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Conspecific density and environmental complexity impact behaviour of turquoise killifish (*Nothobranchius furzeri*)

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Abstract

Fish models are essential for research in many biological and medical disciplines. With a typical lifespan of only 6 months, the Turquoise killifish (*Nothobranchius furzeri*) was recently established as a time- and cost-efficient model to facilitate whole-life and multigenerational studies in several research fields, including behavioural ecotoxicology. Essential information on the behavioural norm and on how laboratory conditions affect behaviour, however, is deficient. In the current study, we examined the impact of the social and structural environment on a broad spectrum of behavioural endpoints in *N. furzeri*. While structural enrichment affected only fish boldness and exploratory behaviour, fish rearing density affected the total body length, locomotor activity, boldness, aggressiveness and feeding behaviour of *N. furzeri* individuals. Overall, these results contribute to compiling a behavioural baseline for *N. furzeri* that increases the applicability of this new model species. Furthermore, our findings will fuel the development of improved husbandry protocols to maximize the welfare of *N. furzeri* in a laboratory setting.

KEYWORDS

animal welfare, behavioural variation, environmental enrichment, model organism, rearing density

1 | INTRODUCTION

Model organisms are essential to biological and medical research. The bulk of our current scientific insight into the fundamental principles of life came from research on model organisms, including fish (Fields & Johnston, 2005; Alfred & Baldwin, 2015). In biomedical research, fish models principally owe their popularity to their physiology, which is largely similar to that of humans, allowing for easy extrapolation of research results from nonhuman animal models to implications for humans (Schartl, 2014). Moreover, laboratory fish are relatively small, can be maintained in large numbers and are amenable to high-throughput experimental manipulation (Schartl, 2014; Russell *et al*., 2017). As fish became widely used models, a vast body of genomic and physiological data on fish models became available (Fields & Johnston, 2005). Classic fish models include zebrafish (*Danio rerio*), medaka or Japanese rice fish (*Oryzias latipes*), fathead minnow (*Pimephales promelas*), three-spined stickleback (*Gasterosteus aculeatus*) and rainbow trout (*Oncorhynchus mykiss*) (Ankley & Villeneuve, 2006; Cresko *et al*., 2007; Schartl, 2014).

In addition, the Turquoise killifish (*Nothobranchius furzeri*) was established as a time- and cost-efficient model organism for whole-life and multigenerational studies in the last decade (Harel *et al*., 2015; Philippe *et al*., 2017, 2018a). This annual fish originates from temporary freshwater pools in south-eastern Africa that are characterized by a seasonal desiccation and inundation, leading to an alternation between a completely dry phase and a wet phase (Cellerino *et al*., 2015; Polačik *et al*., 2016). Annual fish, including *Nothobranchius* killifish, cope with these harsh environmental conditions through specific adaptations, such as the production of drought-resistant eggs that remain dormant in the sediment until the next inundation (Furness *et al*., 2015; Pinceel *et al*., 2015; Grégoir *et al*., 2017a). Hatched fish grow rapidly to reach maturity and produce the next

generation of drought-resistant eggs before their habitat dries (Cellerino *et al*., 2015; Polačik *et al*., 2016). As *Nothobranchius* killifish are limited by the length of the wet phase to complete the aquatic stage of their life cycle, short inundation lengths select for fast maturation and a short lifespan (Terzibasi *et al*., 2008, 2013; Cellerino *et al*., 2015). Under optimal conditions, *N. furzeri* can mature within 3 weeks after hatching and has a lifespan that averages about 5–6 months (Blažek *et al*., 2013; Cellerino *et al*., 2015).

N. furzeri combines the perks of classic fish models with the short generation time of invertebrate model species (Cellerino *et al*., 2015; Polačik *et al*., 2016). With a generation time of about 3 months, the popularity of *N. furzeri* as a model organism for biological research mainly derives from its fast life cycle (Blažek *et al*., 2013; Cellerino *et al*., 2015). Moreover, *N. furzeri* fish have a high reproductive output, daily producing 20–50 eggs per female (Haas, 1976; Cellerino *et al*., 2015). Dormant *N. furzeri* eggs can be stored "on the shelf", without water, for years until fish are needed. Although at present it is not fully understood how storing may impact hatched fish, if at all, it may eliminate the need for a costly and labour-intensive continuous culture that is associated with most vertebrate models (Shedd *et al*., 1999; Nguyen and Persoone, 2000). Moreover, while killifish eggs can remain viable for many years, the rate of embryonic development can easily be manipulated and shortened so that eggs 2–3 weeks post fertilization can be hatched (Polačik *et al*., 2016). When ready-to-hatch eggs are inundated, juvenile fish hatch within 12 h (Polačik *et al*., 2016; Philippe *et al*., 2018b).

N. furzeri was initially launched as a model for gerontology studies to improve our understanding of biological ageing in vertebrates (Reichwald *et al*., 2015; Kim *et al*., 2016). However, the species rapidly gained popularity and is now used in many biological research fields, including genomics and genetics (Cellerino *et al*., 2015; Valenzano *et al*., 2015), ecology (Pinceel *et al*., 2015; Grégoir *et al*., 2017a, 2017b; Reichard *et al*., 2014) and evolutionary biology (Blažek *et al*., 2013). Because of its popularity, an increasing body of genomic, physiological and ecological background information on the species is available. Continued research on *N. furzeri* has resulted in useful research tools, including a whole brain atlas on the species (D'angelo, 2013), age-related histopathological analyses and an annotated genome and transcriptome (Di Cicco *et al*., 2011; Reichwald *et al*., 2015; Valenzano *et al*., 2015). Moreover, different laboratory strains have been developed, including a range of transgenic lines (Hartmann & Englert, 2012; Valenzano *et al*., 2015), with CRISPR/Cas9-mediated genome editing technology recently being established for the species (Reuter *et al*., 2018).

Recently, *N. furzeri* has also been introduced as a complementary model for ecotoxicological research, mainly with regard to whole-life and multigenerational studies. Ecotoxicology combines ecology and toxicology to study the effects of pollutants on organisms and ecosystems (Dell'Omo, 2002; Hellou, 2011) through sensitivity tests on different model organisms (Brady *et al*., 2017; Philippe *et al*., 2017). Because of the inconveniently long lifespan and generation time of classic fish models, full life-cycle and multigenerational assays (*e.g*., OECD guidelines for the testing of chemicals, OECD, 234, 240) are highly resource and time expensive and are therefore hardly performed for regulatory or research purposes (Ankley & Villeneuve, 2006). After an initial screening of congeneric species such as *N. rachovii* (van der Hoeven *et al*., 1982) and *N. guentheri* (Shedd *et al*., 1999), *N. furzeri* has been introduced as a more favourable model organism to facilitate such research agendas (Philippe *et al*., 2018a, 2018b, 2018c; Thoré *et al*., 2018a). Elaborate protocols for acute and chronic ecotoxicity testing with *N. furzeri* have been developed (Philippe *et al*., 2018b). Recently, traditional ecotoxicological testing has been expanded by also including sensitive behavioural responses to more accurately assess the environmental impact of pollution, especially with regard to pharmaceutical contaminants (Thoré *et al*., 2018a, 2019, 2020). To date, however, a lack of fundamental baseline data and the "behavioural norm" of fish models hampers rigorous ecological risk assessment of chemical compounds, and examining how environmental conditions affect baseline behavioural expression is imperative (Harris *et al*., 2014; Thoré *et al*., 2018a; Tanoue *et al*., 2019).

Public concern about the welfare of laboratory animals has increased in recent decades and there is a general call for minimization of animal distress (Walker, 2008). Current ethical guidelines follow the "three R" guiding principles of Russel and Burch: Replacement, Reduction and Refinement (Russell & Burch, 1959; Fenwick *et al*., 2009). Whenever possible, animal models should be replaced by alternative methods ("Replacement") and the number of used animals should be minimized ("Reduction"). When laboratory animals are essential, husbandry and experimental procedures should ensure minimal animal suffering and distress ("Refinement"). Improved husbandry protocols contribute to enhance the welfare of animals throughout their entire lives. Recent attempts to improve animal welfare have included environmental enrichment with both social (*i.e*., contact and interaction with conspecifics) and structural (*i.e*., physical complexity) components (Näslund & Johnsson, 2016). While both types of enrichment are now more or less common practice for mammal laboratory cultures, they are far less common in fish cultures. With increasing demands and regulations concerning environmental enrichment in laboratory animal science comes a need to understand how such procedures actually impact model organisms, fish in particular. This is especially relevant for new models, such as *N. furzeri*, for which standardized husbandry protocols are currently being developed.

In the current study, we examine the impact of enrichment of the rearing environment on fish growth and a broad behavioural repertoire in the fish model *N. furzeri*, based on paradigms that are commonly used to assess behaviour in classic fish models. Specifically, two experiments were conducted to assess the impact of (a) conspecific density and (b) structural complexity on fish body size, spontaneous locomotor activity parameters, boldness and exploratory behaviour, habitat choice, sociability, aggressiveness, feeding behaviour and the antipredator response of young adults. In other fish species, early rearing conditions are known to impact patterns of gene expression and neurophysiology with potential behavioural consequences (Champneys *et al*., 2018). Therefore, we also expect enrichment of the rearing environment to impact behaviour in *N. furzeri*. Not

only will these results contribute to an improved understanding of the behavioural baseline of *N. furzeri* and increase its value as a model, they will also directly contribute to the development of improved husbandry protocols and welfare.

2 | MATERIALS AND METHODS

2.1 | General experimental setup

Two separate experiments were conducted to assess the impact of (a) conspecific density and (b) structural complexity on behavioural expression in adult *N. furzeri*. To assess the impact of conspecific density, fish were reared in barren 10 l tanks (50 cm long × 20 cm wide × 17.5 cm high) at low (1 fish/10 l, n = 43), medium (5 fish/10 l, n = 55) or high (10 fish/10 l, n = 60) conspecific density starting 7 days post hatching and until the end of the experiment. Likewise, to assess the impact of structural complexity, fish were reared in barren control environments (10 fish/10 l, n = 40) or environments enriched with artificial plants (five per tank; each plant consisting of green plastic strips attached to a stainless-steel INOX nut) and plastic T-shaped tubes (one per tank; grey PVC tube with a 5 cm diameter) (10 fish/10 l, n = 40) (Figure 1) starting 9 days post hatching and until the end of the experiment. Mature fish of both experiments were individually subjected to a battery of behavioural tests to assess fish activity level, boldness or exploration tendency, habitat choice, aggressiveness, sociability, feeding behaviour and antipredator response.

Four days before the first behavioural test, total body length (from the tip of the snout to the tip of the tail, dorsal view) was assessed for each individual. To this end, individual fish were briefly transferred to a Petri dish with a sufficient amount of water for the fish to sustain a natural dorso-ventral posture. Size-calibrated photographs were taken (Samsung Galaxy S8+ dual-pixel 12.0MP AF F/1.7 camera, with fish centred in the camera frame to ensure correct measurement) and digitally analysed using the open source image processing software ImageJ Ver 1.50i (Schneider *et al*., 2012).

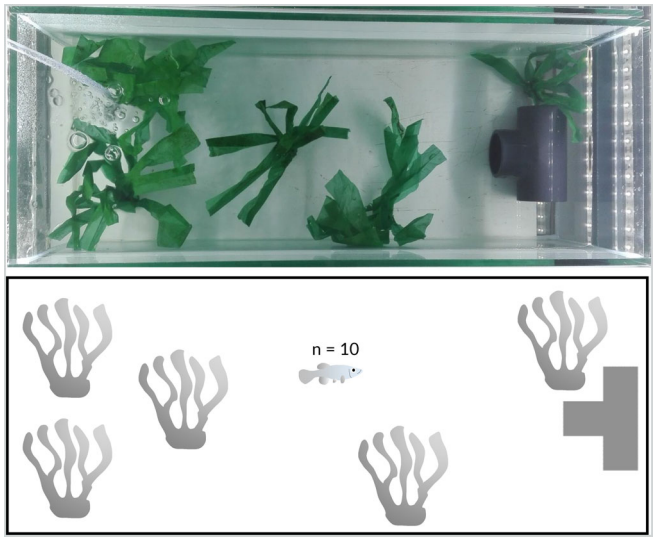

FIGURE 1 The enriched environment used in the structural complexity experiment. Each tank had a fish density of 10 fish/10 l

2.2 | Fish maintenance

Throughout both experiments, fish tanks were kept in a temperature-controlled water bath system to ensure a constant water temperature of 24°C, under a light:dark regime of 14:10 h. At the onset of both experiments, fish of the homozygous laboratory strain GRZ-AD were hatched by inundating "ready-to-hatch" eggs (Stage 43, *sensu* Wourms 1972) to a height of 2 cm with reconstituted water at a conductivity of 600 μS cm^{-1} (Instant Ocean salt mix added to type III RO water) enriched with 1 g l^{-1} humic acid (53,680; Sigma-Aldrich, Overijse, Belgium), after the protocol of Polačik *et al*. (2016). The water level was doubled on days 1 and 3 post hatching. Seven (conspecific density experiment) or nine (structural enrichment experiment) days post hatching, individuals were randomly assigned to a conspecific density condition or structural enrichment condition, respectively. To this end, fish were transferred to 10 l tanks (50 cm long × 20 cm wide × 17.5 cm high). Tanks were visually separated from each other by means of white opaque partitions, and were filled with reconstituted water at 600 μS cm^{-1} conductivity.

Juvenile fish were fed twice a day with *Artemia franciscana* nauplii (Ocean Nutrition, Essen, Belgium) to satiation. Three weeks post hatching, fish diet was supplemented with frozen *Chironomus* larvae (Ocean Nutrition). Starting 5 weeks post hatching and until the end of the experiment, fish were fed once a day with frozen *Chironomus* larvae to satiation. The quantity of food was adjusted to fish density to ensure standardized food availability *per capita*.

Water was renewed once a week. After every cleaning event, individual fish were randomly repositioned in a tank of their respective experimental condition to prevent any 'tank' effects throughout the experiment. Excess food was removed on a daily basis with a pipet to maintain good water quality.

2.3 | Behavioural setup

Starting 53 days (conspecific density experiment) and 84 days (structural complexity experiment) post hatching, individual fish were subjected to a range of behavioural tests to assess the behavioural impact of conspecific density and structural enrichment, respectively. These tests included a mirror test, an open field assay, an emergence test, a sociability test, a maze-test, a life-skills test and a habitat-choice test. The tests were performed in this order, fish were only subjected to one behavioural test per day and there was a minimum of 24 h between each test.

For each test, individual fish were transferred to the experimental arena and allowed to acclimate for 5 min prior to observation. Fish behaviour was recorded (top view) using Logitech C920 HD Pro webcam digital cameras. After each behavioural test, fish were transferred

back to their respective housing tanks. Video recordings were manually analysed afterwards (observer-blind), except for open field data which was analysed using Ethovision XT Ver 14.0 video-tracking software (Noldus Information Technologies, Wageningen, The Netherlands).

2.3.1 | Mirror test

A mirror test was used to evaluate fish aggressiveness (cf. Ansai *et al*., 2016). Fish were individually transferred to a 10 l tank (50 cm long × 20 cm wide × 17.5 cm high) that was divided into two equally sized compartments (one fish per compartment). Each compartment was provided with a mirror that was attached to the side (Figure 2a). Fish were visually separated from each other by means of an opaque plastic divider. Fish behaviour was recorded for 20 min while fish were allowed to visually interact with their mirror image. As a proxy for fish aggressiveness, the time spent in proximity to the mirror (25% of the arena closest to the mirror, corresponding to the zone within 6.5 cm of the mirror, see Figure 2a) was assessed.

2.3.2 | Open field assay

Spontaneous locomotor activity and boldness were assessed using an open field assay (cf. Ansai *et al*., 2016; Thoré *et al*., 2018a, 2018b). Fish were individually transferred to a 2.5 l tank (18 cm long × 12 cm wide × 11.5 cm high) with a water level of 2 cm (water volume of 0.5 l). Travelled distance and total moving time during a 20-min time period were assessed as measures for fish activity level. Locomotor activity was further characterized by assessing swimming velocity and maximum acceleration. In addition, the tank was virtually divided in a centre zone (50% of length and width) upon which time spent in the centre zone, latency time to enter the centre zone and mean distance to the centre of the arena were assessed as measures for fish boldness.

2.3.3 | Emergence test

An emergence test was used to study fish boldness and exploration tendency (cf. Thoré *et al*., 2018a, 2018b). Individual fish were

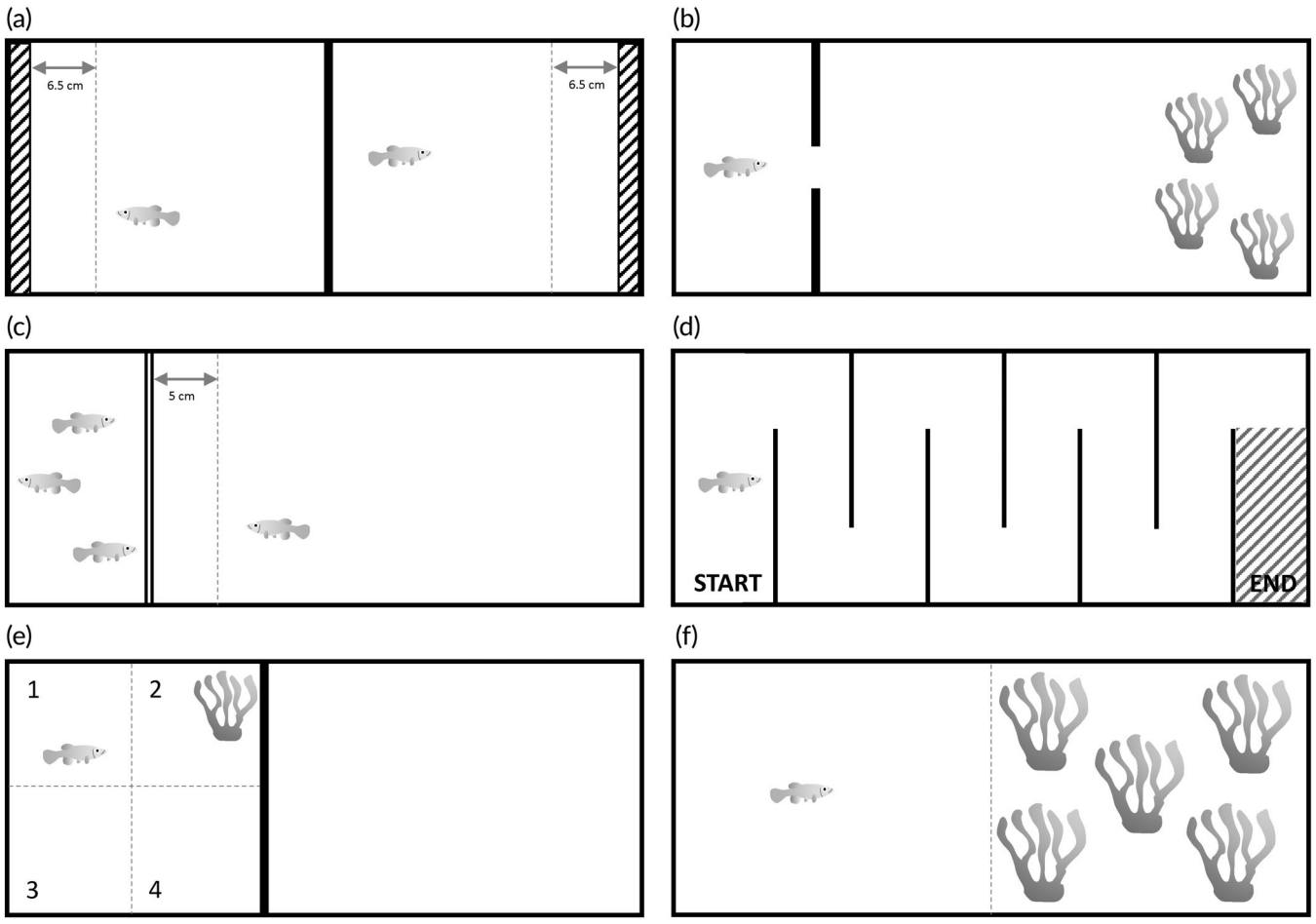

FIGURE 2 Experimental setup for the (a) mirror test, (b) emergence test, (c) sociability test, (d) maze test, (e) life-skills test and (f) habitat choice test. The experimental setup for the open field test is not shown

transferred to a 10 l tank (50 cm long × 20 cm wide × 17.5 cm high) that was divided in a smaller "start" compartment (9.5 cm long × 20 cm wide) and a larger "novel" compartment (Figure 2b). Both compartments were separated from each other by means of an opaque divider. Upon introduction and acclimation in the start compartment, a doorway (circular, diameter 3 cm) to the novel environment was opened to allow the individual to leave the small compartment and explore the newly available compartment. Latency time to enter the novel environment was recorded during the next 45 min as a measure for fish boldness. Individuals that did not enter the novel environment during this time received the maximum score of 2700 s (45 min).

2.3.4 | Sociability test

To assess social behaviour towards conspecifics, fish were individually transferred to a 10 l tank (50 cm long × 20 cm wide × 17.5 cm high) that was divided into a small compartment (9.5 cm long × 20 cm wide) and a larger compartment, separated from each other by means of a transparent divider (Figure 2c). The small compartment held three nonexperimental fish of the same age (mixed sex, and not always the same three individuals between tests). Upon introduction and acclimation of the focal individual in the larger compartment, the fish was allowed to visually interact with the conspecific group for 20 min. As a proxy for fish sociability, the time spent in proximity to the conspecific group (within 5 cm of the transparent divider, see Figure 2c) was assessed.

2.3.5 | Maze test

Exploration tendency was assessed by means of a maze test. Fish were individually transferred to a 10 l tank (50 cm long × 20 cm wide × 17.5 cm high) that was divided in a smaller start compartment and a larger compartment with a PVC maze (Figure 2d). After acclimation, access to the maze was allowed and the latency time to reach the end of the maze was recorded during the next 45 min as a measure for fish exploration tendency. Individuals that did not reach the end of the maze during this time received the maximum score of 2700 s (45 min).

2.3.6 | Life-skills test

Feeding behaviour in a novel situation and antipredator response were assessed using a life-skills test. Fish were individually transferred to a 10 l tank (50 cm long × 20 cm wide × 17.5 cm high) in which there was a smaller test compartment (20 cm long × 20 cm wide). The test compartment was virtually divided into four zones of equal size (Figure 2e). When the focal fish entered either zone 1 or zone 4, the test was initiated and food (frozen *Chironomus* larvae) was administered in zone 3. The latency time to start feeding during the next 15 min was assessed. Fish that failed to feed within this time (two and 12 fish in the conspecific density and structural enrichment experiment, respectively) received the maximum score of 900 s (15 min). When the fish initiated feeding (nonfeeding fish were excluded), a suspended, weighted 15 ml falcon tube (opaque, beak-shaped) was dropped and allowed to touch the water surface to simulate an avian predator attack (cf. Bell & Sih, 2007; Hedgespeth *et al*., 2016; Thoré *et al*., 2018a, 2018b). Subsequently, the time to resume feeding was recorded during the next 30 min. Fish that failed to resume feeding within this time received the maximum score of 1800 s (30 min).

2.3.7 | Habitat choice test

To assess fish habitat choice, fish were individually transferred to a 10 l tank (50 cm long × 20 cm wide × 17.5 cm high) that was divided in two equal parts: an open, barren part and a part provided with artificial plants for shelter (Figure 2f). Fish were introduced to the centre of the open part and allowed to swim freely throughout the test arena. Habitat preference was recorded for 20 min as the proportion of time spent in the open part compared to the total duration of the test (cf. Thoré *et al*., 2018a,b).

2.4 | Ethical statement

All experimental procedures and methods are in accordance with the legal requirements for animal research in Belgium and were approved by the ethical committee of KU Leuven (file number: P070/2016). The condition and health of each individual fish was monitored at least twice a day by two researchers (E.S.J. Thoré and L. Celie) independently. Optimized conditions were provided (cf. Polačik *et al*., 2016) and water quality was monitored daily (7.8 pH, ammonium <0.2 mg l^{-1}, nitrite <25 mg l^{-1}). Disturbance and handling were kept to a minimum to prevent and limit stress.

2.5 | Statistical analyses

Statistical analyses were conducted in R 3.3.1 (R Development Core Team, 2016) at a significance level of alpha = 0.05. Model assumptions, including distributional fit and homogeneity of variances, were verified graphically (complemented with a Shapiro–Wilk test for normality) for all analyses.

Total body length (mm) and all behavioural response variables were analysed using linear models with Gaussian error distribution. Sex (male, female) and condition (low, medium or high density for the density experiment; barren control or enriched environment for the enrichment experiment) were modelled as fixed factors. The significance of the fixed effects was tested by calculating F-tests, and *post hoc* differences were assessed using Tukey-corrected pairwise comparisons.

To improve distributional fit, log-transformations were performed for latency time to reach the end-zone (maze-test), latency time to

resume feeding (life-skills test), latency time to enter the centre zone (open field test) and acceleration (open field test) for both experiments. Latency time to initiate feeding was double log transformed. In addition, emergence time (emergence test) was log-transformed for the conspecific density experiment.

Notably, although Tukey-corrected pairwise comparisons are used to control the probability of making type I errors within each test, there is still a 5% chance of type I errors among tests that should be taken into consideration upon interpretation of the results.

3 | RESULTS

3.1 | Conspecific density experiment

Male fish (mean 31.4 mm, S.E. ±0.4) were larger than female fish (mean 28.5 mm, S.E. ±0.3) ($F_{1,149}$ = 29.465, **P < 0.001**). Solitary fish were larger than individuals from social groups ($F_{2,148}$ = 144.362, **P < 0.001**), irrespective of the sex of the fish ($F_{2,144}$ = 0.106, P = 0.899) (Figure 3).

Fish aggressiveness did not differ between sexes ($F_{1,135}$ = 0.146, P = 0.703). Solitary fish were more aggressive compared to fish from social groups ($F_{2,134}$ = 38.402, **P < 0.001**) (Figure 4a), irrespective of the sex of the fish ($F_{2,131}$ = 0.853, P = 0.428).

Latency time to emerge in the emergence test did not differ between sexes ($F_{1,144}$ = 2.099, P = 0.150). Fish that were reared in medium conspecific density emerged faster than individuals from high conspecific density and solitary fish ($F_{2,143}$ = 3.958, **P = 0.021**). However, the difference with solitary fish was only marginally supported by *post hoc* analysis (Tukey P = 0.055) (Figure 4b). There was no sex-specific effect of conspecific density on latency time to emerge ($F_{2,140}$ = 0.650, P = 0.524).

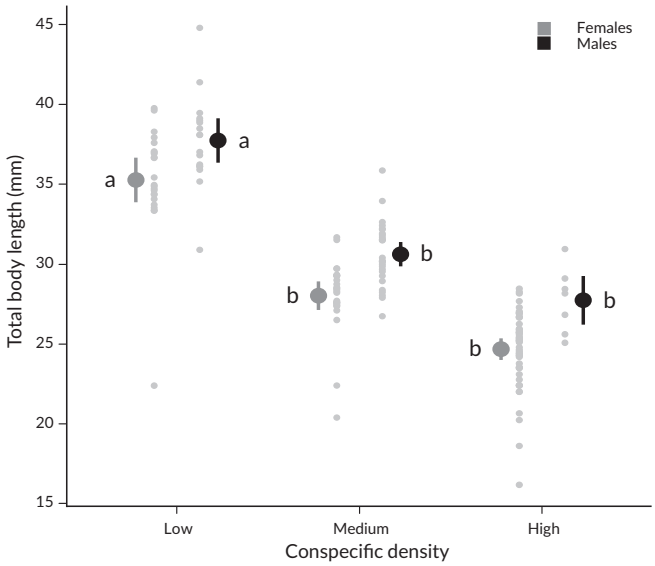

FIGURE 3 Male and female body size for solitary fish, fish from medium density and fish from high density. Whiskers delineate the upper 95% confidence limit. Significant differences are based on Tukey-corrected *post hoc* tests and are indicated with letters. (■) females; (■) males

Latency time to reach the end-zone in the maze test differed marginally between males and females ($F_{1,145}$ = 3.696, P = 0.056), with males taking longer to reach the end-zone (Supporting Information Figure S1). There was no difference between fish from different conditions to reach the end-zone ($F_{2,144}$ = 1.859, P = 0.159), nor was there a sex-specific effect of conspecific density ($F_{2,141}$ = 0.073, P = 0.930).

Solitary males were slower to start feeding compared to males from social groups, whereas no such effect could be observed for female fish ($F_{2,137}$ = 8.165, **P < 0.001**) (Figure 4c). Latency time to start feeding differed overall between sexes ($F_{1,141}$ = 319.749, **P = 0.011**) and conditions ($F_{2,140}$ = 5.581, **P = 0.005**). A similar sex-specific effect of conspecific density was observed for time to resume feeding after simulated predator attack, although this was only marginally supported ($F_{2,135}$ = 2.833, P = 0.062). *Post hoc* analysis revealed that solitary males took longer to resume feeding compared to males from high conspecific density (Tukey **P = 0.006**; Figure 4d). Latency time to resume feeding differed overall between sexes ($F_{1,139}$ = 10.211, **P = 0.002**) and conditions ($F_{2,138}$ = 4.237, **P = 0.016**).

Time spent in the open zone during the habitat choice test did not differ between sexes ($F_{1,146}$ = 0.026, P = 0.871) and conditions ($F_{2,145}$ = 0.571, P = 0.566), nor was there a sex-specific effect of conspecific density ($F_{2,142}$ = 0.503, P = 0.606). Likewise, sociability did not differ between sexes ($F_{1,145}$ = 1.932, P = 0.167) and conditions ($F_{2,144}$ = 1.803, P = 0.169), and there was no sex-specific effect of conspecific density ($F_{2,141}$ = 2.533, P = 0.083).

Swimming velocity did not differ between sexes ($F_{1,140}$ = 2.168, P = 0.143) and conditions ($F_{2,139}$ = 1.990, P = 0.141) overall. However, a higher swimming velocity for solitary fish compared to fish from high densities was found, although only for females ($F_{2,136}$ = 3.171, **P = 0.045**) (Figure 5a).

Maximum swimming acceleration did not differ between sexes ($F_{1,140}$ = 0.395, P = 0.531). Fish from medium densities had a lower maximum acceleration compared to solitary fish ($F_{2,139}$ = 5.141, **P = 0.007** (Figure 5b), irrespective of sex ($F_{2,136}$ = 0.316, P = 0.729).

Female fish from higher rearing densities swam a smaller distance ($F_{2,136}$ = 4.231, **P = 0.016**) (Figure 5c) and spent more time moving compared to solitary fish ($F_{2,136}$ = 5.231, **P = 0.006**) (Figure S2a). Overall, travelled distance did not differ between conditions ($F_{2,139}$ = 1.029, P = 0.360) or sexes ($F_{1,140}$ = 3.050, P = 0.083), nor were there overall differences in total time moving between conditions ($F_{2,139}$ = 0.517, P = 0.597) or sexes ($F_{1,140}$ = 2.182, P = 0.142).

Latency time to enter the centre zone of the open field ($F_{2,139}$ = 6.870, **P = 0.001**) (Supporting Information Figure S2b) as well as mean distance to the centre of the open field ($F_{2,139}$ = 19.378, **P < 0.001**) (Supporting Information Figure S2c) was lower for solitary fish compared to fish from medium or high densities. Likewise, solitary fish spent more time in the centre zone of the arena compared to fish from medium or high densities ($F_{2,139}$ = 20.474, **P < 0.001**) (Figure 5d). The effect of rearing density on the time to enter the centre zone ($F_{2,136}$ = 0.447, P = 0.640), mean distance to the centre ($F_{2,136}$ = 0.340, P = 0.712) and time spent in the centre

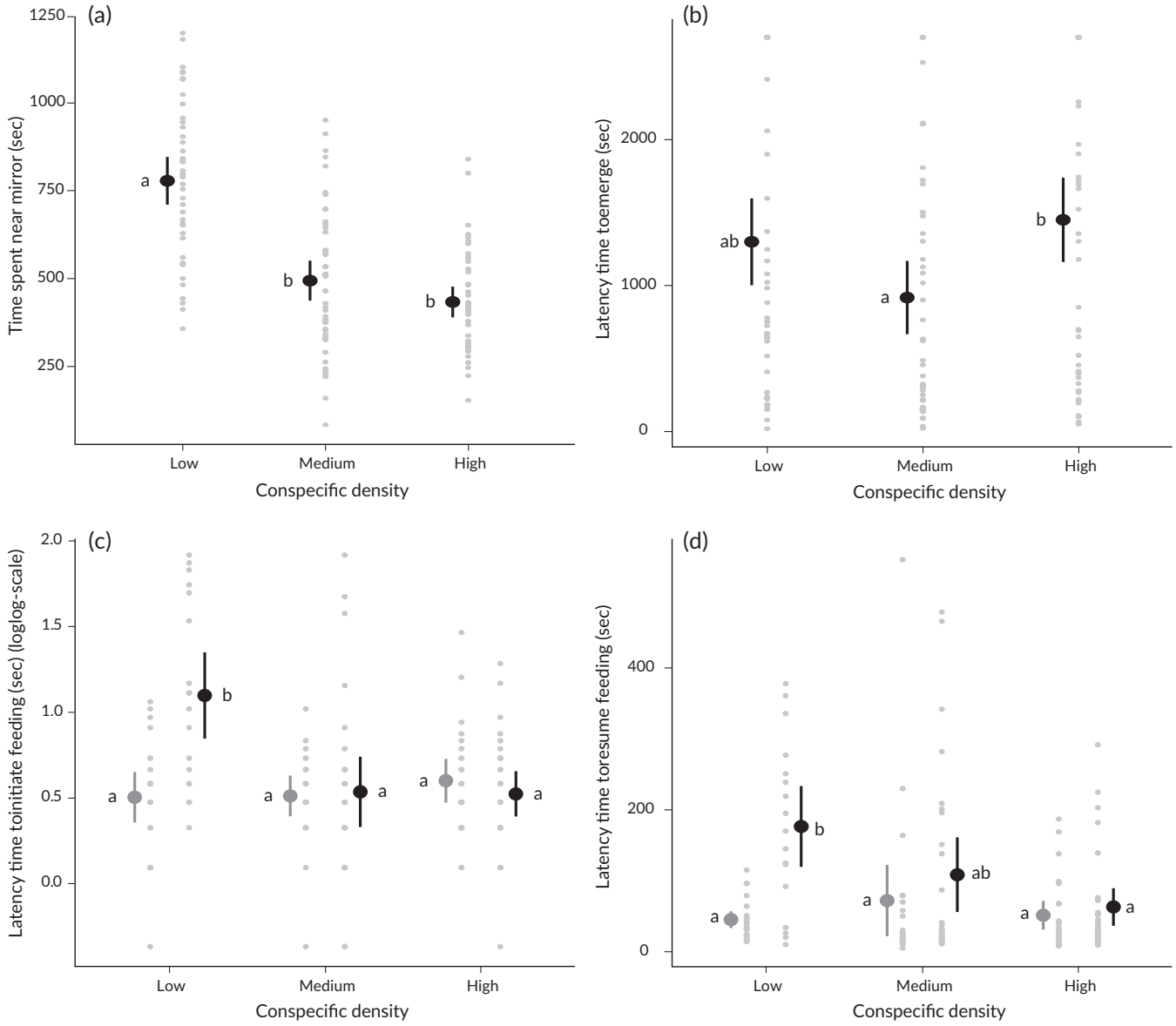

FIGURE 4 The impact of conspecific rearing density on (a) time spent near the mirror as measure for fish aggressiveness, (b) latency time to enter a novel environment in the emergence test as measure for fish boldness, (c) latency time to initiate feeding [(■) females; (■) males] and (d) latency time to resume feeding after a simulated predator attack for males and females [(■) females; (■) males]. Whiskers delineate the upper 95% confidence limit. Significant differences are based on Tukey-corrected *post hoc* tests and are indicated with letters

($F_{2,136}$ = 1.831, P = 0.164) did not depend on sex. There were no overall differences in latency time to enter the centre ($F_{1,140}$ = 0.745, P = 0.389), mean distance to the centre ($F_{1,140}$ = 0.057, P = 0.811) and time spent in the centre ($F_{1,140}$ = 1.365, P = 0.245) between sexes.

Mean value, standard deviation, and minimum and maximum values for all behavioural response variables are presented in Supporting Information Table S1, separated by sex and condition.

3.2 | Structural complexity experiment

Male fish (mean 30.7 mm, S.E. ±0.4) were larger than female fish (mean 28.0 mm, S.E. ±0.4) ($F_{1,74}$ = 24.294, **P < 0.001**). Fish from barren and enriched conditions did not differ in total body length ($F_{1,74}$ = 0.463, P = 0.498), irrespective of the sex of the fish ($F_{1,72}$ = 0.002, P = 0.966).

Fish aggressiveness did not differ between sexes ($F_{1,74}$ = 0.471, P = 0.495) and conditions ($F_{1,74}$ = 1.189, P = 0.279), nor was there a sex-specific effect of structural enrichment on aggressiveness ($F_{1,72}$ = 0.063, P = 0.803).

Fish from an enriched environment were faster to enter the novel zone in the emergence test setup compared to fish from a barren environment ($F_{1,74}$ = 31.979, **P < 0.001**) (Figure 6a). The effect of structural enrichment on latency time to emerge did not depend on sex ($F_{1,72}$ = 0.235, P = 0.629). Moreover, there was no overall difference in latency time to emerge between sexes ($F_{1,74}$ = 0.521, P = 0.473).

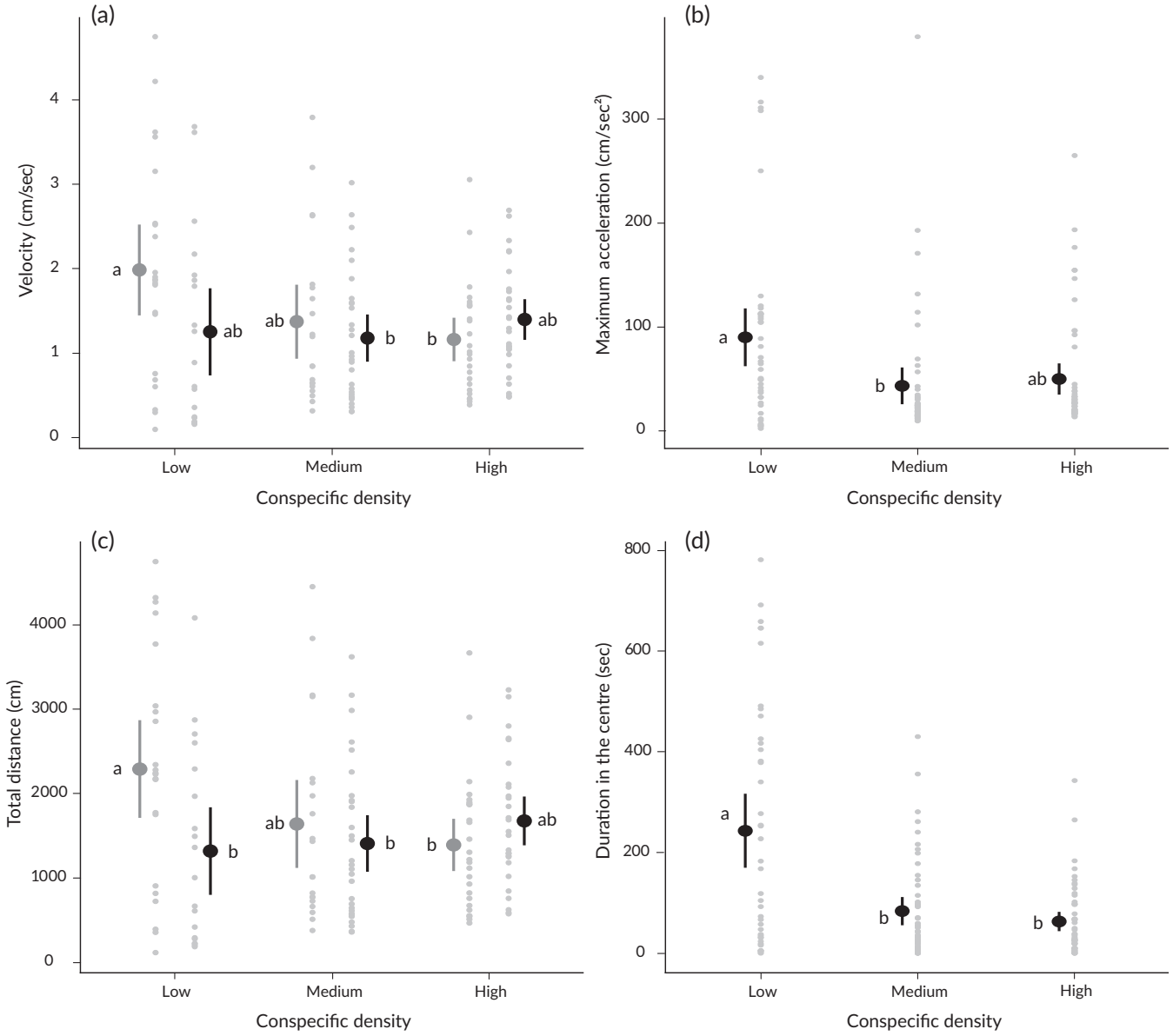

FIGURE 5 The impact of conspecific rearing density on (a) swimming velocity [(■) females; (■) males], (b) maximum acceleration, (c) travelled distance during the open field test as measure for fish activity [(■) females; (■) males] and (d) time spent in the centre of the open field as measure for fish boldness. Whiskers delineate the upper 95% confidence limit. Significant differences are based on Tukey-corrected *post hoc* tests and are indicated with letters

Latency time to reach the end-zone in the maze test did not differ between conditions ($F_{1,73}$ = 3.173, P = 0.079) (Figure 6b). There was no sex-specific effect of structural enrichment on time to reach the end-zone ($F_{1,71}$ = 0.019, P = 0.891), nor did the time differ between sexes overall ($F_{1,73}$ = 1.480, P = 0.228).

Latency time to initiate feeding ($F_{1,71}$ = 0.028, P = 0.866) and resume feeding ($F_{1,59}$ = 3.490, P = 0.067) after a simulated predator attack did not differ between sexes. Initiating ($F_{1,71}$ = 2.326, P = 0.132) and resuming feeding ($F_{1,59}$ = 0.108, P = 0.743) did not depend on the rearing environment, and there was no sex-specific effect of structural enrichment on either initiating ($F_{1,69}$ = 0.004, P = 0.952) or resuming feeding ($F_{1,57}$ = 0.132, P = 0.747) after disturbance.

Time spent in the open zone of the habitat choice test was marginally smaller for fish from an enriched compared to a barren environment ($F_{1,72}$ = 3.855, P = 0.053) (Figure 6c). This did not depend on sex of the fish ($F_{1,70}$ = 0.113, P = 0.737), nor was there an overall difference in time spent in the open zone between sexes ($F_{1,70}$ = 2.138, P = 0.148).

Sociability did not differ between sexes ($F_{1,74}$ = 0.063, P = 0.803) or conditions ($F_{1,74}$ = 0.338, P = 0.563) and there was no sex-specific effect of structural enrichment on fish sociability ($F_{1,72}$ = 0.268, P = 0.606).

Swimming velocity did not differ between sexes ($F_{1,74}$ = 0.712, P = 0.401) or conditions ($F_{1,74}$ = 2.202, P = 0.142) and there was no sex-specific effect of structural enrichment on swimming velocity

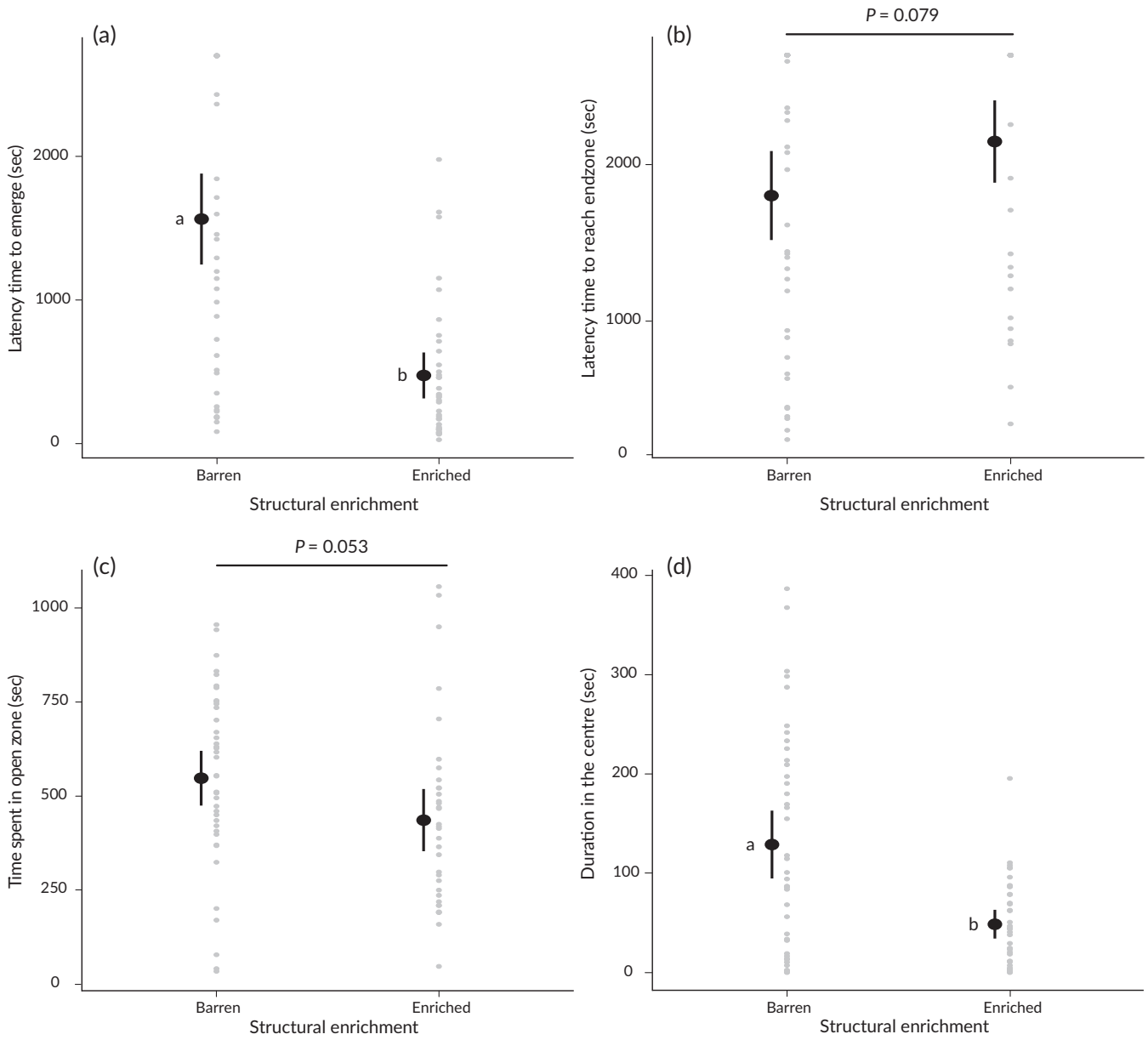

FIGURE 6 The impact of conspecific rearing density on (a) the latency time to enter a novel environment in the emergence test as measure for fish boldness, (b) the latency time to enter the end-zone in the maze test, (c) the time spent in the open zone in the habitat choice test and (d) the time spent in the centre of the open field as measure for fish boldness. Whiskers delineate the upper 95% confidence limit. Significant differences are based on Tukey-corrected *post hoc* tests and are indicated with letters

($F_{1,72}$ = 0.533, P = 0.468). Maximum acceleration was higher for males than for females ($F_{1,74}$ = 6.362, **P = 0.014**) (Supporting Information Figure S3), but did not differ between conditions ($F_{1,74}$ = 0.008, P = 0.929). Moreover, there was no sex-specific effect of structural enrichment on maximum acceleration ($F_{1,74}$ < 0.001, P = 0.988).

Travelled distance ($F_{1,74}$ = 0.724, P = 0.397) and total moving time ($F_{1,74}$ = 0.615, P = 0.435) in the open field test did not differ between sexes. Travelled distance ($F_{1,74}$ = 2.303, P = 0.133) and total moving time ($F_{1,74}$ = 1.739, P = 0.191) did not depend on the rearing environment, and there was no sex-specific effect of structural enrichment on either travelled distance ($F_{1,72}$ = 0.522, P = 0.472) or total moving time ($F_{1,72}$ = 0.585, P = 0.447).

Latency time to enter the centre zone of the open field ($F_{1,74}$ = 8.190, **P = 0.005**) (Supporting Information Figure S4a) as well as mean distance to the centre of the open field ($F_{1,74}$ = 18.262, **P < 0.001**) (Supporting Information Figure S4b) was lower for fish from a barren environment compared to fish from an enriched environment. Likewise, fish from a barren environment spent more time in the centre zone of the arena compared to fish from structurally enriched environments ($F_{1,74}$ = 16.345, **P = 0.001**) (Figure 6d). The effect of structural enrichment on latency time to enter the centre zone ($F_{1,72}$ = 0.068, P = 0.795), mean distance to the centre ($F_{1,72}$ = 0.359, P = 0.551) and time spent in the centre ($F_{1,72}$ = 0.326, P = 0.569) did not depend on sex. There were no overall differences

in latency time to enter the centre ($F_{1,74}$ = 0.042, P = 0.838), mean distance to the centre ($F_{1,74}$ = 0.031, P = 0.860) and time spent in the centre ($F_{1,74}$ = 0.188, P = 0.665) between sexes.

Mean value, standard deviation, and minimum and maximum values for all behavioural response variables are presented in Supporting Information Table S2, separated by sex and condition.

4 | DISCUSSION

We investigated the impact of the social and structural environment on total body length and a broad repertoire of behavioural traits in *N. furzeri*. Fish rearing density affected total body length, locomotor activity, boldness, aggressiveness and feeding behaviour, while structural enrichment only affected classic measures of fish boldness and exploratory behaviour.

Overall, these results contribute to drawing a behavioural baseline for *N. furzeri*, which increases the applicability of this new model species. Furthermore, our findings will fuel the development of improved husbandry protocols to maximize the welfare of *N. furzeri* in a laboratory setting.

4.1 | Conspecific density of the rearing environment impacts *N. furzeri* behaviour

In line with our expectations, boldness and exploratory behaviour were impacted by both the conspecific density and structural complexity of the rearing environment. Conspecifics in the rearing environment impacted fish boldness and exploratory behaviour in a density-dependent manner. While fish that were reared at medium densities started exploring the novel environment faster in the emergence test compared to fish that were reared in isolation, such an effect did not emerge for fish that were reared at a high density. This result indicates that fish reared in isolation or at high densities are less bold compared to fish reared at a medium density. Champneys *et al.* (2018) found similar results for Nile tilapia (*Oreochromis niloticus*): neophobic behaviour and risk-averse behaviour increased for fish that were reared at high densities compared to low densities. At high densities fish had higher chronic stress levels and were argued to have a reactive stress coping style compared to tilapia reared at a low density with a proactive stress coping style. Crowding-associated stress likely also underpins the reduction in boldness in high-density fish compared to medium-density fish in our study. Surprisingly, a higher level of boldness in the open field test was observed for solitary fish compared to fish reared in social groups. Likewise, solitary fish displayed higher levels of aggressiveness compared to fish reared in social groups. This result is consistent with earlier studies that examined the impact of conspecific density on behavioural indicators of animal welfare. For instance, Laursen *et al.* (2013) showed that farmed rainbow trout (*Oncorhynchus mykiss*) displayed more aggressive behaviour with decreasing conspecific density of the rearing environment. In addition, they reported a negative relationship between oxygen consumption (as a proxy for metabolic rate) and conspecific rearing density, which was likely reflective of increased levels of spontaneous activity in low-density fish. Likewise, Champneys *et al.* (2018) reported higher aggressiveness and mobility levels in Nile tilapia reared at low compared to high conspecific density. Although a negative association between spontaneous locomotor activity level and conspecific density could not be confirmed for *N. furzeri* in the current study, female swimming velocity was negatively associated with fish density. Also, maximum swimming acceleration for solitary fish was higher compared to fish from a medium density environment. As total body length decreased with increasing conspecific density, biophysical differences between fish from different rearing conditions possibly underpin these observations. Alternatively, a social novelty effect may contribute to these findings. During observation trials, fish from social environments were suddenly deprived of conspecifics whereas this was not the case for fish that were reared in isolation. This might at least partly explain why behavioural traits are enhanced in solitary fish but not in fish from social environments. If this is true, then the novelty effect was greater for fish reared in social groups compared to solitary fish and suggests that habituation to the test might differ between fish of different social rearing environments. A similar effect has been found for juvenile Eurasian perch (*Perca fluviatilis*). Individuals that experienced the greatest loss of conspecifics upon observation displayed the highest increase in boldness between consecutive repeated trials (Goldenberg *et al.* 2014).

Fish that were reared in isolation also exhibited a longer latency time to initiate feeding compared to fish reared in social groups. Likewise, time to resume feeding after a simulated predator attack increased with increasing fish rearing density. In contrast to these findings, Brockmark *et al.* (2010) showed that the ability to find prey in a maze increased in fry of brown trout (*Salmo trutta*) that were reared at lower compared to higher fish densities. Interestingly, however, a negative association with latency time to feed and fish rearing density only emerged for male fish in our study and solitary males had a longer latency time to feed compared to females. Likely, the high energy demand for egg production of females compared to males may be partly responsible for this observation. In addition, because of their bright colouration, male *N. furzeri* are much more conspicuous to visual predators, such as birds, than dull-coloured females. Therefore, more risk-averse feeding behaviour of males compared to females may reflect an adaptation to minimize the risk of avian predation (Reichard *et al.*, 2009). This sex-related difference in feeding behaviour disappears when fish are reared in social groups. Possibly, predation-risk dilution, predator confusion and increased levels of overall vigilance result in an increased sense of security in group-reared individuals (Goldenberg *et al.*, 2014) that is translated to more risk-prone feeding behaviour of individual fish. Alternatively, fish reared in social groups may be conditioned into faster feeding because of intra-specific competition for resources. In this respect, it is worth noting that, although food availability was standardized *per capita*, competition for food could partly underpin the observed differences in total body length between fish from different density conditions.

4.2 | Structurally complex rearing environments impact boldness and exploratory behaviour

Fish from a structurally complex rearing environment exhibited a lower latency time to enter the novel zone in the emergence test, which may suggest a higher boldness and exploration tendency compared to fish reared in a barren environment. This result could be consistent with earlier studies that reported enhanced behavioural traits when fish were exposed to structural environmental variability in the rearing environment. Braithwaite and Salvanes (2005), for instance, showed that cod (*Gadus morhua*) individuals that were exposed to variable spatial cues in the early rearing environment were faster to explore a novel environment compared to fish that were reared in plain hatchery tanks (although these effects might also be driven by a changeable environment rather than complexity *in se*). However, it should be noted that in the current study the novel environment in the emergence test had artificial plants similar to those used in the structurally enriched tanks. Therefore, the lower latency time to explore the novel environment in the emergence test for fish from structurally complex environments might in fact also reflect an increased tendency of the fish to visit familiar environmental structures or an increased tendency to seek shelter due to increased fearfulness. If this is the case, then a lower latency time to emerge would likely reflect a decreased rather than an increased boldness.

Similarly, Roberts *et al*. (2011) showed that juvenile Atlantic salmon (*Salmo salar*) reared in environmentally enriched tanks with natural prey and subjected to simulated predator attacks were less willing to leave shelter and had a higher latency time to leave shelter compared to control fish. This was suggested to reflect more natural behaviour than that of fish reared under standard hatchery conditions. In further support of these observations, in the current study fish from a structurally complex rearing environment spent less time in the centre zone, had a higher latency time to enter the centre zone and a higher mean distance to the centre of the open field compared to fish from a barren environment. These results indicate that *N. furzeri* individuals that experienced structurally complex rearing conditions are less bold compared to fish from barren environments.

Notably, we cannot exclude that behavioural alterations induced by the rearing-environment are expressed in a context-dependent manner which may lead to diverging results between tests. Such context-dependency has also been described for other fish species. For instance, Lee and Berejikian (2008) showed that juvenile steelhead trout (*Oncorhynchus mykiss*) reared in a structurally complex environment exhibited increased exploratory behaviour compared to fish reared in a barren environment, but this effect was not expressed when fish experienced a high predation threat. Likewise, Näslund *et al*. (2013) found that Atlantic salmon (*Salmo salar*) from structurally complex tanks were more prone to seek shelter than fish from barren tanks, although this effect was only evident for fish that were tested in isolation and did not emerge when fish were tested in small groups.

4.3 | Compiling the *N. furzeri* behavioural baseline to aid the development of husbandry protocols

Because *N. furzeri* has only recently been established as a model organism in a range of biological disciplines, the behavioural baseline of the species so far remains poorly characterized. While the current study should be considered as an essential first step to fill this hiatus, it is important to note that more research is needed to further establish the *N. furzeri* behavioural norm (see, *e.g*., Thoré *et al*. (2018b) for an exploratory study on behavioural repeatability in *N. furzeri*). Here, *N. furzeri* behavioural expression was assessed using a battery of classic fish behavioural tests that are commonly used in other fish models, including zebrafish (Ansai *et al*., 2016). However, behavioural expression may reflect a variety of underlying motivational and cognitive mechanisms that may be species-specific (Budaev & Brown, 2011). Therefore, classic behavioural tests should be validated with *N. furzeri* to facilitate interpretation of the underlying motivators of behavioural expression with a higher level of certainty. For instance, mirror-tests are typically used to examine fish aggressiveness (Balzarini *et al*., 2014; Ansai *et al*., 2016) but their generality across species has been questioned (Scherer *et al*., 2016), with some studies even suggesting mirror tests as a suitable measure for fish sociability (Cattelan *et al*., 2017). In the current study, we observed no mirror biting and lateral display as indicators of aggressiveness, which advocates caution in interpreting the observed behaviour and further suggests that classic behavioural tests should be validated for *N. furzeri*. Furthermore, behavioural expression might be influenced by recent experiences (*i.e*., carryover effects) (Bell, 2013), suggesting that also, for example, the order of behavioural assays and acclimation period may impact *N. furzeri* behaviour. Lastly, it is important to note that the current study design did not allow for assessment of covariance among behaviours, nor for direct assessment on how body size affects behavioural expression.

Although the behavioural baseline of *N. furzeri* needs further scrutiny, optimized and ethically sound animal husbandry protocols also need to be developed. To allow for individual monitoring and to adhere to the highest level of standardization, fish are often housed individually in laboratory conditions. This is, for instance, the case for a range of standard ecotoxicological tests (Philippe *et al*., 2018a, 2018b, 2018c). Ecotoxicological test procedures and husbandry protocols for *N. furzeri* are currently being developed or updated to maximize standardization, ease of cultivation and manipulation while also taking into account animal welfare. Structural complexity and social enrichment of the rearing and test environment add to the ecological relevance of animal testing and facilitate the translation of laboratory conditions to natural conditions. Simultaneously, they are also important measures to accommodate animal welfare by avoiding the development of maladaptive or unwanted traits and through promoting the expression of a complete repertoire of natural behaviours (Näslund & Johnsson, 2016; Champneys *et al*., 2018). Since behavioural endpoints are increasingly promoted as sensitive indicators of pollution, studies like this one that look into how housing conditions impact the baseline behavioural expression of a model organism are highly relevant.

From this perspective, it is also noteworthy that the benefits of environmental enrichment may differ between species and life stages, and environmental enrichment that is not tailored to the circumstances may even create a low-welfare environment and result in distress and injury (Näslund & Johnsson, 2016). Hence, future research is recommended to thoroughly link different enrichment strategies with *N. furzeri* welfare. Overall, the current study provides fundamental data on how enrichment of the rearing environment impacts behavioural development in *N. furzeri* which, in turn, will help to develop husbandry protocols for this recently established model organism. An important next step will be to assess the robustness of these findings against methodological differences between studies (*e.g.*, different endpoints, differences in environmental enrichment), as well as to assess their generality across different populations and strains of *N. furzeri*.

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CONTRIBUTIONS

E.S.J.T. designed and performed the experiments, analysed the data and wrote the manuscript. T.P. designed the experiments and wrote the manuscript. L.B. supervised the project.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data is accessible at the FigShare repository (https://doi.org/10.6084/m9.figshare.12925493.v1).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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