Inhibitory control in zebrafish, *Danio rerio*

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Abstract

We assessed whether zebrafish, *Danio rerio*, display inhibitory control using a simple and rapid behavioural test. Zebrafish were exposed to a prey stimulus placed inside a transparent tube, which initially elicited attack behaviour. However, zebrafish showed a rapid reduction in the number of attacks towards the prey, which indicated the ability to inhibit their foraging behaviour. Zebrafish also exhibited mnemonic retention of foraging inhibition, as indicated by a reduced number of attacks in a subsequent exposure to the unreachable prey. The ability to inhibit the foraging behaviour varied across three genetically separated wild-type strains and across different individuals within strains, suggesting that zebrafish show heritable within-species differences in inhibitory control. Our behavioural test might be suitable for screening large zebrafish populations in mutational studies and assessing effects of pharmacologically active substances on inhibitory control.

Keywords: behaviour; fish cognition; inhibitory control; learning; memory.

Introduction

Cognitive psychologists have collected substantial evidence that a set of core cognitive processes called executive functions allow one to control one's behaviour and achieve complex cognitive tasks (Diamond, 2013). Inhibitory control is the executive function in charge of blocking internal predispositions, prevailing over external lures, and controlling attention and behaviour in order to emit responses that are more appropriate to the context (Diamond, 2013). There is large variability in inhibitory control across individuals (Garavan *et al.*, 2006; Shamosh *et al.*, 2008), which has often been related to cognitive deficits, including pathological disorders. For example, reduced inhibitory abilities negatively affect working memory, text comprehension, mathematical learning, and problem solving (Cain, 2006; Gilmore *et al.*, 2013; Passolunghi *et al.*, 2001) and have been associated with attention-deficit/hyperactivity and schizophrenic disorders (Enticott *et al.*, 2008; Nigg, 2001), aggressive behaviours (Chen *et al.*, 2008), and drug addiction (Baler *et al.*, 2006; Colzato *et al.*, 2007). Evidence suggests that non-human animals possess inhibitory control as well (mammals: Amici *et al.*, 2008; birds: Meier *et al.*, 2017; reptiles: Szabo *et al.*, 2019; teleost fish: Lucon-Xiccato *et al.*, 2017). Arguably, the development of animal models might be important in understanding the neural substrates and molecular bases of inhibitory control and in developing remedies for its deficits. For example, research on rats showed that the medial striatum, the ventral hippocampus, and the serotonin pathway are involved in inhibitory processes (Abela *et al.*, 2012; Eagle and Robbins, 2003a; Homberg *et al.*, 2007), and studies on dogs have demonstrated that inhibitory control can be improved with specific training (Barrera *et al.*, 2019).

The zebrafish is a small teleost fish that is gaining more and more importance in several fields of biological research, including the study of cognition and neurobiological disorders (Best and Alderton, 2008; Blaser and Vira, 2014; Guo, 2004; Stewart *et al.*, 2014). The zebrafish is favoured in research due to its abundant and rapid reproduction, the quick development, and the limited costs for maintenance (Stewart *et al.*, 2014). Moreover, the zebrafish genome has been fully sequenced, and regions of homology with humans have been identified (Howe *et al.*, 2013; Stewart *et al.*, 2014). Several genetic research tools are available for the zebrafish and have enabled production of more than 1000 mutant and transgenic lines so far (Stewart *et al.*, 2014), also for the study of central nervous system disorders (Santana *et al.*, 2012; Xi *et al.*, 2011). Many studies have also described zebrafish's cognitive abilities and developed behavioural procedures to assess them. A number of simple conditioning paradigms permit measurement of general learning processes (e.g., Blank *et al.*, 2009; Morin *et al.*, 2013, Xu *et al.*, 2007), and a few procedures measure specific, high-level cognitive abilities (e.g., Hamilton *et al.*, 2018). However, in zebrafish, there is no evidence of several cognitive abilities detected in other teleost fish, such as the discrimination of complex visual stimuli (i.e., faces) shown by archerfish (Newport *et al.*, 2016), concept and matching-to-sample learning reported for cichlid fish (Gierszewski *et al.*, 2013; Schluessel *et al.*, 2012), and complex spatial maze learning (Lucon-Xiccato and Bisazza, 2017a), serial reversal learning (Fuss and Witte, 2019; Lucon-Xiccato and Bisazza, 2014), and problem solving of poeciliids (Lucon-Xiccato *et al.*, 2019).

Inhibitory control is also poorly investigated in zebrafish. Studies by Parker and colleagues (2013, 2014, 2015) have developed a paradigm that might reflect inhibitory

abilities. Zebrafish had to enter a chamber among 5 available alternatives following a cue. This task allows for measuring attention and, by scoring the tendency to make a choice before the cue, impulsivity (Bari *et al.*, 2008). Impulsivity and inhibitory control are usually considered different cognitive functions (Claes *et al.*, 2006; Jasinska *et al.*, 2012; Schachar *et al.*, 1993), but some studies have reported that impulsivity might be related to certain measures of inhibitory control (Enticott *et al.*, 2006). Specific procedures for studying inhibitory control in fish have also been developed, exploiting the response to transparent objects like those adopted in human infants, other primates and birds (Diamond, 1990). In the barrier test, the fish has to detour a transparent barrier to reach a group of conspecifics (Gatto *et al.*, 2018; Lucon-Xiccato and Bisazza, 2017b; Lucon-Xiccato *et al.*, 2017), which implies inhibiting the strong tendency to swim directly towards the target. However, the barrier test is likely affected by social motivation (Etheredge *et al.*, 2018; Lucon-Xiccato and Dadda, 2017), which varies among individual fish (Cattelan *et al.*, 2017; Pham *et al.*, 2012; Suriyampola *et al.*, 2016) and is altered by psychoactive substances (Araujo-Silva *et al.*, 2018; Fontana *et al.*, 2018). A second alternative procedure is the cylinder test (Keagy *et al.*, 2019; Lucon-Xiccato *et al.*, 2017; Lucon-Xiccato *et al.*, 2019; Santacà *et al.*, 2019). The fish is first trained to enter an opaque cylinder to find a food reward; upon learning, the fish is presented with food inside a transparent cylinder. The cylinder test is complex to execute and requires training each subject for several weeks. Moreover, it is potentially affected by olfactory cues that can guide the fish to the entrance of the cylinder (Santacà *et al.*, 2019). In a third inhibitory control task developed for fish (hereafter, the 'tube task'), the subject is presented with live prey, brine shrimp nauplii, sealed inside a transparent tube. Reduction in

the number of attacks towards the unreachable prey is taken as a measure of inhibition. A prior study analysed in detail the behaviour of fish in this task (Lucon-Xiccato and Bertolucci, 2019). Control trials with an empty tube indicated that the tube task is not affected by neophilia. Moreover, features of habituation learning, such as increased learning speed with increasing the stimulation (Rankin *et al.*, 2009), did not affect the task.

Because of its characteristics, the tube task seems highly promising for applied research on inhibitory control. In the present study, we applied the tube task methodology to the zebrafish. Because the critical property of inhibitory control in humans is inter-individual variability, we also addressed whether zebrafish show individual and heritable differences. For this purpose, we compared different zebrafish strains and observed the performance of each individual twice, an approach that allows for identifying individual differences (Lucon-Xiccato and Bisazza, 2017c).

Materials and methods

Subjects and maintenance

We tested 36 adult (6 months old) wild-type zebrafish from three different strains: 16 zebrafish from a strain regularly bred in our laboratory at University of Ferrara ('Ariosto' strain), 10 AB, and 10 Tubingen. We chose these three strains because they represent the most commonly used zebrafish in laboratory experiments. Ariosto zebrafish were descendant of fish bought from a local shop, and many laboratories use similar commercially available fish (e.g., Flynn *et al.*, 2016; Lima *et al.*, 2016). The Ariosto stock consists of approximately 500 individuals and was originated in 2011 (corresponding to at least 20 generations in the

laboratory) from 100 zebrafish. To keep the line outbred, reproductions were performed with zebrafish haphazardly selected from various maintenance tanks, and twice per year, we added 30-50 new zebrafish to the stock. AB and Tubingen strains are widely diffused in laboratories working on zebrafish, and they have been extensively used in genetic (e.g., Haffter *et al.*, 1996; Wakamatsu *et al.*, 2019) and behavioural research (e.g., Mathur et al., 2011). The genome of zebrafish has been sequenced using the Tubingen strain (de Esch *et al.*, 2012; Howe *et al.*, 2013; Séguret *et al.*, 2016; Wright *et al.*, 2006). For each strain, half of the individuals tested were males and half were females. Before the experiment, we kept the zebrafish in standard tanks (60 × 40 × 35 cm) with water at 26 ± 1 °C, 12 h light-12 h dark photoperiod, and water filters. We daily provided the zebrafish with food flakes and live prey (brine shrimps, *Artemia salina*, nauplii).

Experimental procedures

Apparatus

We tested each zebrafish in a plastic tank (33 × 13 × 15 cm) filled with 4 L water and provided with green plastic walls, air stone for water oxygenation and heaters set at 26 ºC. We built 18 identical apparatuses to simultaneously test multiple subjects. Each subject was housed in the apparatus for the entire duration of the experiment. A transparent plastic lid placed over the tank prevented water evaporation. The lid presented a hole (Ø 1.2 cm) in proximity of one of the short sides (Figure 1). Fifty centimetres above each tank, we placed a strip of warm-white LED (photoperiod 12 h: 12 h), perpendicular to the long wall of the tank, and a Logitech webcam connected to a computer running custom-made recording software.

Habituation phase

We used a two-step procedure. During the initial phase (habituation), we trained the subject to receive food at one extremity of the tank for three consecutive days. On day 1, we moved an individual subject into the experimental tank and immediately delivered a small amount of crumbled flakes (the same used during maintenance) mixed with water by inserting a Pasteur pipette into the hole of the lid. We dispensed food until the zebrafish started to feed. We fed flakes rather than brine shrimps in the habituation phase because in a pilot experiment, we found that some of the prey escaped from the zebrafish and were consumed later. This might reduce the effectiveness of the habituation. After 1 h, we repeated this feeding procedure. On day 2 and day 3, we similarly fed the subject 4 and 6 times, respectively. However, we progressively started to release the food only when the fish spontaneously approached the pipette. Following habitation, fish usually learn to reach the pipette and feed from it within 5 s, which was used as a learning criterion for admission to the testing phase.

Testing phase

On day 4, we performed the second step of the procedure, the testing phase, in which we assessed inhibitory control. At 1000 h, we inserted a standard glass tube (length: 10 cm; Ø: 1.2 cm; Figure 1) filled with brine shrimps into the tank, through the hole in the lid. The tube was kept in place by a support and was suspended in the water column. This setting allowed the zebrafish to see the prey but prevented the use of non-visual sensory cues. A pilot

experiment has revealed that absence of food olfactory cues in the tank did not detectably affect fish performance, in line with studies based on food rewards (Santacà *et al.*, 2019). The brine shrimp nauplii used as the stimulus prey were a type of food routinely provided to zebrafish during maintenance. We presented 4 mL of solution containing approximately 500 brine shrimps. Because we assessed zebrafish behavioural changes, it was important to ensure that brine shrimps' activity and visibility were constant over the testing time. In a preliminary study, we recorded the behaviour of brine shrimps in the tube and we counted the number of times that one of them crossed the median line of the tube. We found that the activity was high in the first minute after insertion in the tube. Thereafter, the activity became constant, with approximately 120 brine shrimps crossing the median line (and therefore fully visible to the fish) each minute (Lucon-Xiccato and Bertolucci, 2019). Therefore, in the present experiment, we kept the brine shrimps in the tube 2 min for acclimation before presenting them to the subjects. After the tube was inserted, the camera recorded the zebrafish behaviour for 20 min. We performed a second trial after a 2-h interval (1100 h), with the same procedure. This second trial allowed us to study individual differences in inhibitory control with a correlation approach, as well as learning and memory. To score subjects' behaviour, we played back the recordings on a computer using the VLC media player (Videolan, https://www.videolan.org/vlc/index.html). To score inhibitory control, we counted the number of attacks towards the brine shrimps per each minute of the test. We considered as an attack every event in which the fish contacted the glass of the tube with its snout.

Statistical analysis

The dependent variable was the number of attacks performed by subjects in each minute of the test. We performed statistical analysis in R (version 3.4.0) using two-tailed tests and a significance threshold set at $P = 0.05$. In our main analysis, we used a generalised mixed-effects model with Poisson error structure (*glmer* function of the *lme4* R package; Bates *et al.*, 2014) because the dependent variable had a Poisson distribution. We fit the model with subject ID as a random effect and minute, trial, and strain as fixed effects. As evidence of inhibition, we expected to detect a decrease in the number of attacks within a trial and between trials (significant main effects of minute and trial). For post-hoc testing on the first minute of each trial, we similarly used generalised linear models fitted with strain as a fixed effect and Poisson error distribution. We used Pearson correlations (log-transformed data) to assess whether the individual performance in the first and second trial was related. For this analysis, we calculated the number of attacks per trial of each individual as the sum of number of attacks in each minute of the trial.

Ethical statement

Experiments followed the law of the country in which they were performed (IT D.L. 4 Marzo 2014, n. 26; EU Directive 2010/63/EU for animal experiments) and were approved by the Ethical Committee of University of Ferrara (protocol n. TLX 2-2018-PR). All subjects were laboratory reared and after the experiments were released in maintenance tanks. None of the subjects was injured or showed signs of distress.

Results

In the habituation phase, all the subjects but one Ariosto fish reached the learning criterion indicating that they accustomed to feed from the pipette. In the test phase, all the subjects attempted to capture the brine shrimps inside the tube, with 61.47 ± 59.06 attacks per individual.

The model to analyse the number of attacks revealed a significant main effect of minute (χ^2_1 = 1088.502, P < 0.001): the number of attacks decreased over the testing time (Figure 2). The effect of minute was also significant in a model run on the data of trial 1 only (χ^2_1 = 729.059, P < 0.001). In addition, there was significant main effect of trial (χ^2_1 = 178.867, P < 0.001): the number of attacks in trial 2 (21.75 ± 25.49; Figure 2b) was smaller compared to the number of attacks in trial 1 (39.72 ± 37.21; Figure 2a). However, in the first minute of trial 2, fish performed a number of attacks that was higher compared to the last minute of trial 1 (χ^2_1 = 3355.20, P < 0.001).

There was no significant main effect of strain (χ^2_2 = 4.825, P > 0.05). However, strain qualified the effects of minute and trial (strain × minute: χ^2_2 = 52.708, P < 0.001; strain × trial interaction: χ^2_2 = 17.759, P < 0.001). Therefore, the reduction of attacks varied according to subjects' strain (Figure 2). This effect was confirmed by post-hoc analysis: there was a strain difference in the number of attacks in the first minute of both trial 1 (χ^2_2 = 69.410, P < 0.001) and trial 2 (χ^2_2 = 112.070, P < 0.001; Figure 2). The minute × trial interaction and the three-way interaction were not significant (χ^2_1 = 2.308, P > 0.05; and χ^2_2 = 2.931, P > 0.05, respectively).

The correlation analysis showed a positive relationship between the number of attacks in the first trial and the number of attacks in the second trial in the Ariosto strain (r_{14} = 0.568,

$P < 0.05$; Figure 3) and in the Tubingen strain ($r_8 = 0.862$, $P < 0.01$; Figure 3). In the AB strain, we found a similar trend but the test did not reach the threshold for statistical significance ($r_8 = 0.585$, $P > 0.05$; Figure 3).

Discussion

We showed that zebrafish exhibit inhibitory control in a foraging context and that the tube task is suitable for studying this cognitive function and, potentially, learning and memory. Moreover, we detected substantial inter-individual variability in inhibitory control, suggesting that individuals and genetically separated strains with reduced inhibitory control are present in this species.

Zebrafish initially attempted to attack the prey sealed in the transparent tube. However, given the impossibility of reaching the prey, zebrafish showed a marked reduction of attack attempts over time. This reduction was particularly evident in the first trial. Subjects could not feed on the brine shrimps; therefore, satiation was not involved in this behavioural trend. The reduction in the number of attacks could therefore be ascribed to zebrafish inhibiting their foraging tendency (Lucon-Xiccato and Bertolucci, 2019). A similar behavioural trend has been observed in another teleost species, the guppy, *Poecilia reticulata*, (Lucon-Xiccato and Bertolucci, 2019) and in an invertebrate, the cuttlefish (Agin *et al.*, 1998; Cartron *et al.*, 2013).

Learning might have also played a role in inhibiting foraging behaviour. For example, zebrafish had to learn that the brine shrimps presented in the testing phase were somehow different from those usually administered during maintenance (i.e., they were not freely

available). Similar involvement of learning is often detected in experiments on animals' inhibitory control (Kabadayi *et al.*, 2017; Gatto *et al.*, 2018). The main approach to measure a specific cognitive ability, controlling for other factors, in human psychometry consists of analysing participants' scores in a battery of tasks (e.g., Enticott *et al.*, 2006). Batteries of cognitive tasks are less used outside humans, with the exception of a few studies on primates and birds (Beran and Hopkins, 2018; Shaw *et al.*, 2015). They are indeed time consuming and suffer carryover effects across the tasks. With this regard, we recently collected data in another teleost fish showing positive covariation between performance in the tube and in the cylinder task (Montalbano *et al.*, 2020). Accordingly, it might be possible to adopt a two-step approach in zebrafish: first, screen large populations with quick procedures such as the tube task; then, investigate in detail their specific cognitive functions with batteries of tasks.

The number of attacks in the second trial was significantly lower than that of the first trial. This finding suggests that zebrafish retained the learned inhibition, further strengthening the interpretation that the effect detected in the first trial was not a transitory change in motivation or other non-cognitive factors. In addition, it indicates that the present paradigm might allow for assessing memory in zebrafish (i.e., how long foraging inhibition lasts). Laboratory experiments in which zebrafish were exposed to the stimuli for 25 min (an interval of time comparable to that of the present study) have reported a memory window of 24 h (Lucon-Xiccato and Dadda, 2014). However, other studies have demonstrated that fish memory can last for more than 11 months (Brown and Warburton, 1997; Triki and Bshary, 2019).

The strain of zebrafish had a clear effect on the rate of inhibition learning: in the initial minute of the test, Ariosto zebrafish performed 15 attacks on average, whereas AB and Tubingen zebrafish showed approximately half as many attacks. There also was an effect on inhibition retention: in the second trial, AB and Tubingen zebrafish showed a reduced number of attacks since the early minutes of exposition, whereas Ariosto zebrafish showed a renewed high number of attacks. One may argue that differences in boldness or metabolism, which affects motivation, could explain the effect of strain. However, it should be noted that all fish admitted to the testing phase showed strong attraction to the food in the habituation phase, with no strain difference in motivation or boldness in approaching the food (i.e., only 1 fish from the Ariosto strain failed to meet the criterion). Similarly, a study in another teleost fish did not find a correlation between motivation to reach the food and inhibitory control performance (Lucon-Xiccato *et al.*, 2020). Physiological studies also agree with this interpretation because AB, Tubingen, and zebrafish obtained from pet shops show a standard decrease in whole blood glucose in response fasting, a measure of metabolism (Meyer *et al.*, 2013). Therefore, strain differences in the tube task are unlikely accounted by differences in boldness or metabolism. Because the lines of zebrafish tested are genetically separated (Meyer *et al.*, 2013), our result was most likely due to genetic differences in cognition between the strains. Literature provides similar evidence of behavioural and cognitive variability across zebrafish populations, including laboratory strains (de Esch *et al.*, 2012; Quadros *et al.*, 2016; Roy and Bhat, 2016; Roy and Bhat, 2018; Spence *et al.*, 2011). In particular, a systematic screening found differences in locomotion activity, startle, exploratory behaviour, and circadian rhythmicity between Tubingen and AB zebrafish

(Vignet *et al.*, 2013). One difference was also cognitive: AB fish learned a colour discrimination faster. However, it is not clear whether this learning difference contributed to the results of our study given that it did not involve discrimination. Overall, our result is promising for research on reduced inhibitory control: it suggests that it might be possible to detect significant differences in the tube task between mutant lines with a putative deficit in inhibitory abilities and control lines. The same could be true for groups treated with diverse psychoactive compounds that might affect inhibitory control.

We also found evidence of variability in inhibitory control within strains, at least in the Ariosto and Tubingen strains. For the AB strain, the statistic was not significant despite the relatively high correlation coefficient ($r > 0.5$). The tube test could allow for detecting subtle individual differences in inhibitory control of zebrafish. This paves the way to translation research aimed at understanding the molecular and genetic basis of reduced individual inhibitory control. Likewise, many studies have recently evidenced individual differences in other cognitive abilities in teleost fish, such as cognitive flexibility, problem solving, spatial abilities, and numerical abilities (reviewed in Lucon-Xiccato and Bisazza, 2017c). Future studies should assess whether zebrafish also possess these individual differences. The presence of cognitive variability in a species with high translational potential such as the zebrafish will certainly improve research on cognitive disorders.

The tube task applied to zebrafish presents several interesting features. It requires little time for habituating and enables assessment of inhibition learning in 20 min, far less time than other procedures based on training, such as the cylinder test. Most of the material used in the apparatus is commonly found in a fish laboratory. As the experimental tank, it is

possible to use a standard mouse cage; the stimulus prey was the food normally used in zebrafish facilities; and the tube to present the prey was a laboratory glass tube. For the video recording, standard, cheap webcams are suitable, and the computer does not require large computational power nor particular features. Consequently, the time and economic investment necessary for preparing this set-up is very low. Because of its simplicity, the tube task might be suitable for studying inhibitory control in very young zebrafish, even larvae, provided the apparatus is scaled appropriately. The cylinder test and the barrier test are less promising in this regard: the former seems too complex for larvae, and the latter requires subjects to be socially motivated, but during the larval stage, the sociality of zebrafish is still under development (Dreosti *et al.*, 2015). For all these reasons, the tube task might be used to understand the neurobiological basis of inhibitory control and to develop zebrafish models for pathologies related to reduced inhibitory control in humans (e.g., Gilmore *et al.*, 2013; Nigg, 2001).

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Contributions

Both authors developed the study concept and design, and approved the final version of the manuscript. T.L.-X. analysed the data and drafted the manuscript.

Significance statement

Inhibitory control is a cognitive function that enables blocking behaviour when it is not appropriate. Humans with reduced inhibitory control often suffer social and health issues. We demonstrated that the zebrafish, the fish most used as model for neurobiological disorders, possesses inhibitory control and that it can be assessed with a rapid test. Moreover, we found substantial individual differences in zebrafish inhibitory control. This paves the way to developing models for inhibitory control using zebrafish.

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Figure 1

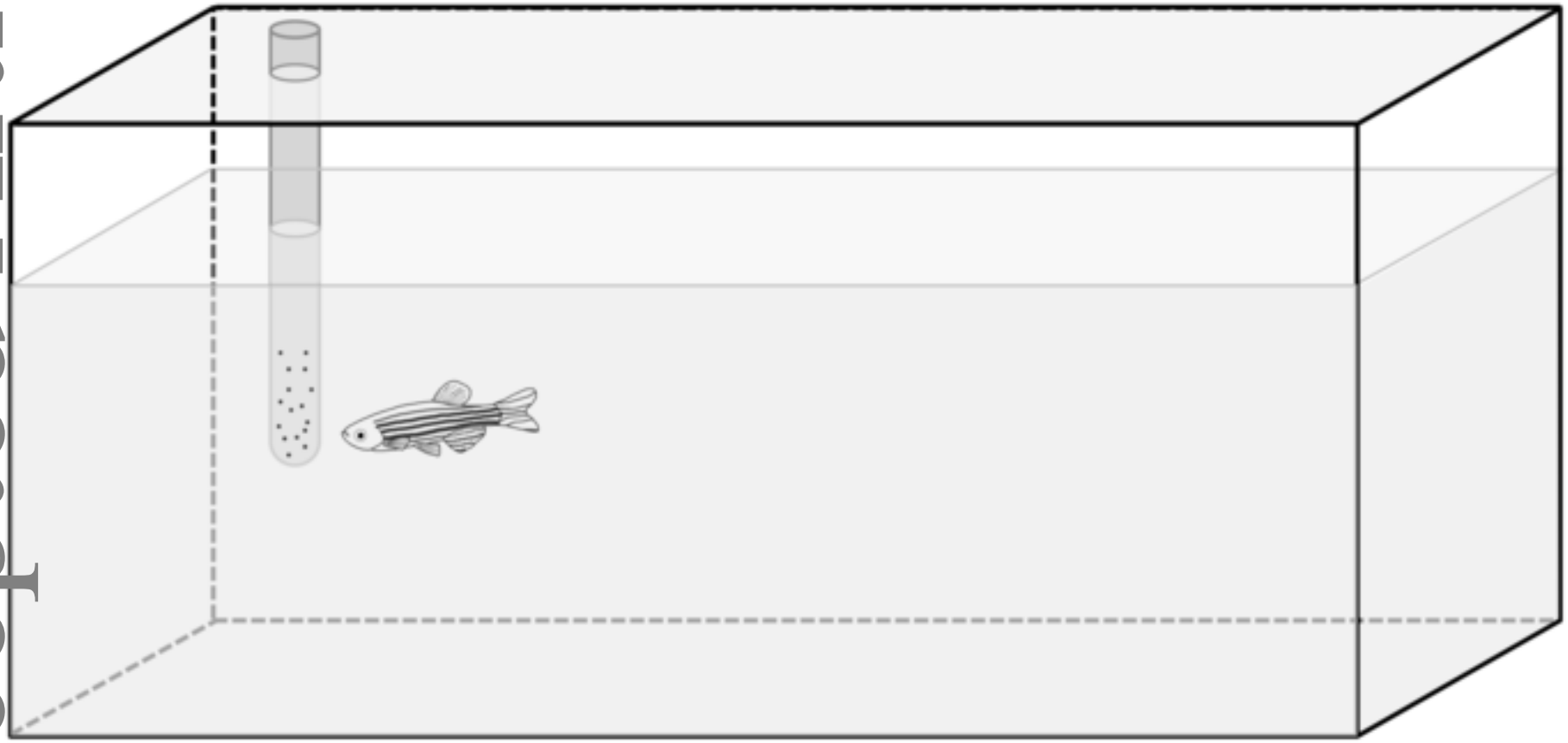
Experimental apparatus. Lateral view of the experimental apparatus with the tube containing the stimulus prey.

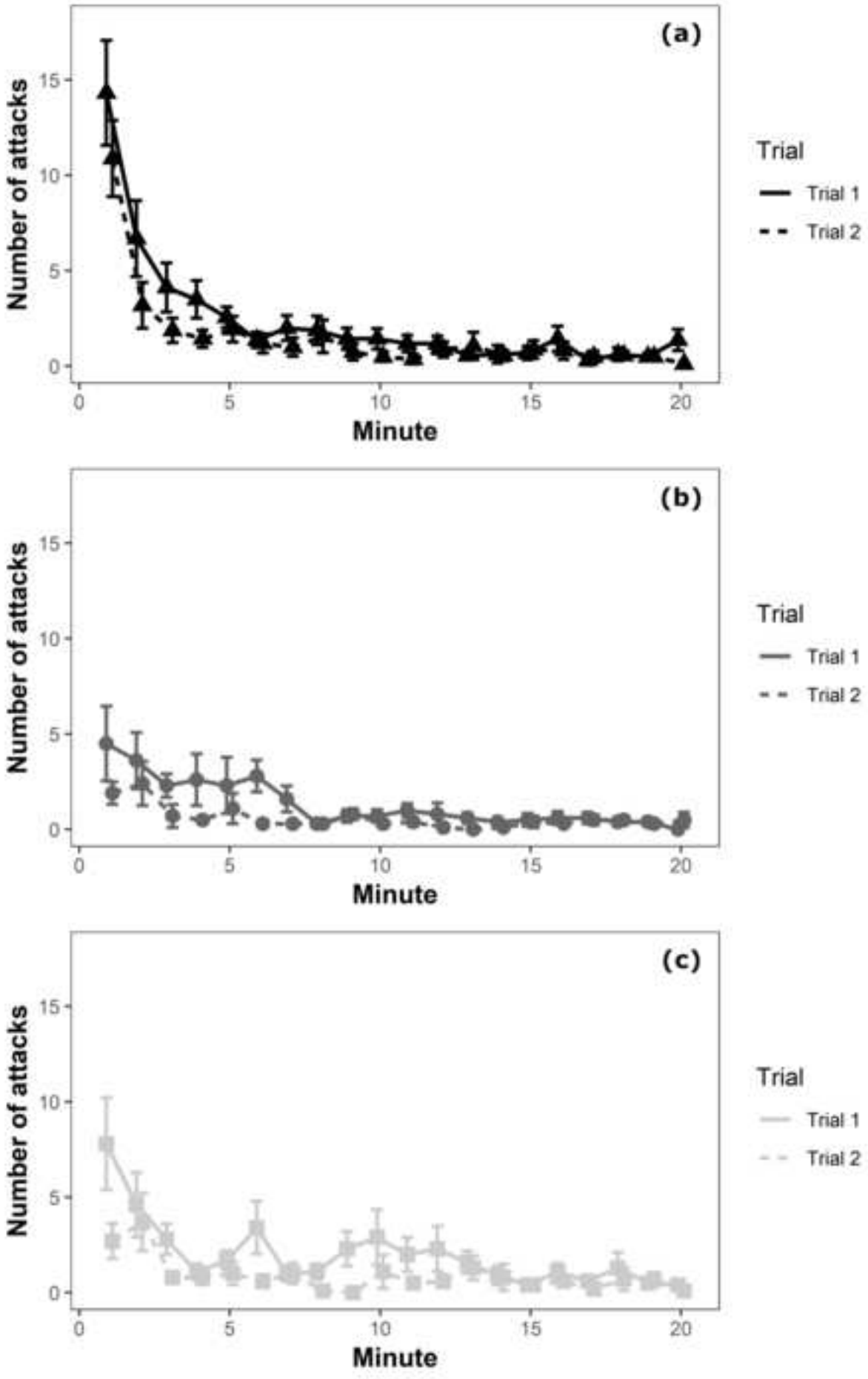
Figure 2

Inhibitory performance of the three zebrafish strains. Number of attacks of (a) Ariosto, (b) AB, and (c) Tubingen zebrafish the 20 min of the trial 1 (solid lines) the trial 2 (dotted lines). Dots represent means; error bars represent standard errors.

Figure 3

Individual differences in inhibitory control. Scatterplot of the number of attacks in trial 1 versus the number of attacks in trial 2. Lines represent regression lines computed for each strain separately.





Figure