

Substrate-treated water: a method to enhance fish activity in laboratory experiments

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ABSTRACT: Fast acclimatisation of experimental animals to laboratory test conditions is crucial for effective research. During highly standardized experiments, confounding parameters must be minimized which might lead to animals behaving unnaturally. The objective of the present study was to develop a simple method to enhance activity of fishes under laboratory test conditions. Many fishes intensively interact with substrate in their natural environment; however, providing substrate during experiments might impair observation or may otherwise confound the results. Therefore, the behaviour of the cichlid *Pelvicachromis taeniatus* was examined in water that was exposed to substrate only prior to the experiment. As a control, fish behaviour in tap water was investigated. Fish activity (i.e. covered distance) was evaluated during a period of 2 h. In substrate-treated water fish were significantly more active than in tap water. Furthermore, males in the substrate-treated water increased their activity over time, whereas males in tap water did not. In contrast, activity of females did not change significantly over time. No significant relationship between fish activity and body size or condition was found. Furthermore, standard chemical water parameters were not altered by the substrate treatment. Thus, substrate is suggested as a means to improve the olfactory environment, promoting fish welfare and activity. In conclusion, this method is suggested as a standard with wide applicability across benthic fish species to improve acclimatisation of fishes to experimental conditions in the laboratory.

KEY WORDS: *Pelvicachromis taeniatus* · Odour · Sand · Cichlid · Activity patterns

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INTRODUCTION

Chemical cues have been shown to play an important role in the life of fishes. Olfaction is, e.g., involved in homing (Atema et al. 2002, Gerlach et al. 2007, Dixon et al. 2008), food finding (Bardach et al. 1967, Kasumyan 2002, Barata et al. 2009), species recognition (Ojanguren & Brana 1999, Wong et al. 2005, Guevara-Fiore et al. 2010), kin recognition (Thünken et al. 2009, Mehlis et al. 2010) and predator recognition (Brown et al. 2000, Mikheev et al. 2006, Fincel et al. 2010). In cichlids, odours play a role in species recognition (Plenderleith et al. 2005) and in the recognition of parents and offspring (Nelissen 1991, Wisenden & Dye 2009), mates (Reebs 1994), kin (Thünken et al. 2009, 2011) and predators (Foam et al. 2005), indicating that they have a well-developed olfactory sensory system. Similar to many benthic fishes, cichlids frequently interact with the substrate in their aquatic habitat (Barlow 2000). By their digging behaviour, they influence the

distribution of sand across patches in rivers and lakes (Statzner et al. 2003). Also, they often transfer sand outside the nest during courtship and breeding; this behaviour might serve as a signal in conspecific interaction or as an anti-predator defence (Ochi & Yanagisawa 1999). Extreme forms of such non-bodily signals are the sand craters built by several species of East African cichlids like *Cyathopharynx furcifer* (Schaedelin & Taborsky 2010).

Therefore, cichlid welfare is positively influenced by access to substrate (Galhardo et al. 2008), and cichlids prefer sandy over other environments (Galhardo et al. 2009).

However, under experimental conditions, the presence of substrate might be disadvantageous. In experiments, fish then often show a lack of contrast when observed from above, which makes the evaluation of their behaviour difficult (e.g. by analyzing video-recordings). Additionally, the digging behaviour of cichlids (Ochi & Yanagisawa 1999, Statzner et al. 2003) might impair ob-

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ervation. Hence, a natural setting during experiments has to be carefully traded off against difficulties of observation. As a result, substrate is often removed from experimental setups at the cost that fish might behave unnaturally.

The aim of the present study was to develop a non-invasive method of increasing fish activity in a novel environment. Instead of providing sand as a substrate, water previously exposed to sand ('sand-treated water') was used in the experiment, and fish behaviour in sand-treated water was compared with that in tap water. Experiments were conducted with males and females of the cichlid *Pelvicachromis taeniatus* (Boulenger, 1901). It has a well-developed sense of smell and is able to discriminate between its own and familiar kin chemical cues (Thünken et al. 2009). Also, *P. taeniatus* males interact intensively with available sand and construct sand piles in front of their breeding caves (Baldauf 2006).

Fishes are known to reduce their swimming behaviour (i.e. speed or distance) in response to unfavourable or novel conditions (Godin 1997, Brown et al. 2001, Huntingford et al. 2006, Wilson et al. 2010). Therefore, the covered distance was measured as a proxy for activity and acclimatisation. Among cichlids, males are the predominant sex in substrate interaction, especially in the context of nest building (Baldauf 2006, Mendonça et al. 2010, Schaedelin & Taborsky 2010). Thus, one would predict that male *P. taeniatus* show a stronger response than females to sand-treated water.

MATERIALS AND METHODS

Experimental animals. Fish were descendants of wild-caught *Pelvicachromis taeniatus* from the Moliwe River near Limbe, Cameroon (04° 04' N, 09° 16' E). Prior to the experiment, the experimental fish were kept in mixed sex groups with up to 30 individuals in five 60 × 45 × 30 cm (length × width × height) tanks, equipped with java moss *Taxiphyllum barbieri* and sand as substrate. The tanks were positioned on a white polystyrene layer and separated from each other by opaque plastic sheets. Illumination was provided in a 12 h light:12 h dark cycle (from 09:00 to 21:00 h) by fluorescent tubes (Lumilux Plus Eco L36W/21-840, Osram) 8 cm above the tank. Water temperature was kept constant at 25 ± 1°C. Fish were fed daily ad libitum with a mix of defrosted food that contained mosquito larvae of the genera *Chironomus*, *Culex* and *Chaoborus*, as well as *Artemia* sp. in a ratio of 2:1:0.25:1.

Water preparation. Preparation of the water used for the treatment groups was conducted in four 50 × 30 × 30 cm (length × width × height) tanks, called 'prepara-

tion tanks' from now on. One day prior to the experiment, tanks were filled with 40.5 l of tap water. To create sand-treated water, three 105 ml cups filled with substrate were added to half of the preparation tanks. The substrate used in the experiment was commercially obtained Rhine sand that consists of >90% quartz sand. The substrate consisted of approximately 5% fine sand (particles <0.18 mm), 75% of the volume was medium sand (0.18 to 0.50 mm) and 10% was coarse sand (0.50 to 1.50 mm). The last 10% of the total volume consisted of fine gravel (1.50 to 3.50 mm). The 315 ml volume used for each sand-water preparation tank weighed on average (±SD) 413.32 ± 19.06 g (n = 10). Sand in the holding tanks was of the same origin as that used in the experiments. The tanks used for preparing sand-treated water and tap water were cleaned after conducting all experiments each day. Subsequently, treatments were reassigned to the tanks randomly; a sand-water tank was always placed next to a tap-water preparation tank.

Experimental design. The experiments were conducted in four 30 × 20 × 20 cm tanks placed in a square fashion. Each tank was covered with a 30 × 20 cm transparent, 1 cm thick Plexiglas pane.

Furthermore, all tanks were surrounded by a white polystyrene layer to prevent visual contact between fish. This setup was placed on a 74 cm high table. Tanks were illuminated by a fluorescent tube (Lumilux Plus Eco L36W/21-840, Osram) placed 70 cm above the tanks. For video recording, a web camera (QuickCam Pro 9000, Logitech) was placed 57 cm above the experimental setup.

Experimental procedure. Each day, the tanks were filled with 9 l of the previously prepared water in such a way that diagonally positioned tanks became the same treatment. Prior to filling the experimental tanks, the temperature of the prepared water was adjusted to 25.0°C. Prepared water came from the same preparation tank only once per day.

Fish holding tank origin was randomized such that fish of the same sex and from the same holding tank did not meet the same conditions (water type, preparation tank, experimental tank). Four experimental fish were carefully captured with a hand net and put into a 15.5 × 9 × 11 cm transportation box each filled with 1 l of tap water. Each fish was then, alternately for the 2 treatments, introduced into its experimental tank. Subsequently, the behaviour of all 4 fish was recorded for 2 h. Per treatment group 6 males and 6 females were tested. Each fish was only used once, resulting in 24 experiments.

Body measurements and behavioural analysis. Subsequent to each experiment, fish standard length (SL: body size from snout to the beginning of the tail fin) was measured to the nearest millimetre, and body

mass (M) was determined (LC 221S, Sartorius). The body condition factor (CF) was calculated after Bolger & Connolly (1989) such that:

$$CF = 100 \times M \times (SL^3)^{-1}$$

Recordings of the experiments were analyzed by tracking the fish with the software BioObserve. Track length was used as an operational variable to infer activity. For every fish, the total track length covered in 2 h was calculated. In order to analyze activity changes during the experiment, track length was also computed in 1 min intervals over the duration of the experiment.

Water analysis. Furthermore, chemical water parameters were obtained from 6 different instances of either treatment. The pH and electrical conductivity (EC) values were obtained with a digital meter (HI 98129, Hanna Instruments, measurement errors: pH 0.05 units, EC 2%). Alkalinity (carbonate hardness) (kH, measurement error 0.009 mmol l⁻¹), total or general hardness (gH, measurement error 0.004 mmol l⁻¹), nitrate (NO₃⁻, measurement error 2.5 mg l⁻¹), nitrite (NO₂⁻, measurement error 0.005 mg l⁻¹) and ammoniac (NH₃/NH₄⁺, measurement error 0.025 mg l⁻¹) values were identified using drip tests (JBL). Also, the amount of diluted oxygen (O₂) was obtained twice in a 45 min interval per water analysis using an oxygen probe (OxiCal-SL, WTW, measurement error 0.5%) connected to a digital meter (OxiCal 842, WTW). The values of both oxygen measurements were averaged for statistical analysis. Carbon dioxide saturation (CO₂) was calculated based on the pH and kH values using the formula:

$$CO_2 \text{ (mg l}^{-1}\text{)} = kH \text{ (mmol l}^{-1}\text{)} \times 44 \times 10^{(pK - pH)}$$

with a calculated mean pK of 6.321 by incorporating temperature and EC according to Norm DIN 38404-10 (1995).

Statistical analyses. All statistics were computed using the R 2.5.1 statistical package. In order to examine whether the treatment (i.e. tap water or sand-treated water) influenced fish activity, linear models (LM) were conducted. Tests of statistical significance were based on likelihood ratios (LRT). Hence, degrees of freedom always differed by 1. Non-significant factors and interactions were removed from the analysis (Engqvist 2005). The total track length the test fish covered was entered as a dependent variable, and 'treatment' and 'sex', as fixed factors. Furthermore, the interaction between 'treatment' and 'sex' was included in order to test whether males and females responded differently to the treatment. For analyzing activity changes over time, for each of the 24 experiments Spearman rank correlation tests between track length per minute and time were conducted. The correlation

coefficients obtained were entered in a LM as the dependent variable, and 'treatment' and 'sex', as fixed factors. To examine sex-specific differences, the interaction between 'treatment' and 'sex' was included. Parametric tests were applied only when the data were normally distributed according to the Shapiro-Wilk test. When data were not normally distributed, non-parametric tests were applied. Test probabilities are 2-tailed throughout.

RESULTS

The mean (\pm SD) total track length covered by *Pelvicachromis taeniatus* in the sand-treated water group was almost twice as high as that in the control group (LRT, $F = 4.991$, $df = 1$, $p = 0.036$; Fig. 1). This difference was independent of sex (interaction sex \times treatment, LRT, $F = 1.032$, $df = 1$, $p = 0.332$). Also, the sexes did not cover significantly different total distances (LRT, $F = 0.207$, $df = 1$, $p = 0.654$). Activity changes over experimental time were significantly explained by the interaction between treatment and sex (LRT, $F = 4.595$, $df = 1$, $p = 0.045$; Fig. 2). Female activity did not significantly change over time in either treatment (tap water: LM, intercept, $t = 1.476$, $df = 5$, $p = 0.200$; sand-treated water: LM, intercept, $t = 1.518$, $df = 5$, $p = 0.189$; Fig. 2), nor did male activity in tap water (LM, intercept, $t = 1.485$, $df = 5$, $p = 0.198$; Fig. 2). However, males significantly increased their activity over time in sand-treated water (LM, intercept, $t = 5.805$, $df = 5$, $p = 0.002$; Fig. 2). Body measures did not differ significantly

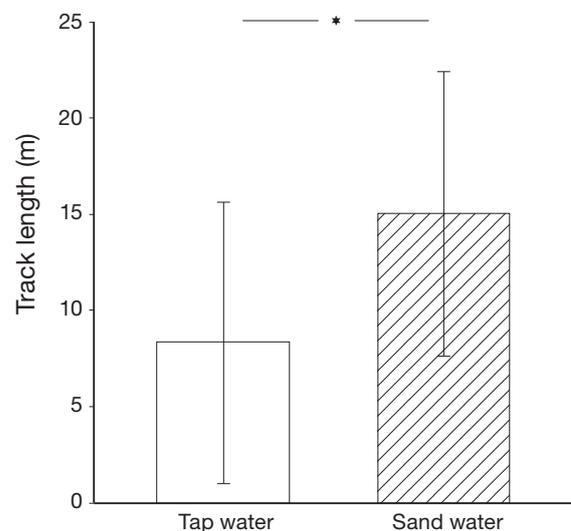


Fig. 1. *Pelvicachromis taeniatus*. Mean (\pm SD) total track length the fish covered during 2 h in tap water (white) and sand-treated water (hatched). The asterisk above the bars indicates $p < 0.05$

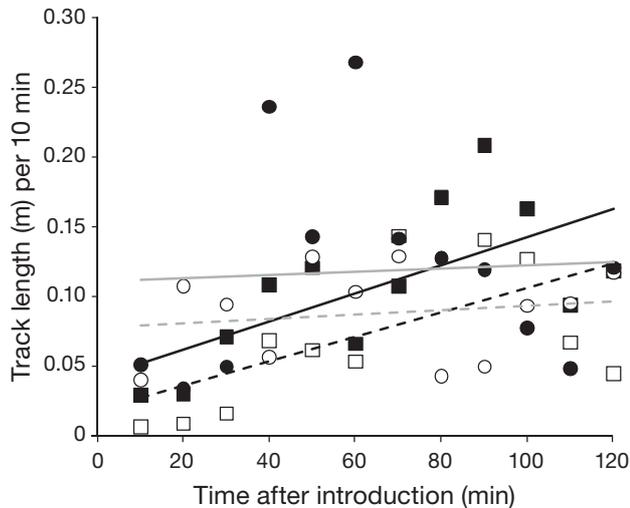


Fig. 2. *Pelvicachromis taeniatus*. Track length per successive 10 min intervals by males (squares, black regression lines) and females (circles, grey regression lines) in tap water (open symbols, dashed regression lines) and sand-treated water (filled symbols, solid regression lines) during the 2 h experiment

between the fish in the sand-treated water and control treatments (standard length: mean \pm SD: 4.91 ± 0.86 vs. 4.96 ± 0.92 cm, independent Wilcoxon test, $W = 75.5$, $p = 0.862$; body mass: mean \pm SD: 3.01 ± 1.21 vs. 3.16 ± 1.43 g, independent Wilcoxon test, $W = 74$, $p = 0.932$; condition factor: mean \pm SD: 2.51 ± 0.43 vs. 2.50 ± 0.43 , independent t -test, $t = -0.035$, $df = 22$, $p = 0.973$). Also, activity (i.e. total track length) was not significantly correlated with standard length (Spearman rank correlation, $r = -0.203$, $n = 24$, $p = 0.341$), body mass (Spearman rank correlation, $r = -0.123$, $n = 24$, $p = 0.564$), or condition factor (Pearson correlation, $r = 0.083$, $n = 24$, $p = 0.471$). The physical water parameters were not significantly different between treatments (Table 1).

Table 1. Comparison of water parameters from sand-treated water ($n = 6$) and tap water ($n = 6$). Shown are means (\pm SD), test statistics and p-values. Nitrite (NO_2^-) and ammoniac ($\text{NH}_3/\text{NH}_4^+$) concentrations were below measurement thresholds in all cases, and thus, statistics could not be performed. The nitrate (NO_3^-) values were identical in each measurement and, thus, were not statistically analyzed. kH: carbonate hardness; gH: general hardness

Parameter	Tap water	Substrate-treated water	Test statistic	p-value
pH	8.37 ± 0.05	8.36 ± 0.07	$W = 18$	>0.999
Conductivity (μS)	369.17 ± 25.31	370.16 ± 25.47	$t = 0.068$	0.947
kH (mmol l^{-1})	1.53 ± 0.19	1.59 ± 0.26	$t = 0.452$	0.662
gH (mmol l^{-1})	1.03 ± 0.12	1.03 ± 0.12	$W = 11$	>0.999
NO_3^- (mg l^{-1})	5 ± 0	5 ± 0	–	–
NO_2^- (mg l^{-1})	<0.01	<0.01	–	–
$\text{NH}_3/\text{NH}_4^+$ (mg l^{-1})	<0.05	<0.05	–	–
O_2 (mg l^{-1})	8.34 ± 0.33	8.20 ± 0.26	$t = -0.791$	0.448
CO_2 (mg l^{-1})	0.65 ± 0.22	0.60 ± 0.10	$W = 18.5$	>0.999

DISCUSSION

Generally, the activity of *Pelvicachromis taeniatus* was positively influenced by substrate-induced alteration of tap water. Fish were significantly more active in sand-treated water than in tap water independent of sex. Variation in body size, mass, or condition did not significantly correlate with activity, which contrasts to some other studies (Budaev et al. 1999, Reeb 2002). Significant differences between the sexes were found in activity changes over time. Males started with a somewhat lower activity than females, especially compared to females in sand-treated water. The males in the sand-treated water significantly increased their activity over time, whereas those in the control group did not. In the case of females, neither group developed significantly higher activity over time. The different responses of the sexes may be not surprising, because substrate is a resource more relevant for *P. taeniatus* males, which construct sand piles in front of their breeding caves (Baldauf 2006). Also, recent research has determined that in other cichlid species predominantly males use substrate for nest building (Mendonça et al. 2010, Schaedelin & Taborsky 2010). Sexes may also differ in learning ability and thereby in activity (Sneddon 2003). Another explanation is that males might be the choosier sex in *P. taeniatus* (Baldauf et al. 2009) and thus be more cautious.

Substrate treatment did not alter any measured chemical water parameters significantly. Also, microorganisms could not be detected in water samples, whereas microscopically small pieces of organic particles were present in higher quantity after substrate treatment (D.M., personal microscopic observations, magnification 400 \times). The results suggest that the difference in activity is not due to an effect of substrate on water-quality parameters (Broom & Johnson 1993), but rather implies that fish recognized the chemical trace of the substrate. Furthermore, it is likely that this odour is mediated through microscopically small organic particles. In other experiments, different authors found increased fish activity in tanks containing substrate (Webster & Hart 2004, Galhardo et al. 2009). Thus, recognition of substrate odours in the water might similarly influence fish welfare positively even without actual substrate interaction. Since the same type of substrate was available in the holding tanks, one may assume that the substrate odours represented a familiar environment (Gerlach et al. 2007).

The present research showed that activity of the cichlid *Pelvicachromis*

taeniatus was positively influenced by using water that had been pre-treated with sand. Providing olfactory enrichment by using this simple method may be widely applicable in the study of different fish species that inhabit sandy habitats in order to facilitate acclimatization to minimalistic experimental setups.

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