width × 7 cm in length × 2.5 cm in height). At 60 dpf, the device consisted of a dark, covered compartment (8 cm in width × 10 cm in length × 11 cm in height) connected by an opening (2.5 cm in diameter) situated at the centre of the connecting face of the compartment to another one that was lit (15 cm in width × 10 cm in length × 11 cm in height) (Rey *et al.*, 2013).

Regardless of age, the risk-taking test started by gently introducing the group of fish ($n = 15$) into the sheltered compartment. After an acclimation period of 15 min, a door was opened, allowing fish to exit the sheltered compartment to enter the risky lit compartment for a period lasting 10 min. At the end of the experiment (*i.e.*, 10 min after opening the door) or after half of the fish had left the sheltered area (if less than 10 min), the connection between the two compartments was cut. The 10 min duration was used based on Tudorache *et al.* (2015). Larvae (8 or 21 dpf) were gently caught using a 3 ml pipette (with a wide-cut opening) and placed individually into a Petri dish (3.3 cm in diameter × 2.5 cm in height). At 60 dpf, fish were netted and placed individually in a 1 l tank (AquaSchwarz, Germany) before further behavioural experiments. Fish that left the sheltered compartment were considered to be bold, and fish that stayed in the covered compartment were considered to be shy. The proportion of bold and shy fish (%) was calculated for each group and age.

2.3.2 | Individual open-field test and novel tank diving test

The day after the risk-taking test was conducted, fish were tested individually to evaluate behavioural consistency across contexts and identify a behavioural syndrome by measuring activity and thigmotaxis/vertical positioning (8 dpf: $n = 48$ bold, $n = 48$ shy; 21dpf: $n = 50$ bold, $n = 55$ shy; 60 dpf: $n = 24$ bold, $n = 23$ shy). Depending on the age of the fish, three different devices and experimental procedures were used, but all three belonged to the family of novel environment test devices, either open-field or novel tank diving tests.

The open-field test for 8 dpf larvae was adapted from Tudorache *et al.* (2015). Larvae were individually transferred into one well of a 24-well plate (Krystal 24, opaque wall and clear bottom microplate) and placed into the DanioVision device. After an acclimation period of 5 min in darkness, the light was switched on and a video was recorded in top view for 10 min. The open-field arena was virtually separated into two areas of equal volume (centre and periphery), according to Schnorr *et al.* (2012). The distance travelled (cm) and the proportion of time spent in the peripheral half (%) were calculated to evaluate activity and thigmotaxis, respectively. At 21 dpf, the open-field test was adapted from Ahmad and Richardson (2013). Larvae were individually transferred to a Petri dish (3.3 cm in diameter × 1 cm in height) containing 5 ml E3 medium and placed into the DanioVision device. A video was recorded in top view for 10 min. The open-field arena was virtually separated into two areas of equal volume (centre and periphery). The distance travelled (cm) and the proportion of time spent in the peripheral half (%) were recorded to evaluate activity and thigmotaxis, respectively. At 60 dpf, a novel tank diving test adapted from Egan *et al.* (2009) was used. Fish were individually transferred into a novel tank (trapezoidal 1.5-l tank, Aquatic Habitats) and a video was recorded in side view for 4 min. The novel tank arena was virtually separated into two areas of equal volumes (top and bottom) according to Egan *et al.* (2009). The distance travelled (cm) and the proportion of time spent in the bottom half (%) were recorded to evaluate activity and bottom dwelling, respectively.

2.3.3 | Marking procedure

After behavioural characterization (*i.e.*, at 8 or 21 dpf for the larval and juvenile experiments, respectively) groups of fish identified as either bold or shy were marked using alizarine red S and the procedure described below (section 2.4.1). Thus, both bold- or shy-marked fish were reared under common garden conditions (*i.e.*, in a common tank) with unmarked larvae of the opposite coping style until the next assessment (see Figure 1).

2.4 | Vital staining by alizarin red S, reading procedure and LPMR test

This section presents the marking procedure used for the experiment described earlier, as well as the preliminary experiments carried out to validate the use of alizarin red S as a vital stain.

2.4.1 | Marking procedure

Alizarin red S is a fluorochrome certified by the Biological Stain Commission for *in vivo* marking. The marking procedure of Bensimon-Brito *et al.* (2016) has been validated in this study by first verifying that marking had no effect on development, survival or larval behaviour by comparing these features between control and alizarine red S marked larvae. Triplicates ($n = 3 \times 40$ larvae) were assembled for each treatment for both larval and juvenile experiments. Treated larvae were bathed in the 1 l system in water containing 0.01% alizarin red S (Sigma-Aldrich, St Quentin Fallavier, France) buffered with sodium hydroxide (50 μ l of 0.5 M NaOH) for 150 min. After the bathing period, larvae were rinsed in clean water twice before they were reintroduced into the rearing tank. Larvae marked at 8 dpf were then observed at 21 dpf (larval experiment), and larvae marked at 21 dpf were observed at 60 dpf (juvenile experiment) to verify the efficiency of the marking protocol. The control groups were treated in the same way, except they were not exposed to alizarine red S, and were reared in separate tanks under the same conditions. Survival and persistence of the staining were monitored up to the end of the test period.

2.4.2 | Reading procedure

Larvae or juveniles were anaesthetized with benzocaine [50 μ l of a stock solution (50 g.l^{-1} in 100% ethanol); Sigma-Aldrich] for vital staining reading using a stereo microscope (Olympus SZX9) under green fluorescent light (510–550 nm, X-Cite, 120Q EXFO). Images were captured using a camera (DFX31AU03, The Imaging Source, Germany) and IC Capture 2.4 software (The Imaging Source).

2.4.3 | Monitoring of larval behaviour after alizarin red S marking

To ascertain that the marking procedure has no behavioural effects, the larval photomotor response (LPMR) test was performed (Schnorr *et al.*, 2012; Vignet *et al.*, 2015) on a sub-sample of larvae ($n = 30$ per treatment). The day after alizarin red S marking (*i.e.*, 9 dpf), larvae were transferred individually into the wells of 24-well plates (Krystal 24, opaque wall and clear bottom microplate) for acclimation, and the plates were transferred to the experimental room, as described earlier. An equal number of larvae from each treatment were introduced into each plate to avoid a potential trial effect. Plates were successively placed into a DanioVision device. After an initial 10-min acclimation period in the DanioVision device, the LPMR, consisting of the following 5-min steps, was performed: Light on-1 (LON1, 70 lx), Light off (LOFF, < 1 lx) and Light on-2 (LON2, 70 lx), with constant infra-red light maintained during video recording. The distance travelled (cm) was recorded and summed for each 5-min step.

2.5 | Statistical analyses

Statistical analyses were performed using R 3.1.0 software (R Core Team, 2013). All statistical analyses were carried out at a 95% level of significance. Values are represented as the mean \pm S.E. Normality of the data was verified using the Shapiro test before applying either parametric (ANOVA) or non-parametric tests (Wilcoxon rank sum test), depending on whether normality was verified.

The proportion of fish that left the covered area (bold) during the risk-taking tests was compared between ages using the chi-squared test, followed by pair-wise multiple comparisons. *P*-values were adjusted using the Holm method.

Principal component analysis (PCA) was performed within each age group for the open-field and novel tank diving tests using the informative variables for activity [the distance travelled (cm)] and thigmotaxis or vertical positioning [*i.e.*, proportion of time spent in the peripheral area (%) at 8 and 21 dpf and the proportion of time spent in the bottom area (%) at 60 dpf] using the ade4 package (Dray and Dufour, 2007). Individual PC scores were downloaded for the first axis (PCA axis 1) to extract individual behavioural scores, as described in numerous studies (Castanheira *et al.*, 2013, 2016; Ferrari *et al.*, 2015; Millot *et al.*, 2014). Consistency across contexts (risk taking vs. open-field/novel tank diving tests) was estimated using the Wilcoxon rank sum test on individual behavioural scores to compare coping styles (*i.e.*, bold vs. shy according to the risk-taking of the corresponding age) within each age group of the two experiments.

Finally, the exact binomial test was performed to evaluate behavioural consistency in the risk-taking test between two successive ages (*i.e.*, between 8 and 21 dpf and between 21 and 60 dpf) with random theoretical probability within each coping style ($P = 0.5$, *i.e.*, the equal probability of being characterized as bold or shy in the second test). The chi-squared test was also performed to compare behavioural consistency in risk taking between coping styles within each experiment.

In parallel, survival was compared between bathed and control larvae using the log-rank Mantel-Cox test to confirm the harmlessness of vital staining. For the LPMR test, the distance travelled was compared between treatments (control vs. marked) with a repeated-measures ANOVA (with three periods, *i.e.*, LON-1, LOFF, LON-2) followed by Tukey's HSD *post hoc* test.

3 | RESULTS

3.1 | Validation of the alizarin red S marking procedure

In preliminary test groups, 100% of larvae bathed in 0.01% alizarin red S for 150 min at 8 and 21 dpf showed visible marks in the otolith and calcified tissues of the caudal peduncle at 21 and 60 dpf, respectively (Figure 2a,b). Survival in the stained groups was above 80% and identical to that of the control groups (Mantel Cox: $\chi^2 = 1.470$, $df = 1$,

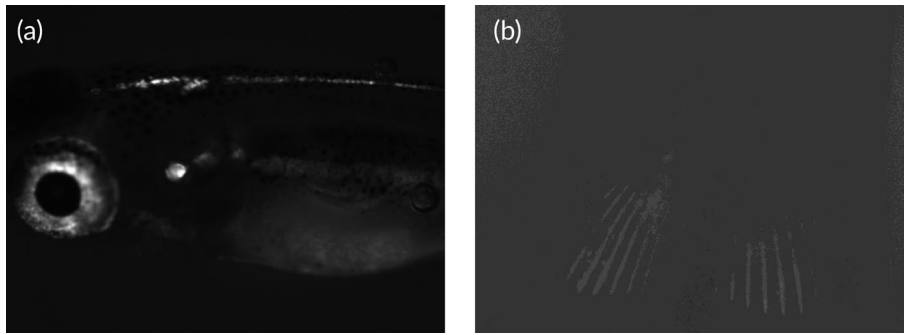


FIGURE 2 Example of alizarin red S marks observed in anaesthetised individuals in (a) otolith at 21 dpf and (b) in caudal peduncle rays at 60 dpf

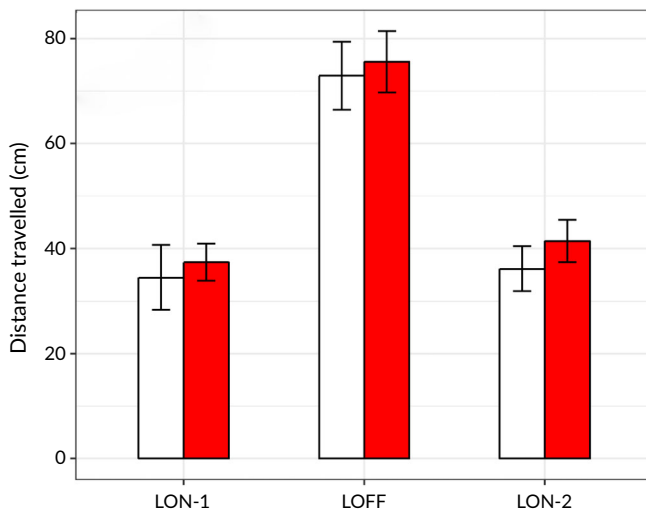


FIGURE 3 Distance travelled during each 5-min steps (Light on-1, Light off, Light on-2) of the larval photomotor response performed at 9 dpf for control ($n = 30$, white bars) and marked larvae ($n = 30$, red bars). Values are mean \pm S.E. (□) control and (■) marked

$P = 0.23$ and $\chi^2 = 0.005$, $df = 1$, $P = 0.94$ for marking at 8 and 21 dpf, respectively).

The distance travelled in the LPMR test was higher during the LOFF than during LON1 and LON2 periods, regardless of the treatment (control vs. marked) (repeated measures ANOVA, $df = 2$, $F = 72.5$, $P < 0.001$) and there was no effect of treatment ($F = 0.4$, $df = 3$, $P = 0.5$) or interaction between treatment and period ($F = 0.1$, $df = 3$, $P = 0.9$; Figure 3).

3.2 | Risk-taking test

The proportion of bold fish, individuals which left the covered compartment during the risk-taking test, was significantly different between ages ($\chi^2 = 62.7$, $df = 3$, $P < 0.001$; Figure 4). Pair-wise comparison showed that the proportion of bold fish was lower at 8 dpf (14.5%) than at 21 dpf (35%) during the larval experiment ($P < 0.001$). The proportion of bold fish at 21 dpf did not differ between the larval and juvenile experiments (35% in the larval experiment and 48% in

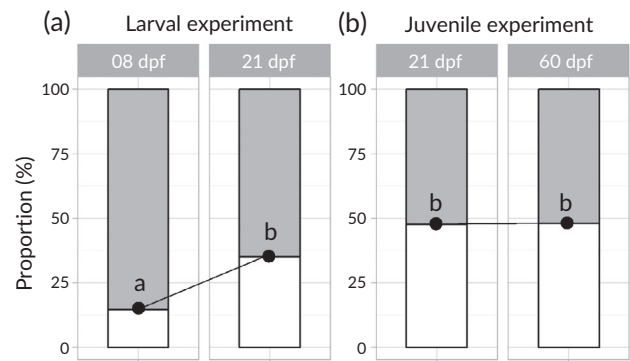


FIGURE 4 Proportion of bold (white bars) and shy (dark grey bars) individuals during risk-taking test for (a) larval experiment (8 dpf: $n = 48$ bold, $n = 282$ shy; 21 dpf: $n = 21$ bold, $n = 39$ shy) and (b) juvenile experiment (21 dpf: $n = 50$ bold, $n = 55$ shy; 60 dpf: $n = 24$ bold, $n = 26$ shy). Pair-wise comparison of proportions: different letters indicate significant difference for proportions of proactive fish across ages. (□) bold and (■) shy

juvenile experiment; $P = 0.47$). Finally, the proportion of bold fish in the juvenile experiment did not differ between 21 and 60 dpf (48% at both ages; $P = 1$).

3.3 | Behavioural consistency across contexts

PCA axis 1 explained 69 and 75% of the variability observed in the behavioural responses at 8 and 21 dpf, respectively, during the larval experiment. PCA axis 1 explained 70 and 58% of the variability observed in the behavioural responses at 21 and 60 dpf, respectively, during the juvenile experiment. PCA axis 1 was equally explained by the distance travelled (cm) and the proportion of time spent in the peripheral/bottom area (%) for each age group. Individuals with a high behavioural score expressed higher activity and higher thigmotaxis than those with low behavioural scores (Table 1).

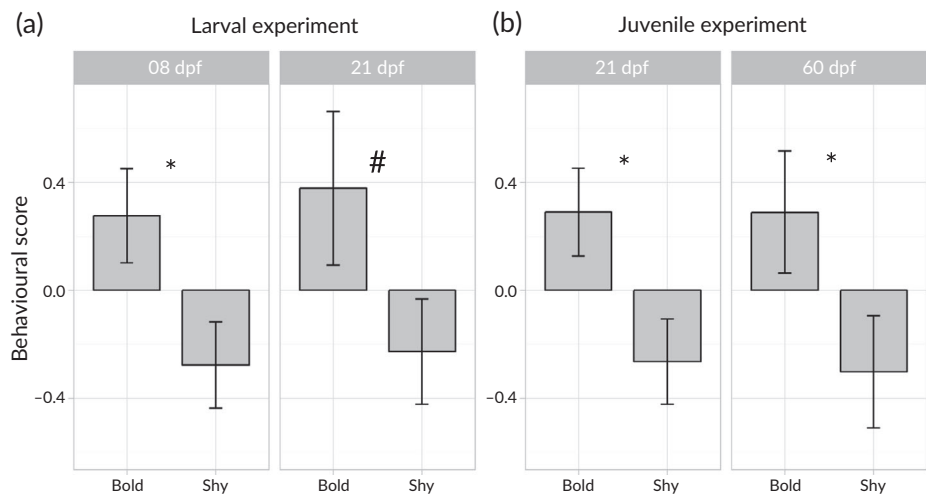
During the larval experiment, PCA axis 1 scores were significantly higher for bold fish than shy fish at 8 dpf ($W = 1435$, $P = 0.04$) and a "trend" was observed at 21 dpf ($W = 468$, $P = 0.09$). During the juvenile experiment, bold fish had higher behavioural scores than shy fish at both 21 and 60 dpf ($W = 1046$, $P = 0.04$ and $W = 374$, $P = 0.04$,

TABLE 1 Loadings of behavioural variables for the PCA axis 1 performed during larval experiment (8 and 21 dpf) and juvenile experiment (21 and 60 dpf)

Variables in PCA axis 1	Larval experiment		Juvenile experiment	
	8 dpf	21 dpf	21 dpf	60 dpf
Distance travelled (cm)	0.83	0.87	0.84	0.76
Proportion of time (%) spent in periphery (8 and 21 dpf) or bottom area (60 dpf)	0.83	0.87	0.84	0.76
Variance explained (%)	69	75	70	58

Note: dpf, days post fertilization; PCA axis 1, principal component analysis axis 1.

FIGURE 5 Individual behavioural scores (principal component analysis axis 1) of bold and shy fish for (a) larval experiment (8 dpf: $n = 48$ bold, $n = 48$ shy; 21 dpf: $n = 21$ bold, $n = 35$ shy) and (b) juvenile experiment (21 dpf: $n = 50$ bold, $n = 55$ shy; 60 dpf: $n = 24$ bold, $n = 23$ shy). Wilcoxon sum rank test between coping styles within age #: $P < 0.10$ and *: $P < 0.05$. Values are mean \pm S.E.



respectively; Figure 5). This shows that bold and shy fish characterized using the risk-taking test differed in their behavioural responses during the open-field and novel tank diving tests. Bold fish displayed higher activity and higher thigmotaxis than shy fish, both during the larval and juvenile experiments, except for a "trend" for 21 dpf during the larval experiment.

3.4 | Behavioural consistency over age

The same procedure was repeated, marking either bold fish in some replicates ($n = 3$ for both larval and juvenile experiments) or shy fish in others ($n = 2$ and $n = 3$ replicates for the larval and juvenile experiments, respectively), to account for potential bias induced by marking. The data were pooled, as no differences were observed in survival or behavioural responses (see the "Validation of alizarin red S marking procedure" section). Approximately 33% of bold and 61% of shy fish expressed behavioural consistency during the risk taking-test between 8 and 21 dpf. Between 21 and 60 dpf, 41% of bold and 61% of shy fish showed behavioural consistency (Figure 6). The proportion of consistent behaviour did not differ from theoretical random probability for bold ($P = 0.12$) or shy fish ($P = 0.28$) between 8 and 21 dpf. Between 21 and 60 dpf, the proportion of consistent behaviour also did not differ from theoretical random probability for bold ($P = 0.52$) or shy fish ($P = 0.40$). Nonetheless, shy

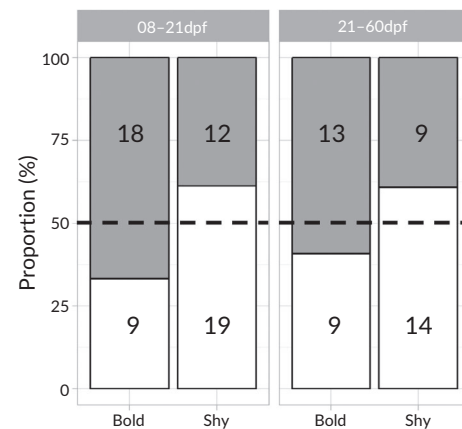


FIGURE 6 Proportion of fish with consistent (white bars) and non-consistent (dark grey bars) coping style during risk-taking test for each age in bold and shy fish (8–21 dpf: $n = 27$ bold, $n = 31$ shy; 21–60 dpf: $n = 22$ bold, $n = 23$ shy). Dashed black line represents random theoretical probability to be categorized as bold or shy in the following risk-taking test. (□) consistent and (■) not consistent

fish tended to display more consistent behaviour than bold fish between 8 and 21 dpf ($\chi^2 = 3.5$, $df = 1$, $P = 0.06$). This trend disappeared between 21 and 60 dpf ($\chi^2 = 1.1$, $df = 1$, $P = 0.29$; Figure 6).

4 | DISCUSSION

Coping style is assumed to be consistent across contexts and over time. Nonetheless, this has been poorly studied during early life stages. In parallel to development, early experience can affect individual coping capacity, including in fishes (Poisson *et al.*, 2018; 2019; Vindas *et al.*, 2019). The present study aimed to evaluate coping style in fish, starting at the larval stage, and to evaluate the consistency of the coping style over age, *i.e.*, by comparing the results between the larval and juvenile stages. Thus two distinct experiments were conducted, a larval experiment from 8 to 21 dpf and a juvenile experiment from 21 to 60 dpf. Within each experiment, two behavioural tests were repeated (*i.e.*, the group risk-taking test and the novel environment test) at the two start and end ages to evaluate consistency both across contexts and over time.

The first step in both experiments was to evaluate boldness using the group risk-taking test. In the larval experiment, the proportion of bold larvae exiting the shelter was 14.5% of tested individuals at 8 dpf. This result contrasts with those of previous studies, which reported a proportion of more than 90% of larvae exiting the shelter (Tudorache *et al.*, 2015). This difference may be due to the fish strain used (the TU strain); the previous studies used a hybrid of the AB and TL strains. Indeed, several studies have reported significant behavioural differences between classical wild-type zebrafish strains, such as AB and TL (Mustafa *et al.*, 2019; van den Bos *et al.*, 2017), including at early stages, as shown for AB and TU (Vignet *et al.*, 2013). Another explanation for the difference may be the lighting level of the room. Indeed, the higher the difference in lighting between the sheltered and open areas, the higher the level of required boldness. Such a difference in response to the differing lighting levels in a behavioural test has already been shown for zebrafish larvae (Schnorr *et al.*, 2012). In the juvenile experiment, 48% of the individuals were characterized as bold at 21 dpf, which is slightly higher than that determined for zebrafish adults (26%) by Rey *et al.* (2013). Nonetheless, this proportion is in the range of what is generally observed during group risk-taking tests in other fish species (*e.g.*, Castanheira *et al.*, 2013; Alfonso *et al.*, 2019; Carbonara *et al.*, 2019). Overall, the results from the larval and juvenile experiments show that the proportion of bold fish in the zebrafish population increased from 8 to 21 dpf (from 14.5 to ~35–48%, respectively) and was then stable, at least until 60 dpf. These results suggest that the proportion of fish with bold/shy traits is more variable during early life, before stabilizing around 21 dpf, at the time of mid-metamorphosis. Further experiments with closer time points will be needed to precisely quantify the time of stabilization of the bold/shy proportion of the population.

In both experiments, the group risk-taking test was followed the day after by a novel environment test to evaluate consistency across contexts and to determine a behavioural syndrome. Bold fish in both the larval and juvenile experiments showed higher activity and higher thigmotaxis during the open-field and novel-tank diving tests, respectively. Nonetheless, weaker behavioural consistency was observed across contexts at 21 dpf in the larval experiment, suggesting that the group risk-taking test was less discriminant for this age group (*i.e.*,

higher proportion of “false” bold fish). This could also be attributed to the previous experience and manipulation of the fish at 8 dpf or smaller sample size at this experimental age. Such a behavioural syndrome, higher activity in bold individuals, is indeed well described in the literature, including in fish (Castanheira *et al.*, 2017; Geffroy *et al.*, 2015; Moretz *et al.*, 2007; Réale *et al.*, 2010), whereas the link between boldness and thigmotaxis is less well documented and still debated (Ariyomo *et al.*, 2013; Burns, 2008; Dahlbom *et al.*, 2011; Thörnqvist *et al.*, 2019). In this study, bold fish showed higher thigmotaxis. Given that shy individuals are considered to be more plastic than bold ones (Ruiz-Gomez *et al.*, 2008; Chapman *et al.*, 2010; Coppens *et al.*, 2010; Laubu *et al.*, 2016), it is possible that shy fish may more easily adapt to the open field or a novel tank than bold fish, which could explain the lower thigmotaxis in shy fish. Another explanation could be that shy individuals display freezing behaviour in the centre of the arena after introduction into the novel environment. Overall, this consistency in behavioural responses across contexts defined exiting fish as proactive, whereas the others were defined as reactive individuals, regardless of life stage.

The same two tests were repeated 13 days later in the larval experiment and 40 days later in the juvenile experiment to evaluate consistency over time (*i.e.*, across life stages). Overall, no behavioural consistency was observed between ages, either in the larval experiment or juvenile experiment for bold or shy fish. This contrasts with strong behavioural consistency previously reported in zebrafish adults over a period of 10 months (Rey *et al.*, 2013). This study's results are, however, in accordance with those of other studies, which analysed consistency over time across different ontogenic stages. In nine-spine sticklebacks, starting at 30 dph, feeding behaviour was shown to be consistent within ontogenic stages (juveniles or adults) but not across stages (Herczeg *et al.*, 2013). A similar result was obtained with Eastern mosquitofish (*Gambusia holbrooki*), which displayed very little consistency in personality across ontogenic stages (Polverino *et al.*, 2016). The same inconsistency in individual behaviour has been shown for three-spine sticklebacks *Gasterosteus aculeatus* during the juvenile, sub-adult and adult stages (Bell and Stamps, 2004) and for sea bream *Sparus aurata* between the juvenile and adult stages (Castanheira *et al.*, 2016). Finally, the only other study that follows behavioural consistency from the larval to juvenile stages also concluded that there was no consistency between the two life stages in mangrove killifish (Edenbrow and Croft, 2011): the onset of behavioural consistency occurring near sexual maturity during the juvenile stage. Nonetheless, in this study, the fish were raised in isolation, which can introduce biases, including individual stress and/or divergence in physicochemical rearing conditions. In addition, this study reported the absence of cross-context consistency before this same age. This is consistent with the fact that no evidence for personality was found in 30-day-old juvenile Eastern mosquitofish vs. sub-adults and adults [60 and 120 dph, Polverino *et al.* (2016)]. This work, however, leads to a different conclusion, as consistency was observed across contexts at all studied ages, with a clear behavioural syndrome already visible at the larval stage. This is in accordance with a report showing that 8 dpf zebrafish larvae exhibit a coping style (Tudorache *et al.*, 2015).

Overall, this study suggests that important changes that occur in the organism during transition between life stages may trigger shifts in coping styles. Metamorphosis is a complex process that includes several morphological and physiological changes that are more or less synchronized. Further, and beyond metamorphosis, experience and learning capabilities also mature, and it has been shown that both classical and operant learning become efficient at approximately 3 weeks of age (Valente *et al.*, 2012). The comparison of results between 8 and 21 dpf (larval experiment) or 21 and 60 dpf (juvenile experiment) did not find behavioural consistency between the successive ages. These suggest that during all the larval stages, the continuous biological processes that shape the morphological and physiological changes of fish between the two stages also modify the personality that will become consistent later during zebrafish life. Further studies are needed to refine the time window of the onset of stable personality traits in zebrafish. This could probably be near sexual maturity, as found by Edenbrow and Croft (2011) for mangrove killifish.

In addition, alizarin red S was used to mark the fish and thereby mark them between the two successive ages of each experiment. The marks were visible in the zebrafish bones and otoliths at least 39 days after marking. Alizarin red S marking (at the concentration chosen) did not induce particular mortality or developmental defects in zebrafish, as reported in previous studies with other fish species (Caraguel *et al.*, 2015; Lü *et al.*, 2015; Stanczak *et al.*, 2015). Marking did not induce behavioural disruption in larvae, as shown by the LPMR test, or a differential response after determination of the coping style. These results thus validate alizarin red S for non-invasive marking, suitable for individual or group identification starting at early life stages in fish. The use of alizarin red S provides new perspectives for the further study of the longitudinal evolution of various traits, including behaviour, across life stages.

In conclusion, boldness in zebrafish, as measured using the group risk-taking test, is lower at 8 dpf than at 21 and 60 dpf. Fish categorized as bold or shy using the group risk-taking test display differential behavioural responses in the open-field or novel tank diving tests at 8, 21 and 60 dpf. Bold fish are more active and show higher thigmotaxis than shy fish, regardless of age, starting at least at 8 dpf. There was no behavioural consistency, however, between different ages, suggesting behavioural plasticity across life stages. Coping styles are thus present at the larval stage but are not fixed and may evolve through individual experience and physiological changes (Castanheira *et al.*, 2016; Ferrari *et al.*, 2016).

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AUTHOR CONTRIBUTION

S.A. conceptualized the experiment and methodology, performed the experiments, analysed the data, wrote the original draft and reviewed

and edited the manuscript. M.P. performed the experiments and analysed the data. M-L.B. and X.C. acquired the funding, conceptualized the experiment and methodology, supervised the experiments and data analyses, reviewed and edited the manuscript.

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