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Substrate colour guides turquoise killifish's (*Nothobranchius furzeri*) choice of preferred spawning habitat

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Abstract

Turquoise killifish (*Nothobranchius furzeri*) is a promising new model species used in biomedical and ecological laboratory experiments, and should be kept under optimal conditions to ensure fish welfare and the quality of science. While the popularity of this model species is rapidly increasing, we need to improve our understanding of how the species interacts with its environment to optimize its husbandry. Specifically, turquoise killifish are substrate spawners that bury their eggs in the sediment, which can be accommodated under captive conditions, but it is not yet known whether or not turquoise killifish have a preference for a specific sediment colour. Here, we performed a laboratory experiment in which fish could choose between white, orange and black sand for spawning, colours which are relevant in both laboratory and field conditions. We assessed their preference in the context of single breeding pairs, as well as in a social group setting. Additionally, we also assessed the preference of individuals for a white *versus* black background in a nonmating context. Single breeding pairs deposited over 3.5 times more eggs in black compared to orange or white sand. Similarly, fish in social groups deposited over 3.5 times more eggs in black compared to orange sand, which in turn was over two times higher than that in white sand. Fish showed a slight preference for the black compared to the white zone in a nonmating context, but this did not correlate with substrate choice during the spawning tests. The results suggest that turquoise killifish select their preferred spawning location based on the colour of the substrate. These findings contribute to our understanding of the species' biology and can help to guide good welfare and scientific practice.

KEYWORDS
choice, husbandry, model organism, preference, reproduction, welfare

1 | INTRODUCTION

Globally, millions of fish are used for scientific laboratory experiments (Lee *et al*., 2022). Typical study species include, among others, zebrafish (*Danio rerio*), medaka (*Oryzias latipes*) and guppy (*Poecilia reticulata*). Despite their proven value in a wide range of biomedical and ecological disciplines, these species mature relatively slowly and live up to several years, which constrains research that aims to monitor fish across life stages and/or multiple generations (Thoré *et al*., 2023; Thoré, Brendonck, & Pinceel, 2021). To facilitate such agendas, turquoise killifish (*Nothobranchius furzeri*) was recently established as an complementary model that combines the perks of classic fish models with the fast life cycle of short-lived nonvertebrates (Cellerino *et al*., 2016; Russell *et al*., 2017). Turquoise killifish is an annual fish from south-east Africa that inhabits often turbid ephemeral freshwater ponds which only hold water during the rainy season and desiccate entirely each year. Killifish populations survive this periodic drying by virtue of drought-resistant eggs that are buried in the sediment.

These eggs only hatch during a next inundation, after which the fish complete their life cycle in just a few months before the pond dries again (Pinceel *et al*., 2021). As a result, turquoise killifish can mature as fast as within 3 weeks after hatching and have a typical lifespan of about 5–6 months (Blažek *et al*., 2013; Cellerino *et al*., 2016). Because of these unique characteristics, the species was initially developed as a model for vertebrate aging (Harel *et al*., 2016; Reichwald *et al*., 2015; Valenzano *et al*., 2015) but is now also gaining popularity in other disciplines, including, among others, genomics and genetics (Cellerino *et al*., 2016; Valenzano *et al*., 2015), ecology (Blažek *et al*., 2016; Grégoir *et al*., 2018; Reichard *et al*., 2009), behavioural biology (Thoré *et al*., 2018; Thoré, Brendonck, & Pinceel, 2020) and (eco)toxicology (Philippe *et al*., 2018, 2019; Thoré, Philippe, *et al*., 2020).

Researchers have a duty of care for laboratory animals, including turquoise killifish, to ensure they are kept under the best possible conditions (Lee *et al*., 2022). Helpfully, several recommendations on how to care for turquoise killifish were recently made available (*e.g*. Dodzian *et al*., 2018; Philippe *et al*., 2018; Polačik *et al*., 2016). Most of these recommendations are aimed at optimal performance of the fish, and researchers often customize these methods for convenience or to fit specific needs. For example, several methods exist for breeding turquoise killifish under laboratory conditions, which are inspired by the ecology of the species to varying degrees depending on the needs of the laboratory. In the wild, *Nothobranchius* killifish typically mate with multiple partners (*i.e*., polygynandry), with males competing among each other for access to females (Cellerino *et al*., 2016; Haas, 1976). When a male encounters a receptive female, they swim to the bottom of the pond, after which the male uses his dorsal and anal fins to clasp the female, who positions her anal fin close to the surface. The couple then performs a rapid jerking movement to deposit a single externally fertilized egg in the substrate. This spawning action can be repeated so that females usually deposit 20–50 eggs per day (Cellerino *et al*., 2016; Thoré, Brendonck, & Pinceel, 2020). In captivity, various substrates can be used for oviposition, including sand, glass beads, peat moss and coconut fibre (Cellerino *et al*., 2016; Philippe *et al*., 2018; Polačik *et al*., 2016). Fish can also become accustomed to spawn on a false mesh bottom, as commonly used for zebrafish breeding (Cellerino *et al*., 2016; Reichard *et al*., 2022). Furthermore, turquoise killifish can be bred in social groups that are allowed to spawn continuously or, alternatively, egg yield can be maximized by isolating fish and only allowing them to spawn in single breeding pairs for 2 h twice per week (Polačik *et al*., 2016; Reichard *et al*., 2022).

Even though these various practices could be perfectly acceptable in terms of reproductive performance and/or welfare, our understanding of how turquoise killifish interacts with its environment is still in its infancy (Thoré, Brendonck, & Pinceel, 2020). Here, we set out to answer a question that has not yet received any attention so far: does substrate colour determine turquoise killifish's choice of preferred spawning location? For this, we used a laboratory experiment in which adults can choose between white, orange and black sand for spawning, colours which are relevant in both laboratory and field conditions. In the wild, the species typically spawns in specific parts of the pond which are believed to be selected based on the suitability of the substrate for egg survival (Cellerino *et al*., 2016). Specifically, egg survival critically depends on the presence of vertisol soils (Reichard *et al*., 2009; Watters, 2009), which appear as a dark substrate (Cellerino *et al*., 2016). Based on this, we hypothesized that fish will prefer to deposit their eggs (*i.e*., larger clutch size) in the black sand. We assessed this in the context of single breeding pairs as well as in a social group setting, expecting that this will not influence the substrate preference. To assess whether or not fish are (also) habitat-selective in other settings, we furthermore tested the preference of individuals for a white *versus* black background in a nonmating context. While this study does not intend to make recommendations on how (or how not) to breed turquoise killifish, this is the first study to assess if turquoise killifish have a preference for a specific substrate colour for spawning, which will be important to tailor husbandry guidelines and experimental protocols to the biology of the species.

2 | MATERIALS AND METHODS

2.1 | Fish maintenance

The experiment started on 1 May 2022 and ran until 28 July 2022.

Nothobranchius furzeri eggs were recruited from a heterozygous laboratory culture that originates from a natural population in Mozambique (MZCS-222) and has been kept under optimal laboratory conditions for over five generations at the breeding facility of KU Leuven (Belgium). Forty-six fish were hatched by submerging ready-to-hatch eggs (after Polačik *et al*., 2016 and Philippe *et al*., 2018) in reconstituted water (Instant Ocean (Blacksburg, VA, USA) Sea Salt mix added to type III RO water until a conductivity of 600 μS/cm, pH 7.8) with 1 g/l humic acid (53,680; Merck, Darmstadt, Germany). Eight days post hatching (dph), each hatchling was transferred to a 10 l glass tank (50 cm long × 20 cm wide × 17.5 cm high) with aerated reconstituted water, in groups of six or seven fish per tank. At 43 dph, all fish were assumed to have reached sexual maturity ($n = 22$ males, $n = 24$ females) and were individually transferred to a 2 l transparent, glass jar (one fish per jar) until 67 dph for individual monitoring while still allowing visual interaction among fish. Starting from 67 dph and until the end of the experiment, fish were transferred back to 10 l glass tanks in social groups of two males and two females per tank.

Throughout the experiment, tanks and jars were cleaned once and twice per week, respectively. Each time, the water was completely renewed to maintain good water quality. To avoid unnecessary handling of fish, cleaning coincided with spawning tests and/or collection of eggs (see sections 2.2 and 2.3). Water quality was monitored at least twice per week (ammonium <0.2 mg l^{-1}, nitrate <25 mg l^{-l}, nitrite <0.2 mg l^{-1}). Fish tanks/jars were placed in heated water tubs at a constant temperature of 25°C and under a 14 h light:10 h dark photo-regime. These tubs were made from grey polyvinylchloride, so that fish in the jars/tanks were kept on a grey background. Full-spectrum, white LED light was provided at a constant intensity of 2000 lx (jar level) and was switched on and off at 8.00 am and 10.00 pm, respectively.

Fish larvae were fed twice daily (at 8.30 am and 6.30 pm) until satiation with live *Artemia franciscana* nauplii (Ocean Nutrition, Essen, Belgium) from 1 until 21 dph. Starting 14 dph, fish were weaned on frozen *Chironomus* larvae (Ocean Nutrition) that were supplemented to the *Artemia* diet once per day. Starting at 21 dph, fish were fed twice daily until satiation with frozen *Chironomus* larvae and once per day (at 8.30 am) as of 43 dph until the end of the experiment. Each time after feeding, excess food was removed from the tanks/jars using a glass pipette to maintain good water quality.

2.2 | Spawning tests with single male–female pairs

Between 43 and 67 dph, fish were housed individually (see section 2.1) and paired twice per week (one male + one female) to spawn for a total of five spawning sessions per individual (Figure 1). This setup allows fish to be monitored individually without the need for marking, facilitates standardization of environmental and social conditions, and maximizes egg yield (Reichard *et al*., 2022). Per spawning session, each female was transferred to a separate 10 l glass spawning tank (50 cm long × 20 cm wide × 17.5 cm high) provided with three transparent plastic trays (16 cm long × 11 cm wide × 6.5 cm high) positioned randomly in the tank. Each tray was provided with spawning substrate of a different colour (white, orange or black; 130 g per tray, ±1 cm depth): Sansibar White, Orange, or Dark (JBL, Neuhofen, Germany). Each of these consists of fine granite sand that was thoroughly rinsed with water and sieved to a grain size of 0.2–0.5 mm before use. Consistent with standard laboratory practice, we selected a sand grain size that is smaller than killifish eggs to facilitate egg collection by sieving. Additionally, we chose a size that is large enough to avoid turbidity and maintain good water quality. Each female was given 5 min to acclimate after being introduced to the spawning tank before a random male was added to the tank. The couple was then allowed to spawn for 2 h. Afterwards, each fish was transferred back to its housing jar and the sand was sieved (mesh size of 0.5 mm) to count the number of deposited eggs per substrate type. In addition, the water was sieved to account for any eggs that may have been deposited outside of the spawning trays.

Spawning tests always took place between 10.00 am and 3.00 pm to limit confounds related to daily behavioural variation (Thoré, Philippe, *et al*., 2021). For feasibility, fish were divided into two cohorts so that not all spawning tests would have to be conducted on the same day (23 fish per cohort). Fish from cohort 1 were allowed to spawn at 46, 51, 54, 58 and 61 dph, and fish from cohort 2 were allowed to spawn at 50, 53, 57, 60 and 64 dph. Because there were slightly more females than males, a small number of females was sometimes paired with a male from the other cohort. These limited number of reused males were chosen at random each time. Even though male *N. furzeri* are constantly ready to coerce females into spawning (Cellerino *et al*., 2016), males always had a minimum of 24 h rest in between spawning tests.

One female was lost due to early mortality at 60 dph and could only complete three out of five spawning sessions, amounting to a total of 118 spawning sessions of single male–female pairs.

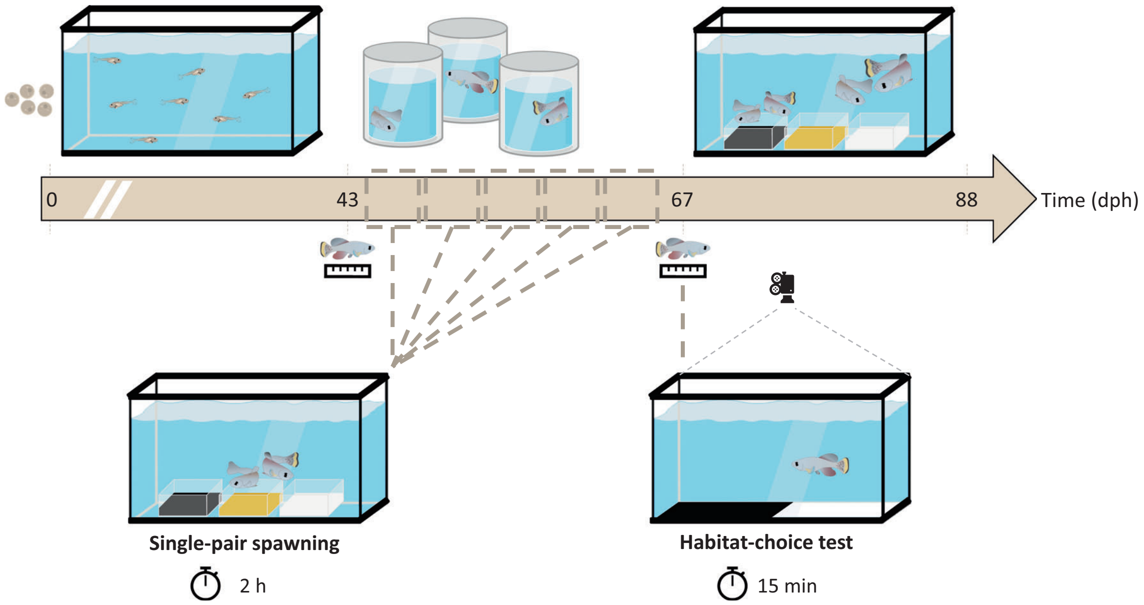

FIGURE 1 The experimental setup. Adult fish were housed individually between 43 and 67 dph, during which time they were paired to spawn for 2 h twice per week (one male + one female, randomly paired), for a total of five spawning sessions per individual. During spawning, fish were provided with three different colours of spawning substrate: white, orange and black sand. At 67 dph, each fish was individually subjected to a 15-min habitat-choice test to assess its preference for a white *vs*. black background in a nonmating context. Afterwards, and until the end of the experiment, fish were housed in social groups of two males and two females per tank, and allowed to continuously spawn for 3 weeks (collecting all eggs every 7 days). The total body length of each fish was assessed at 43 and 67 dph. dph, days post hatching

2.3 | Spawning in social groups

From 67 to 88 dph, 36 randomly selected fish were housed in social groups (see section 2.1) of two males and two females per tank. Individuals for each group were chosen at random and afterwards not reshuffled among tanks ($n = 9$ tanks of four fish each). During this period, each tank was continuously provided with three trays with spawning substrate (one white, one orange and one black sand), *i.e.* the same setup as during the spawning tests with single male–female pairs (see section 2.2 and Figure 1). After 7 days and for a total of three times (at 74, 81 and 88 dph, coinciding with cleaning of the tanks), all sand (and water) was sieved to count the number of deposited eggs per substrate type.

2.4 | Habitat-choice test

At 67 dph, and before fish were transferred to tanks in social groups (see section 2.1, section 2.3 and Figure 1), each individual fish was transferred to a habitat-choice test arena to assess its preference for a white *versus* black background in a nonmating context (Figure 1). The test arena consisted of a 10 l glass tank (50 cm long × 20 cm wide × 17.5 cm high) of which the background was divided into a white half and a black half (randomly oriented). The sides of the arena were covered with a white screen (opaque) to avoid disturbance.

At the start of the habitat-choice test, each fish was allowed to acclimate for 5 min. After that, fish movement was recorded for 15 min (900 s) using a Logitech C920 HD Pro (Lausanne, Switzerland) webcam that was centred above the arena (top-view). All tests were conducted between 10.30 am and 3.00 pm to minimize confounds related to daily behavioural variation (Thoré, Philippe, *et al*., 2021). Afterwards, all recordings were manually analysed to determine the amount of time each fish spent in the white *versus* the black zone.

2.5 | Total body length measurement

The total body length of each fish was measured at 43 dph (*i.e.*, right before individual housing) and 67 dph (*i.e.*, right after the habitat-choice test and before social housing; see also Figure 1). To do this, each fish was briefly placed in a Petri dish with a small amount of water to avoid vertical movement. The fish were centred in the frame (dorsal view) of a Samsung Galaxy S8+ (Seoul, South Korea) dual-pixel 12.0 MP AF F/1.7 camera to take a size-calibrated photograph which was afterwards analysed using ImageJ v. 1.50i (Schneider *et al*., 2012) to assess the total length of each fish (*i.e.*, tip of snout to tip of tail).

2.6 | Animal welfare note

All procedures adhered to the legal requirements for animal research in Belgium and were approved by the ethical committee of KU Leuven (file number: P113-2022). The condition and health of each individual fish was monitored at least twice per day. Fish were kept at optimal water quality and following standard *N. furzeri* husbandry procedures. Any disturbance and handling that was not strictly necessary for the experiment was kept at a minimum to prevent and limit stress.

2.7 | Statistical analyses

All statistical analyses were performed in R version 4.0.5 (R Core Team, 2021) at a significance level of $\alpha = 0.05$. Model assumptions, including distributional fit and homogeneity of variances, were verified graphically. Gaussian error distributions were additionally verified using a Shapiro–Wilk test.

Clutch size (*i.e.*, the number of deposited eggs per substrate type) during the spawning tests with single male–female pairs (43–67 dph) was analysed using a linear mixed-effects model (lme4 package; Bates *et al*., 2017) with Poisson error distribution. Substrate type (white, orange or black sand) and total body length at 43 dph (mean-centred and scaled) were added as fixed factors to the model, including their interaction. The identity of females and session number (referring to the repeated measures: 1–5) were added as random effects. In addition, an observation-level random effect was added to account for overdispersion. Cohort (cohort 1, cohort 2) was initially added as an additional random effect, but was dropped from the final model (based on the Akaike information criterion (AIC) and a likelihood-ratio test comparing the model with and without the cohort random effect structure). Only actual clutches were considered, *i.e.* females had to deposit at least one egg regardless of whether it was deposited in the white, orange or black substrate. When females did not produce a minimum of one egg, the spawning was not considered successful and was treated as a missing value (14 out of 118 spawning sessions).

Clutch size during the spawning in social groups (67–88 dph) was analysed using a similar linear mixed-effect model with Poisson error distribution. Substrate type was added as a fixed factor, and group identity (tank number) and session number (repeated measures: 1–3) were added as random effects. An observation-level random effect was added to account for overdispersion. Type 3 Wald chi-square tests were used to test the significance of the fixed effects and the interaction terms. *Post hoc* differences were assessed by means of Tukey-corrected pairwise comparisons (lsmeans package; Lenth & Love, 2017).

Habitat choice during the habitat-choice test was measured as the time spent in the white zone minus time spent in the black zone (in seconds). A positive score therefore indicates that a fish spent more time in the white compared to the black zone, and *vice versa*. A score of 0 means that the fish spent an equal amount of time in the white and the black zones. To assess how habitat choice may be affected by sex and body length of the fish, a linear model with Gaussian error distribution was constructed, with sex (male, female) and total body length at 67 dph (mean-centred and scaled) as fixed factors, including their interaction. To assess whether or not fish on average prefer one of both habitats (as opposed to having no preference at all), a one-sample *t*-test with $\mu = 0$ was run. To assess if behaviour in the habitat-choice tests correlates to substrate choice in the single-pair spawning tests, we first calculated the proportion of eggs deposited in the black sand compared to the total clutch size per spawning session for each fish (males and females). This value was then averaged for each fish over the five spawning sessions and correlated to the total amount of time that each fish spent in the black zone during the habitat-choice test (Spearman's rank correlation test).

3 | RESULTS

During the single-pair spawning tests, a total of 2230 eggs was produced. The large majority of these eggs (2190 eggs, 98%) was deposited in the spawning trays, while a small minority (40 eggs, 2%) was deposited outside of the spawning trays. When considering the eggs that were deposited in the spawning trays, fish had a clear preference for substrate colour ($\chi^2 = 82.688$, $P < 0.001$; Figure 2a). In total, 1415 eggs were deposited in the black sand whereas only 401 and 374 eggs were deposited in the orange and white sand, respectively. Average clutch size (mean ± s.d. 12 ± 4 eggs) in black sand was over 3.5 times higher (Tukey $P < 0.001$; Figure 2a) compared to that in orange (mean ± s.d. 3 ± 2 eggs) and that in white sand (mean ± s.d. 3 ± 1 eggs). The number of produced eggs ($\chi^2 = 0.165$, $P = 0.684$) and clutch size per substrate ($\chi^2 = 0.796$, $P = 0.671$) were independent of the size of the females.

During the 3-week period of spawning in social groups, a total of 4671 eggs was produced. No eggs were detected outside of the spawning trays. Fish had a clear preference for substrate colour ($\chi^2 = 106.440$, $P < 0.001$; Figure 2b). In total, 3325 eggs were deposited in the black sand whereas only 922 eggs were deposited in the orange sand and only 424 eggs in the white sand. Average weekly clutch size in black sand (mean ± s.d. 123 ± 58 eggs) was over 3.5 times higher (Tukey $P < 0.001$; Figure 2b) compared to that in orange sand (mean ± s.d. 34 ± 32 eggs), which in turn was over two times higher (Tukey $P < 0.001$; Figure 2b) compared to that in white sand (mean ± s.d. 16 ± 18 eggs).

Fish showed a slight habitat preference during the habitat-choice test ($t_{44} = -2.170$, $P = 0.035$; Figure 3). Specifically, fish preferred the black over the white zone, spending on average 517 s (±208 s s.d.) in the black zone (*i.e.*, ~57% of time). Habitat preference did not depend on the sex ($F_{1,41} = 0.954$, $P = 0.334$) or body length of the fish ($F_{1,41} = 0.193$, $P = 0.663$), nor was there any sex-dependent relationship between fish body length and habitat preference ($F_{1,41} = 0.958$, $P = 0.333$). Behaviour in the habitat-choice test did not correlate with substrate choice during the single-pair spawning tests for females ($r_s = 0.321$, $P = 0.135$) or males ($r_s = 0.099$, $P = 0.661$).

4 | DISCUSSION

In this laboratory study, we presented fish with three different colours of sand which they could choose for spawning and found that fish deposit over 3.5 times more eggs in black compared to orange or white sand. This suggests that substrate colour guides turquoise killifish's choice of preferred spawning location, at least in captive conditions. This finding may be important when developing improved husbandry and experimental protocols tailored to the biology of the species.

The finding that fish prefer to deposit their eggs in black (compared to orange or white) substrate is consistent with our

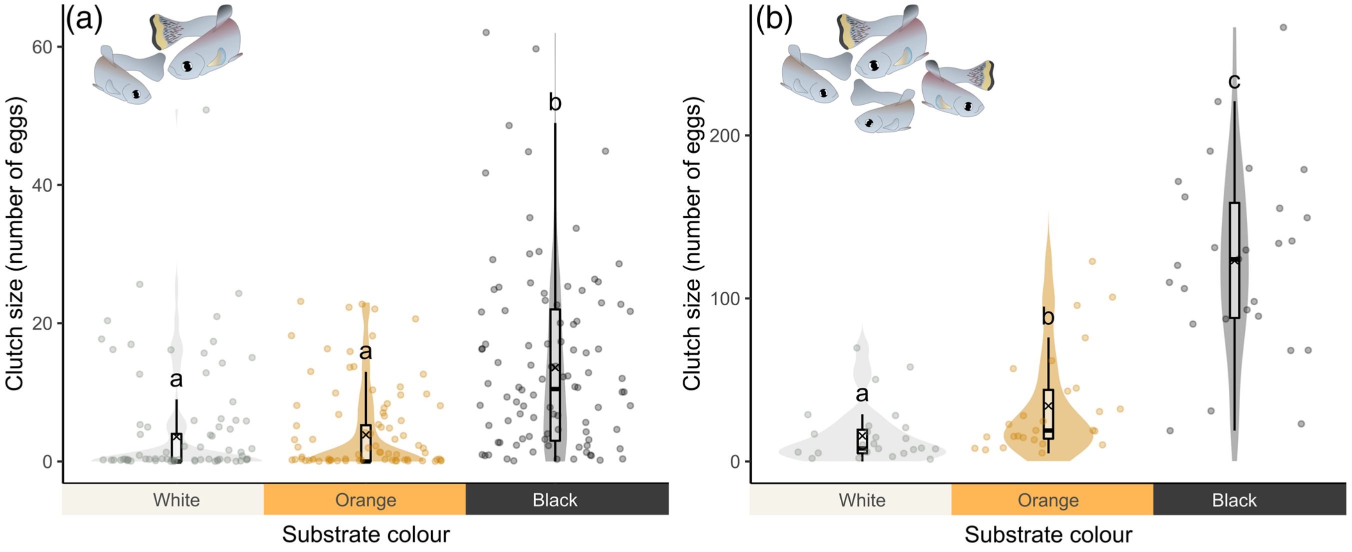

FIGURE 2 Clutch size per substrate type. (a) Number of deposited eggs per substrate type (white, orange or black sand) during 2-h spawning trials with single male–female pairs [46–64 days post hatching (dph)]. (b) Weekly number of deposited eggs per substrate type during social housing of two males and two females (67–88 dph). Boxplots show the 25th, 50th (median) and 75th quartiles. The mean is indicated by 'x'. Different letters indicate significant differences

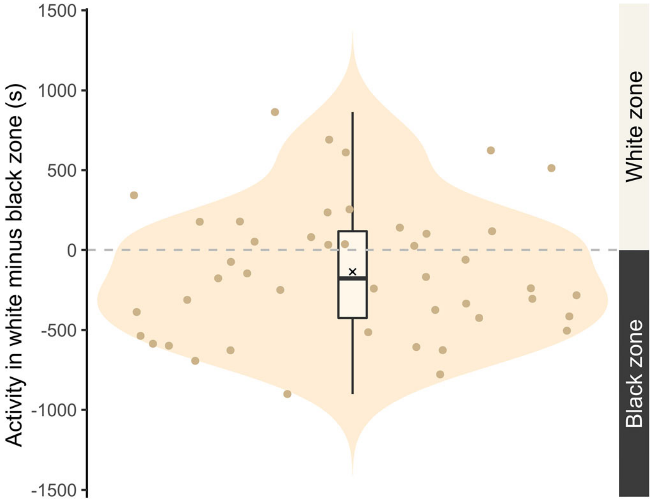

FIGURE 3 Habitat choice, measured as the time spent in the white zone minus time spent in the black zone. A positive score indicates that a fish spent more time in the white compared to the black zone, and *vice versa*. The boxplot shows the 25th, 50th (median) and 75th quartiles. The mean is indicated by 'x'

expectations and may be explained by their natural history. Specifically, the natural distribution of turquoise killifish is strictly related to the presence of black vertisol soils, sometimes referred to as 'black cotton soil', on alluvial deposits (Matias & Adrias, 2010; Reichard *et al*., 2009; Wildekamp, 2004). This is because vertisols have a high water-retaining capacity, which is necessary to ensure the survival of eggs during pond desiccation (Cellerino *et al*., 2016). In contrast, ponds developed on exclusively laterite soils, which are usually rust-coloured and retain less water during dry periods, do not sustain killifish populations (Matias & Adrias, 2010; Reichard *et al*., 2009). Accordingly, only specific parts of the ponds are used for spawning, which are likely selected based on the suitability of the substrate for egg survival (Cellerino *et al*., 2016). The findings of the current study confirm that, at least in captive conditions, turquoise killifish are substrate-selective for spawning and show that colour is an important cue for this.

While it is conceivable that the observed preference for dark substrate is because killifish prefer to deposit their eggs on (black) vertisol soils, it is also possible that it is anxiety-related and that killifish adopt this strategy to, for example, decrease their susceptibility to predation. Indeed, killifish spawning is a conspicuous activity during which fish are vulnerable to visual predators such as birds (Cellerino *et al*., 2016; Grégoir *et al*., 2018). To avoid being detected by predators, many fish species have a natural tendency for scototaxis, *i.e*. a preference for dark *versus* bright areas (De Abreu *et al*., 2020; Kysil *et al*., 2017). For example, zebrafish, guppy, goldfish (*Carassius auratus*), tilapia (*Oreochromis niloticus*) and others have all been shown to markedly prefer dark over light backgrounds (reviewed by Maximino *et al*., 2010). In support of this hypothesis, turquoise killifish not only preferred to spawn on dark substrate but also spent more time on the black *versus* white background in a nonmating context. Nevertheless, this preference was not very pronounced (*i.e*., fish spent on average only ∼57% of time in the black *vs*. white zone), and scototaxis on an individual level did not correlate with their preference for dark substrate during spawning. Ideally, more in-depth studies are needed to identify the exact motivational drivers behind the observed colour preference. It is of note that turquoise killifish are often found in murky ponds (Reichard *et al*., 2009), where the colour of the substrate may conceivably be less important as it is in clearer ponds. Comparing populations from murky and clear ponds, and/or manipulation of water turbidity, could therefore provide valuable further insight into the substrate colour preference of turquoise killifish.

Interestingly, fish kept in social groups deposited an intermediate amount of eggs on orange substrate, *i.e*. 3.5 times less compared to black substrate but two times more compared to white substrate. In contrast, fish that spawned in single breeding pairs deposited as few eggs on orange as on white substrate. While follow-up research would be needed to uncover a biological explanation for this observation, it is not unlikely that this observed difference between social groups and single pairs is, in part, an artefact of the experimental setup. Specifically, male turquoise killifish are not strictly territorial but they do establish a hierarchy (Polačik & Reichard, 2009) which can result in dominant males controlling areas that are best suited for spawning (Cellerino *et al*., 2016). This means that, in social groups, the dominant male may have controlled the spawning tray with black sand while the subordinate male had to resort to the most suitable of two lesser options (with orange sand being closer to the natural situation and/or being less bright than white sand).

It is important to note that the current study has a number of limitations that provide valuable opportunities for follow-up research. First, preference tests, such as the one used here, can be informative because they allow fish to choose what they want but it is important to keep in mind that the choices are restricted to the provided options (Lee *et al*., 2022; Marques Maia & Luiz Volpato, 2016). For example, different substrates can be used for oviposition of turquoise killifish under captive conditions (Cellerino *et al*., 2016), of which particularly sand is often recommended. This is because (1) eggs are easy to sieve from sand whereas isolating them from moss peat or coconut fibre is much less convenient (Philippe *et al*., 2018) and (2) using sand as substrate more closely approximates the natural conditions compared to, for instance, glass beads or spawning trays with a false mesh bottom (Astre *et al*., 2022). Still, analysis of the habitat use of turquoise killifish in the wild showed that the species was associated with pools containing soft muddy substrate (as opposed to sandy substrate) (Reichard *et al*., 2009). Consequently, fish may no longer prefer black sand for spawning when also other soil types (such as clay-rich soil) are presented. Furthermore, fish preference for background colour may change over time. For example, whereas adult zebrafish typically avoid bright backgrounds (De Abreu *et al*., 2020; Lau *et al*., 2011), larval zebrafish are known to be dark-aversive (Bai *et al*., 2016). The experimental design of this study does not account for potential age-related differences in colour preference so that any comparison between the behaviour of single breeding pairs (46–64 dph) *versus* fish in social groups (67–88 dph) is inherently confounded. Another important difference between the single pair and the social group setup is that single pairs were only allowed to spawn for 2 h each time, whereas fish in social groups could spawn continuously. Turquoise killifish tend to cannibalize their eggs (Polačik *et al*., 2016; Reichard *et al*., 2022) and while this behaviour was not observed during the 2-h spawning sessions, the possibility that it occurred during social group housing cannot be excluded. Still, the fact that fish preferred dark spawning substrate in both setups, despite the methodological differences between the scenarios, adds confidence to the credibility of this finding. Lastly, choice behaviours can be hard-wired as well as experience-dependent (Lau *et al*., 2011), which means that the rearing conditions of the current study may have affected the preference for dark spawning substrate. For example, fish were reared on a grey polyvinylchloride background, which could have contributed to their colour preference. Although only 2% of eggs were deposited outside of the spawning trays (*i.e*., on the grey background) during the single-pair spawning sessions (and even though no eggs were detected outside of the spawning trays during housing in social groups), follow-up research to assess how past experience influences substrate preference would be valuable.

To date, little work has been done on the importance of background and/or substrate colour to help define the most suitable housing conditions in terms of the welfare of laboratory fish and the quality of science (Lee *et al*., 2022). At the same time, model species are often decontextualized from their natural history (Alfred & Baldwin, 2015) so that their husbandry is often not optimally tailored to their ecological needs (Lee *et al*., 2022; Tsang *et al*., 2017). For example, zebrafish is among the most successful experimental fish models for which many husbandry recommendations are available (*e.g*., Aleström *et al*., 2020) yet there are still a lot of unknowns when it comes to how zebrafish interact with the biotic and abiotic features of their natural environment (Lee *et al*., 2022; Tsang *et al*., 2017). This is also the case for turquoise killifish, for which a wide range of husbandry procedures exist (Reichard *et al*., 2022) that may not yet consider all relevant features of the species' natural behaviour. The finding that turquoise killifish in laboratory conditions are habitat-selective and that substrate colour guides their choice of preferred spawning location contributes to our understanding of the species' biology and can help to guide good welfare and scientific practice.

AUTHOR CONTRIBUTIONS

E.S.J.T.: Conceptualization, investigation, methodology, formal analysis, visualization, writing – original draft, writing – review and editing. W.M.: Resources, writing – review and editing.

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CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in figshare at https://doi.org/10.6084/m9.figshare.22595989.v1.

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