

Plastic changes in brain morphology in relation to learning and environmental enrichment in the guppy (*Poecilia reticulata*)

Stephanie Fong^{1*}, Séverine D. Buechel¹, Annika Boussard¹, Alexander Kotrschal¹, Niclas Kolm¹

¹ Department of Zoology, Stockholm University, Stockholm, Sweden

*Corresponding author: Stephanie Fong

Department of Zoology, Stockholm University, Stockholm, Sweden

stephanie.fong@zoologi.su.se

Keywords: brain plasticity, cognition, reversal learning, spatial learning

SUMMARY STATEMENT

Modifications of the brain can occur as a form of adaptation to environmental conditions. Environmental enrichment was found to influence relative brain size in guppies.

ABSTRACT

Despite the common assumption that the brain is mainly malleable to surrounding conditions during ontogeny, plastic neural changes can occur also in adulthood. One of the driving forces responsible for alterations in brain morphology is increasing environmental complexity that may demand for enhanced cognitive abilities (e.g. attention, memory and learning). However, studies looking at the relationship between brain morphology and learning are scarce. Here, we tested the effects of both learning and environmental enrichment on neural plasticity in guppies (*Poecilia reticulata*), by means of either a reversal-learning test or a spatial-learning test. Given considerable evidence supporting environmentally-induced plastic alterations, two separate control groups that were not subjected to any cognitive test were included to account for potential changes induced by the experimental setup alone. We did not find any effect of learning on any of our brain measurements. However, we found strong evidence for an environmental effect, where fish given access to the spatial-learning environment had larger relative brain size and optic tectum size in relation to those exposed to the reversal-learning environment. Our results demonstrate the plasticity of the adult brain to respond adaptively mainly to environmental conditions, providing support for the environmental enhancement theory.

INTRODUCTION

Brain morphology shows remarkable variation both across and within species. Despite a massive research effort in the last decades to explain this variation (Herculano-Houzel et al., 2006; Isler and van Schaik, 2009; Reader and Laland, 2002; van Schaik et al., 2012), the processes and mechanisms that have generated this variation in brain size, brain subregion size, neuron number and interconnectivity patterns remain elusive. Hardwired adaptive genetic variation is one important explanation for some of this variation (Hibar et al., 2015; Kotrschal et al., 2013; Peper et al., 2007), but in the last decades, several studies have demonstrated that neural plasticity can also be highly important in generating variation in brain morphology (Fox et al., 2010; Gonda et al., 2009a; Gonda et al., 2009b; Iwaniuk and Nelson, 2003).

While plastic neural changes have been shown to be extremely pronounced during juvenile stages mainly due to the fact that the brain is still undergoing rapid growth (Knickmeyer et al., 2008), structural changes in the adult brain have also been shown to occur as an adaptive response to changing environmental conditions. For instance, the size of mushroom bodies in worker ants has been found to increase with the level of foraging activity (Gronenberg et al. (1996). There is therefore evidence contradicting the notion that brain morphology in adults is mainly hardwired, instead it retains at least some of its plastic ability to respond to external factors (Gonda et al., 2009b; Gonda et al., 2013).

The earliest evidence for plastic changes during adulthood stem from the manipulation of environmental conditions in rats (Bennett et al., 1964; Rosenzweig et al., 1962). Treatments were designed with varying degrees of both environmental and social complexity, and several brain subregions were compared across groups. Rats assigned to the most complex treatment (e.g. group housing in a large cage with toys, regular handling and exposure to various mazes) had the largest increase in weight of cerebral cortex after controlling for body size and genetic factors. The inclusion of two other treatments differing in the extent of stimulation further indicated that numerous facets of the environment could potentially alter brain architecture. Other instances of brain plasticity changes under artificial settings

include caching opportunities in marsh tits, which resulted in the enlargement of the hippocampus of birds allowed to cache relative to those that were not offered similar opportunities (Clayton and Krebs, 1994). The social environment has also been shown to exert a considerable influence with regards to plastic adaptations in brain morphology. The use of social cues, for example, has been extensively studied in nonhuman primates, where social network size has been found to be correlated with increases in grey matter (Sallet et al., 2011).

One particularly interesting aspect of plasticity in the adult brain is how learning can potentially affect brain morphology. Neuronal activation, as in the case of various forms of cognitive stimulation, is thought to be the main instigating factor supporting sustained enlargement of specific brain regions. In light of this, the use-it-or-lose-it hypothesis (Shors et al., 2012; Swaab, 1991) states that neuronal activation of the brain would result in consequent enhancements in brain morphology. Since cognition is costly, in particular when associated with changes in costly neural tissue (Aiello and Wheeler, 1995; Isler and van Schaik, 2006; Isler and van Schaik, 2009; Kotrschal et al., 2016; Kotrschal et al., 2013; Kozlovsky et al., 2014), it could be highly advantageous to avoid spending unnecessary energy on maintaining costly neural tissue when it is not needed, and instead show rapid increases in neural tissue after periods of increased usage. Learning-dependent changes in brain morphology have been demonstrated in humans (Driemeyer et al., 2008; Maguire et al., 2000; Scholz et al., 2009), macaques (Quallo et al., 2009) and rodents (Blumenfeld-Katzir et al., 2011; Lerch et al., 2011). However, more studies are needed to test the generality of the use-it-or-lose-it idea in non-mammalian species.

The guppy (*Poecilia reticulata*) is a suitable model for studies of brain morphology plasticity. As is the case with many fishes, guppies experience continued growth and prolonged neurogenesis throughout their entire lifespan. The occurrences of adaptive plastic responses in neural architecture even in adult animals is therefore not unexpected (Ekström, 1994; Gonda et al., 2009b; Kihlslinger and Nevitt, 2006; Sørensen et al., 2013; Zupanc, 2006). Such plastic responses, where present, have also been shown to occur rather rapidly. In a study by Burns et al. (2009), a reduction in total brain size was evident after a single generation of domestication in the lab. The use of guppies to investigate adaptive neural alterations over a

short period of time could therefore potentially augment gaps in our understanding of the factors driving plastic changes in brain morphology.

Here, we adopt a cross-sectional approach to examine whether successful learning in various cognitive tests would lead to the enlargement of corresponding brain regions associated with each learning task. For this, we included matched controls for each task in order to account for plastic alterations in brain morphology that could be attributed to environmental enrichment effects, rather than cognitive processes. For instance, the addition of small stones to the rearing tanks of salmon juveniles was found to result in the growth of larger cerebella in these fish (Kihlslinger and Nevitt, 2006). Such environmental enrichment effects are common in vertebrates (e.g. (Gonda et al., 2013) and are thus important to control for. Two cognitive assays, a reversal-learning test and a spatial-learning task, were chosen with the intention of assessing separate cognitive traits. Absolute and relative brain size in addition to six major brain sub regions (the olfactory bulbs, telencephalon, optic tectum, cerebellum, hypothalamus and dorsal medulla) were quantified with the objective of testing for plastic changes in neural architecture as a consequence of learning. Ultimately, we wanted to investigate whether and how structural plasticity changes in the brain would occur in response to learning while controlling for environmental enrichment effects.

MATERIALS AND METHODS

Animals

We used guppies from a wild type laboratory-kept population, housed in 200 l aquaria of mixed sex ratios. The fish originated from a high-predation population that had been kept at high population size in Trondheim University for multiple generations, maintaining high genetic diversity (Kotrschal et al., 2013). 80 female guppies were netted out and individually housed in 4 l aquaria with a layer of light-coloured gravel ranging from 0.20 to 1 cm³ in volume, constant aeration, freely-floating java moss (*Taxiphyllum sp.*) spanning approximately 10% of the volume of each tank and > 5 water snails (*Planorbis sp.*) to eliminate organic waste. Tanks were arranged in rows of 6 on each shelf, such that the animals had visual contact with two other individuals on either side in order to minimize

potential stress brought about by social isolation. The visibility of fish placed in tanks at either ends of the shelf was limited to one neighbour in the adjacent tank. To avoid any biases in social stimuli prior to the experiments, individuals in different treatments were equally distributed from both types of tank positions. The Laboratory conditions were maintained to provide a stable water temperature of 25 ± 1 °C under a 12 h light/ 12 h dark photoperiod. Fish were fed with flake food and live *Artemia* (brine shrimp) hatchlings six days of the week. Only sexually mature female guppies were used in the study.

Prior to the start of any experimental procedures, fish were randomly allocated into either of two experiments, reversal-learning (n = 40) or spatial-learning (n = 40). Within the reversal-learning test, we further divided the animals into a treatment group (n = 20) and an environment control group (n = 20). Similarly, fish placed in the spatial-learning test were equally distributed into a treatment (n = 20) and an environment control group (n = 20) (see Fig. 1 for graphical presentation of the different treatments). Animals in the learning environment control groups were treated identically as their respective treatment groups in terms of housing and handling, with the exception of not being trained in each task. This was to test for and control for possible plastic changes in neural anatomy generated by external influences, such as environmental enrichment effects due to exposure to the learning environment itself.

Reversal-learning treatment

Female guppies assigned to the reversal-learning group (n = 40) were equally divided into two separate batches and housed individually in experimental tanks as described by Buechel et al. (2018). Briefly, experimental tanks consisted of a home compartment (25 x 15 cm) and an experimental compartment (15 x 15 cm) at the front of the tank, separated by two guillotine doors, one transparent and one opaque (Fig. 2). Fish were confined to the home compartment outside of test trials, where they had visual contact with individuals in neighbouring tanks to minimize potential stress brought about by social isolation. Visual contact during trials, however, was prevented to avoid any social learning effects. The experimental compartment contained a white plate with 20 identical circular holes (5 mm deep, 10 mm diameter). Frozen *Artemia* were placed in two of the holes, separated by one empty hole in between, and covered with a red or yellow plastic disc (14 mm in diameter).

The designated unrewarded stimulus could not be displaced due to the adherence of a rubber tube under the plastic disc, while the rewarded stimulus could easily be moved by the fish. A food reward was placed under both the rewarded and unrewarded discs to control for olfactory cues. The rewarded colour was counterbalanced across subjects in each treatment group and batch. Half of the animals were first trained to associate red with the reward and the other half were trained on yellow, controlling for any variation in colour bias present in this species. Under natural conditions, female guppies often search for potential prey covered by leaves at the bottom of the rivers, and hence this tendency to displace the discs is part of their innate behavioural repertoire and thus highly ecologically relevant (Houde, 1997).

During the initial training phase, females in the treatment group were first trained to dislodge a green disc in order to gain access to the hidden food reward. Green was chosen as female guppies were shown to display a strong preference for this colour in a previous colour preference test. Fish in the reversal-learning environment control group were kept naïve to the training but were fed similar amounts of frozen *Artemia* as the treatment group with the use of a pipette. Once individuals had learnt to displace the green discs, they were next trained in the colour association phase (i.e. the forward learning phase), whereby either a red or yellow disc was associated with the food reward. The horizontal position of the rewarded disc was randomly assigned for each trial, whereby the rewarded disc was in the same position for no more than two consecutive trials. The first choice made by the fish, defined as any attempt to displace a disc with the snout or mouth, was recorded for each trial. An individual was given up to 5 min to make a choice, after which the rewarded disc was partially opened and the fish was allowed to consume the food. This was done to ensure that all individuals had the same experience associating the specific coloured disc with a food reward but was still noted as a non-choice response. Fish were tested in blocks of 3 trials, up to a maximum of 2 blocks in each given day. This meant that individuals could receive a maximum of 6 trials per day. Since individuals received the food reward at the end of the trial regardless of the first choice made, this ensured that the fish remained motivated in the tests. We randomly allocated either 1 or 2 blocks to each test day, where a maximum of 6 trials per day was permitted for no more than three consecutive days. In total, individuals received 30 trials for this phase.

After all individuals had completed 30 trials, the reward contingency was reversed and training for the reversal-learning phase began. Guppies previously trained on red as the rewarded stimulus in the forward-learning phase now had to displace the yellow disc in order to obtain the food reward, and vice versa. Similar to the forward phase, individuals were allowed up to 5 min to make a choice, and their first choice was noted as correct or as incorrect. A total of 66 trials were presented to the fish in the reversal phase, at which point at least 85% of all individuals had reached the pre-defined 70% learning criterion. In other words, individuals had to get a minimum of 7 out of 10 trials correct, over 6 consecutive blocks of 10 trials. This specific criterion was chosen in order to increase our sample size while keeping experimental times down. All individuals received the exact same number of trials for both the forward (30 trials) and reversal phase (66 trials). However, due to time constraints, the total duration of the entire test for batch one and two took 22 and 19 days, respectively.

Following the completion of the reversal-learning test, fish were euthanized in a water bath containing an overdose of benzocaine (0.4 g/L), and fixed with 4% paraformaldehyde buffered in phosphate buffer saline (PBS) for five days. The fish were then washed twice in PBS and stored in 4°C awaiting dissection.

Spatial-learning treatment

Prior to the experiment, animals were housed individually in 4 l aquaria as described above, and had visual access to fish in neighbouring tanks. Fish in the spatial-learning treatment group were not fed outside of the test to ensure sufficient motivation to forage and perform during the spatial-learning cognitive task. Similar to the reversal-learning environment control group, individuals assigned to the spatial-learning environment control group were fed in their home tanks at the end of the day, thus minimizing the possibility of any positive reinforcement within the maze. The maze was constructed from white acrylic with the dimensions 100 x 50 cm. The walls were opaque to prevent external disturbances and the floor of the maze was lined with 1 cm of light coloured gravel and water depth was kept at approximately 10 cm. The inclusion of two dead-ends in the maze allowed for a separate measure of cognitive performance by quantifying the number of entries into dead-ends (i.e.

number of errors) as well as the time spent within dead-ends (Fig. 2). Between three to five Frozen *Artemia* hatchlings were placed on a small plastic petri dish (6 cm in diameter) located in the goal compartment (Fig. 3B). In order to minimize the influence of olfactory cues on performance, the water used in the preparation of the frozen *artemia* was evenly sprinkled throughout the maze to homogenize the maze in terms of food smell prior to the start of trials. Females were tested once per day over 14 consecutive days, giving a total of 14 trials per fish. To ensure that individuals had approximately 24 h between each trial, the order in which they were tested was maintained. Fourteen trials were chosen because a previous experiment on male guppies had shown that this was sufficient for the animals to learn to navigate through a similar maze while maintaining relevant variation between individuals (Kotrschal et al., 2015b). Trials commenced every morning at 08:30, where an individual was netted from its home tank and placed in a transparent Perspex ring at the start of the maze. A 60 s acclimation period was allowed following transfer. At the end of the acclimation period, the ring was lifted and the individual was given a maximum of 15 min to complete the maze. The fish was noted to have completed the maze once half its body length crossed the final barrier, giving it access to the food reward. Individuals that did not manage to navigate through the entire maze within the allocated 15 min were gently guided to the end with the use of a hand net and given 2 min to consume the food, but were assigned the maximum completion time of 15 min for subsequent statistical analyses. Fish were then transferred back to their home tanks.

Trials were recorded through the use of an overhead video camera, and behaviours were scored using the behavioural observation software, BORIS (Friard and Gamba, 2016). As in the case of the reversal-learning treatment, fish were euthanized in a similar manner one week following completion of the trials. (see Fig. 1 for step-wise progression of tests for each group).

Brain region quantification

The standard length of each individual (from the tip of the snout to the end of the caudal peduncle) was measured to the nearest 0.01 mm using digital callipers. Whole brains were removed under a dissecting microscope (Leica MZFLIII) and images of the brain from four different views (dorsal, right lateral, left lateral and ventral) were taken with a digital camera

(Leica DFC 490). The length, width and height of six major brain regions (i.e. telencephalon, optic tectum, cerebellum, dorsal medulla, hypothalamus and olfactory bulb) were quantified from the digital images using imageJ software (Schindelin et al., 2012). All dissections and measurements were carried out by one person (SF) to minimize inter-observer variability. Given that individuals could only be identified by their numbers, the experimenter remained blind as to the treatment of each individual. Volume of individual brain regions was quantified via the ellipsoid model (Pollen et al., 2007; White and Brown, 2015b):

$$V = (L \times W \times H) \frac{\pi}{6}$$

Total brain volume was subsequently determined by the addition of the volume of all six major brain regions.

Data analysis

To first assess that individuals assigned to the reversal-learning treatment had successfully learnt both the colour associative and reversal-learning phase, we fitted two separate generalized linear mixed models (GLMM) with response as the dependent variable; trial as the fixed effect and individual as a random effect (lme4 syntax for R model_{forward-learning}: `response (correct/incorrect first choice) ~ trial (1-30) + (1 + fish | fish)`, family = binomial, lme4 syntax for R model_{reversal-learning}: `response (correct/incorrect first choice) ~ trial (1-66) + (1 + fish | fish)`, family = binomial). Similarly, to determine performance in the spatial-learning test, we used a linear mixed model (lmer) with completion time as the dependent factor; trial and batch as fixed factors, and fish as a random factor (lme4 syntax for R model: `completion time ~ trial + batch + (1 + fish | fish)`).

In female guppies, body size tends to be indicative of their age. Since sexually mature females of indeterminate age were randomly assigned to the different treatment groups, we wanted to test for any difference body in size across treatments. An analysis of variance (ANOVA) was performed with standard length as a response variable and treatment group as a fixed effect (lm syntax for R model: `standard length ~ treatment`).

In order to examine for any treatment effects on brain morphology, we used an analysis of covariance model (ANCOVA) across all 4 treatment groups, with total brain size or brain subregions as dependent variables. We were only concerned with the comparison of relative

brain size and relative size of the various subregions across treatments. For total brain size, standard length was included in the model as the covariate. As for the separate analysis of each brain subregion, rest of the brain was the only covariate included. The covariate, 'rest of the brain', was specific to each brain subregion analyzed, and was determined based on the total brain volume excluding the volume of the specific region of interest. Batch was included as a fixed factor and treatment effects were averaged across the two batches using the package emmeans (Lenth, 2018). (lm syntax for R model: absolute brain size ~ standard length + treatment group * batch; sub region ~ rest of the brain + treatment group * batch). Non-significant interaction effects ($p > 0.05$) were excluded from the final model.

When treatment effects were found to be significant, post-hoc tests were performed on all possible pairwise comparisons, where p-values were calculated following Holm's adjustment for multiple comparisons. To determine whether learning in either of the cognitive test had an effect on brain morphology, brain measures were specifically compared within each test (i.e. reversal-learning environment control vs. reversal-learning treatment; spatial-learning environment control vs. spatial-learning treatment). To investigate the possibility that a more stringent criterion of 80% in the reversal learning phase could have potentially revealed learning-induced plastic changes in brain morphology, we performed additional comparisons of all brain subregions including only the fish that had achieved the higher criterion. Effects of the learning environment were analysed by comparing the brain morphology of individuals in the reversal-learning environment control vs. spatial-learning environment control, and reversal-learning treatment vs. spatial-learning treatment. All brain measures and body size were log-transformed prior to analysis. Analyses were performed using R statistical software v3.5.1 (Team, 2015).

RESULTS

Individuals successfully learnt to associate either coloured disc with a food reward in both the forward learning (GLMM; trial: $z_{1,30} = 7.85$, $p < 0.001$) and reversal phase of the reversal-learning test (GLMM; trial: $z_{1,66} = 15.3$, $p < 0.001$). End performance levels (\pm s.d.) were comparable across individuals in both the forward-learning phase ($89 \pm 1\%$) and in the reversal-learning phase ($82 \pm 0.8\%$).

The significant decrease in time spent navigating through the spatial-learning setup across trials was indicative of learning across trials (GLMM; trial: $t_{1,14} = -9.51$, $p < 0.001$). The frequency of errors made (i.e. entries into the dead ends) also significantly decreased over trials (GLMM; trial: $z_{1,14} = -5.68$, $p < 0.001$).

There was no significant difference in body size across treatments (GLM: treatment $F_{3,74} = 2.18$, $p = 0.0979$), indicating that females were of similar age. We found an overall treatment effect on relative brain size across all treatment groups ($F_{3,73} = 7.33$, $p < 0.001$). Post-hoc pairwise comparisons, however, indicated the absence of any learning effects on brain morphology within each cognitive test (reversal-learning: $t_{73} = -0.885$, $p = 0.758$; spatial-learning: $t_{73} = -0.849$, $p = 0.758$). In contrast, we found a strong effect of the learning treatment environment on relative brain size ($t_{73} = -3.30$, $p = 0.0074$). Pairwise comparisons revealed that fish in the spatial-learning group (i.e. both treatment and control groups) had overall larger relative brain size than those in the reversal-learning group (refer to Table 2; Fig. 4A).

Similar to relative brain size, no significant differences in any of the relative major brain subregions was observed between the treatment groups and the control groups within each test (refer to Table 1). Additionally, we did not detect any significant effect of learning on brain anatomy in individuals that achieved a higher end performance level in the reversal learning experiment (all p -values > 0.15). We did, however, find a learning environment effect on relative optic tectum size. Individuals exposed to the spatial-learning environment tended to have a larger relative optic tectum size compared to those placed in the reversal-learning test (Table 2; Fig. 4B).

DISCUSSION

We did not find any support for plastic changes in brain morphology during learning in either the reversal-learning or spatial-learning tasks. However, we found a plastic response in relative brain size and relative optic tectum size which both increased in the spatial-learning environment independent from learning. Our results thus suggest that the effects of the physical environment are more important than the effects of learning for rapid plastic changes in brain morphology. We discuss the general implications of these results in more detail below.

The lack of change in brain size or brain morphology in response to learning means that plastic changes do not always occur even after very cognitively challenging tasks. It appears that such short-term skill acquisition, as was the case for each of our cognitive tests, was insufficient to generate visible plastic changes in brain size and brain subregion sizes, at least in our study species. Based upon improvement rates and end performance levels, we are confident that individuals actually learnt each task and hence neural processes important in learning and memory were likely activated during the training. The absence of any evident plastic effects on brain morphology could thus not be explained by lack of activation of cognitive processes during the learning experiments. But why did we not detect any effects of learning on brain morphology in our experiments then? Learning-related plasticity changes in brain morphology have thus far been investigated in only a handful of species, including humans (Boyke et al., 2008; Draganski and May, 2008; Maguire et al., 2000), rodents (Blumenfeld-Katzir et al., 2011; Lerch et al., 2011) and avian species (Patel et al., 1997). The bulk of these studies involve a longitudinal design; where training durations ranged from 5 days to 3 months. Studies involving animal models generally utilize a training regime that fall between 5 days in rodents and 14 days in macaques. Human studies, on the other hand, tend to adopt training schedules spanning over an extended period of time. For cases pertaining to the acquisition of a new skill such as juggling, or a period of extensive learning, studying for a medical examination for instance, individuals were permitted a training/learning duration of approximately 3 months. In the current study, individuals assigned to the reversal-learning treatment group underwent a total of between 19 and 22 days of training whilst those in the spatial-learning treatment group were trained over 14 days. Given that the time allocated for each of the cognitive tasks falls within the range

normally utilized by aforementioned studies, we therefore expected our test duration to be sufficiently long enough for plastic changes in brain morphology to occur.

The perhaps most parsimonious explanation is that all animals used in the experiment had enough neural capacity to learn the cognitive tasks presented to them in the time frame of the study. The cognitive tasks used here were explicitly chosen to be ecologically relevant to provide measurements of naturally occurring cognitive challenges. Under natural conditions, spatial ability is an important aspect of fitness for guppies in terms of encountering and remembering foraging sites, predation avoidance as well as territorial defence (Burns and Rodd, 2008). This is especially crucial for individuals living under conditions of high predation, where there is a significant trade-off between time spent foraging and risk of exposure to predation (Botham et al., 2008). The associative learning task is a variant of spatial learning that is also common in the natural habitat of the guppies since foraging is frequently done under leaves and debris on the bottom of streams (Houde, 1997). The reversal-learning aspect of the experimental design could be representative of a situation in which resources are suddenly depleted from a frequently visited foraging patch, forcing the animal to locate an alternative food source. Hence, if the static part of the guppy brain architecture is sufficient to solve these cognitive tasks, plastic changes may not have been required. This is in contrast to previous studies demonstrating learning-induced plastic changes in neural architecture, which involved tasks that are not part of the natural behavioural repertoire of the species examined. Learning how to use tools in order to obtain a food reward, though frequently employed in studies examining level of innovativeness, is not a typical behaviour exhibited by macaques (Quallo et al., 2009). It is possible that the nature of the tasks implemented in other studies deviate further away from the natural behavioural patterns exhibited by the respective species tested, which therefore required a larger extent of neural reorganization. Along the same lines, it is plausible that the tests implemented in our study were not as cognitively challenging as initially anticipated. Even though spatial learning has been shown to be an important aspect in fish, a certain degree of complexity may be missing in our spatial learning test, given that the start and end positions remain the same throughout the entire experiment. Hence, guppies could have simply utilized an egocentric strategy in order to navigate to the goal compartment after the first few trials. In the case of the reversal learning experiment, forward learning in the form of

distinguishing between two distinct colours could be a fairly easy task. Taking into consideration the natural behaviour of wild guppies, females are often faced with situations involving some aspect of colour discrimination as in the case of foraging and mate choice (Lucon-Xiccato et al., 2019). Given this innate ability to distinguish between colours, successful learning in the associative learning phase may not necessarily require extensive cognitive capabilities. However, the reversal learning phase entails a certain degree of behavioural flexibility, whereby individuals have to learn to repress a previously-rewarded behavior. Previous studies that have adopted this paradigm have shown that this ability to flexibly withhold a particular behaviour tend to be cognitively demanding. We had therefore expected successful learning in the reversal learning phase to be sufficiently challenging to induce plastic changes in brain morphology.

Another possible explanation for the lack of evident plastic changes in brain morphology in response to cognitive tasks could be due to the nature of changes elicited by different learning processes. For instance, plasticity changes in fine-scale neural structure, where present, may not be perceptible at the gross anatomical level investigated here. Studies focusing on learning-induced plastic changes in adult human brains have mainly utilized voxel based morphometry (Draganski et al., 2004; Maguire et al., 2000), a procedure that permits the detection of subtle changes in grey matter. Alterations in underlying neurological processes, such as changes in neuronal density or modified synaptic connections, could be occurring without any consequent changes in overall brain size or in brain subregions. In the study by Lema et al. (2005), the authors noted an increased in rate of cell proliferation without any ensuing changes in the volume of the telencephalon. The employment of more advanced imaging techniques such as classic sectioning or x-ray microscopy could offer a more in-depth insight into more subtle changes undergoing at the cellular level. Ideally, future studies should expand sample sizes and consider the incorporation of additional techniques such as histological staining, microdissections with gene expression analyses, and qPCR analysis to provide a clearer picture regarding underlying neural modifications induced by learning. Employing such a range of techniques would reveal a broader range of potential changes at the neural structure level, for instance rewiring of synapses.

Interestingly, we found a substantial effect of the physical test environment on relative brain size. Fish exposed to the spatial-learning setup had significantly larger relative brain size in comparison to their conspecifics placed in the reversal-learning setup. Navigating through a spatial maze constitutes a form of complex behaviour that requires the integration of multiple pieces of information, (Salvanes et al., 2013), which could account for the larger brains and the larger, albeit non-significant, relative optic tectum size in fish exposed to the spatial-learning setup. This could also be the result of the considerably larger area that individuals had to swim through in order to obtain the food reward in the spatial-learning setup relative to the experimental compartment in the reversal-learning test. Moreover, fish assigned to the reversal-learning group resided in the same aquarium that they were subsequently tested in, and hence the structural enhancement of their environment was not as substantial as that encountered by individuals in the spatial-learning task. The fact that we found an effect of the spatial-learning environment on relative optic tectum size but not relative telencephalon size suggests that the reliance on visual cues may have been sufficient for successful performance. As previously demonstrated by White and Brown (2015a), species living in environments of relatively low complexity had larger optic tecta, which was attributed to the heavy emphasis on visual cues in foraging and predation avoidance. It could be argued that fish placed in the spatial-learning setup might have experienced accelerated growth rates and correspondingly larger brains due to higher activity levels. This activity-growth correlation was found in Atlantic salmon placed under moderate water velocity levels in comparison to conspecifics under conditions of low water velocity (Nilsen et al., 2019). Although the authors were able to establish that higher activity levels prompted increased growth in these fish, the study extended over a duration of minimum duration of 46 days. In light of the fact that our guppies were placed in the spatial-learning setup only 15 minutes daily for a total of 14 days, we do not expect such short-term exposure to have drastically affected their growth rates. We therefore propose that the observed larger relative brain size and the trend for a larger optic tectum in the spatial-learning environment is the result of environmental enhancement effects rather than accelerated growth per se.

The evident strength in the plastic response to the physical environment as opposed to the cognitive tasks could also mean that the mechanisms behind plastic changes in brain morphology are responding mainly to environmental cues (Gonda et al., 2011). It could for instance be such that the level of environmental complexity is always strongly linked to the level of cognitive demands in wild populations in the guppy. The main cognitive challenges for wild guppies include avoiding predation, finding food and finding a mate (Burns and Rodd, 2008). All these three aspects have been previously demonstrated to depend on brain size and cognitive ability (Buechel et al., 2018; Corral-López et al., 2017; Kotrschal et al., 2015a; van der Bijl et al., 2015). And variation in environmental factors are often strongly associated with predation levels (Barbosa et al., 2018; Kotrschal et al., 2017; Templeton, 2004), food resources (Grether et al., 2001; Krause and Liesenjohann, 2012) and mating opportunities (Kelly et al., 1999) in wild guppy populations. Hence, it could be that the potential plasticity in the brain in these types of small-brained vertebrates need only target environmental variation, and this is the reason for the strong environmental effects and lack of response to learning treatments in our study. Although our results clearly show stronger environmental effects on plastic changes in brain morphology as compared to any effects from learning, we note that we did not include a control without both the learning test and the environmental treatment. This could have facilitated the dissociation also of small plastic changes in brain morphology elicited by learning, and would be valuable to add in future studies, in particular for studies that use high resolution methods to quantify brain anatomy. Investigations into the role of environmental factors and learning induced changes in neural anatomy within a single study are currently lacking in studies of brain plasticity modifications. Although it stands to reason that both aspects are likely to contribute to any plastic alterations in brain morphology, the influence that each has on plastic changes may not necessarily be equal. Future efforts should aim to distinguish the effects brought about by environmental versus learning factors, ultimately uncovering the mechanisms behind plasticity in brain structure and function.

Ethics

The experiment was performed in accordance with ethical applications approved by the Stockholm Animal Research Ethical Permit Board (Dnr: N173/13 and 223/15).

Acknowledgements

We thank Wouter van der Bijl for valuable comments and discussion on the manuscript, and Anna Rennie for assistance with animal housekeeping.

Competing interests

The authors declare no competing interests.

Funding

This project was funded by grants to N.K. from the Swedish Research Council (grant 2016-03435) and Knut and Alice Wallenberg Foundation (grant 102 2013.0072).

Data availability

All data will be available on dryad upon acceptance.

REFERENCES

- Aiello, L. C. and Wheeler, P.** (1995). The expensive-tissue hypothesis: the brain and the digestive System in human and primate evolution. *Current Anthropology* **36**, 199-221.
- Barbosa, M., Deacon, A. E., Janeiro, M. J., Ramnarine, I., Morrissey, M. B. and Magurran, A. E.** (2018). Individual variation in reproductive behaviour is linked to temporal heterogeneity in predation risk. *Proceedings of the Royal Society B: Biological Sciences* **285**.
- Bennett, E. L., Diamond, M. C., Krech, D. and Rosenzweig, M. R.** (1964). Chemical and anatomical plasticity of brain. *American Association for the Advancement of Science* **146**, 610-619.
- Blumenfeld-Katzir, T., Pasternak, O., Dagan, M. and Assaf, Y.** (2011). Diffusion MRI of structural brain plasticity induced by a learning and memory task. *PLOS ONE* **6**, e20678.
- Botham, M. S., Hayward, R. K., Morrell, L. J., Croft, D. P., Ward, J. R., Ramnarine, I. and Krause, J.** (2008). Risk-sensitive antipredator behavior in the trinidadian guppy, *Poecilia reticulata*. *Ecology* **89**, 3174-3185.
- Boyke, J., Driemeyer, J., Gaser, C., Büchel, C. and May, A.** (2008). Training-induced brain structure changes in the elderly. *The Journal of Neuroscience* **28**, 7031.
- Buechel, S. D., Bousard, A., Kotrschal, A., van der Bijl, W. and Kolm, N.** (2018). Brain size affects performance in a reversal-learning test. *Proceedings of the Royal Society B: Biological Sciences* **285**.
- Burns, J. G. and Rodd, F. H.** (2008). Hastiness, brain size and predation regime affect the performance of wild guppies in a spatial memory task. *Animal Behaviour* **76**, 911-922.
- Burns, J. G., Saravanan, A. and Helen Rodd, F.** (2009). Rearing environment affects the brain size of guppies: lab-reared guppies have smaller brains than wild-caught guppies. *Ethology* **115**, 122-133.
- Clayton, N. S. and Krebs, J. R.** (1994). Hippocampal growth and attrition in birds affected by experience. *Proceedings of the National Academy of Sciences* **91**, 7410.
- Corral-López, A., Bloch, N. I., Kotrschal, A., van der Bijl, W., Buechel, S. D., Mank, J. E. and Kolm, N.** (2017). Female brain size affects the assessment of male attractiveness during mate choice. *Science Advances* **3**.
- Draganski, B., Gaser, C., Busch, V., Schuierer, G., Bogdahn, U. and May, A.** (2004). Changes in grey matter induced by training. *Nature* **427**, 311.
- Draganski, B. and May, A.** (2008). Training-induced structural changes in the adult human brain. *Behavioural Brain Research* **192**, 137-142.
- Driemeyer, J., Boyke, J., Gaser, C., Büchel, C. and May, A.** (2008). Changes in gray matter induced by learning—revisited. *PLOS ONE* **3**, e2669.
- Ekström, P.** (1994). Developmental changes in the brain-stem serotonergic nuclei of teleost fish and neural plasticity. *Cellular and Molecular Neurobiology* **14**, 381-393.
- Fox, S. E., Levitt, P. and Nelson III, C. A.** (2010). How the timing and quality of early experiences influence the development of brain architecture. *Child Development* **81**, 28-40.
- Friard, O. and Gamba, M.** (2016). BORIS: a free, versatile open-source event-logging software for video/audio coding and live observations. *Methods in Ecology and Evolution* **7**, 1325-1330.
- Gonda, A., Herczeg, G. and Merilä, J.** (2009a). Adaptive brain size divergence in nine-spined sticklebacks (*Pungitius pungitius*)? *Journal of Evolutionary Biology* **22**, 1721-1726.
- Gonda, A., Herczeg, G. and Merilä, J.** (2009b). Habitat-dependent and -independent plastic responses to social environment in the nine-spined stickleback (*Pungitius pungitius*) brain. *Proceedings of the Royal Society B: Biological Sciences*.

Gonda, A., Herczeg, G. and Merilä, J. (2011). Population variation in brain size of nine-spined sticklebacks (*Pungitius pungitius*) - local adaptation or environmentally induced variation? *BMC Evolutionary Biology* **11**, 75.

Gonda, A., Herczeg, G. and Merilä, J. (2013). Evolutionary ecology of intraspecific brain size variation: a review. *Ecology and Evolution* **3**, 2751-2764.

Grether, G. F., Millie, D. F., Bryant, M. J., Reznick, D. N. and Mayea, W. (2001). Rain forest canopy cover, resource availability, and life history evolution in guppies. *Ecology* **82**, 1546-1559.

Gronenberg, W., Heeren, S. and Hölldobler, B. (1996). Age-dependent and task-related morphological changes in the brain and the mushroom bodies of the ant *Camponotus floridanus*. *The Journal of Experimental Biology* **199**, 2011.

Herculano-Houzel, S., Mota, B. and Lent, R. (2006). Cellular scaling rules for rodent brains. *Proceedings of the National Academy of Sciences* **103**, 12138.

Hibar, D. P., Stein, J. L., Renteria, M. E., Arias-Vasquez, A., Desrivieres, S., Jahanshad, N., Toro, R., Wittfeld, K., Abramovic, L., Andersson, M. et al. (2015). Common genetic variants influence human subcortical brain structures. *Nature* **520**, 224.

Houde, A. (1997). Sex, color, and mate choice in guppies. . *Princeton University Press*.

Isler, K. and van Schaik, C. P. (2006). Metabolic costs of brain size evolution. *Biology Letters* **2**, 557.

Isler, K. and van Schaik, C. P. (2009). The expensive brain: a framework for explaining evolutionary changes in brain size. *Journal of Human Evolution* **57**, 392-400.

Iwaniuk, A. N. and Nelson, J. E. (2003). Developmental differences are correlated with relative brain size in birds: a comparative analysis. *Canadian Journal of Zoology* **81**, 1913-1928.

Kelly, C. D., Godin, J.-G. J. and Wright, J. M. (1999). Geographic variation in multiple paternity within natural populations of the guppy (*Poecilia reticulata*). *Proceedings of the Royal Society of London. Series B: Biological Sciences* **266**, 2403.

Kihlslinger, R. L. and Nevitt, G. A. (2006). Early rearing environment impacts cerebellar growth in juvenile salmon. *Journal of Experimental Biology* **209**, 504.

Knickmeyer, R. C., Gouttard, S., Kang, C., Evans, D., Wilber, K., Smith, J. K., Hamer, R. M., Lin, W., Gerig, G. and Gilmore, J. H. (2008). A structural MRI study of human brain development from birth to 2 years. *The Journal of Neuroscience* **28**, 12176.

Kotrschal, A., Buechel, S. D., Zala, S. M., Corral-Lopez, A., Penn, D. J. and Kolm, N. (2015a). Brain size affects female but not male survival under predation threat. *Ecology Letters* **18**, 646-652.

Kotrschal, A., Corral-Lopez, A., Amcoff, M. and Kolm, N. (2015b). A larger brain confers a benefit in a spatial mate search learning task in male guppies. *Behavioral Ecology* **26**, 527-532.

Kotrschal, A., Deacon, A. E., Magurran, A. E. and Kolm, N. (2017). Predation pressure shapes brain anatomy in the wild. *Evolutionary Ecology* **31**.

Kotrschal, A., Kolm, N. and Penn, D. J. (2016). Selection for brain size impairs innate, but not adaptive immune responses. *Proceedings of the Royal Society B: Biological Sciences* **283**.

Kotrschal, A., Rogell, B., Bundsen, A., Svensson, B., Zajitschek, S., Brannstrom, I., Immler, S., Maklakov, A. A. and Kolm, N. (2013). Artificial selection on relative brain size in the guppy reveals costs and benefits of evolving a larger brain. *Curr Biol* **23**, 168-71.

- Kozlovsky, D. Y., Brown, S. L., Branch, C. L., Roth li, T. C. and Pravosudov, V. V.** (2014). Chickadees with bigger brains have smaller digestive tracts: a multipopulation comparison. *Brain, Behavior and Evolution* **84**, 172-180.
- Krause, E. T. and Liesenjohann, T.** (2012). Predation pressure and food abundance during early life alter risk-taking behaviour and growth of guppies (*Poecilia reticulata*). *Behaviour* **149**, 1-14.
- Lema, S. C., Hodges, M. J., Marchetti, M. P. and Nevitt, G. A.** (2005). Proliferation zones in the salmon telencephalon and evidence for environmental influence on proliferation rate. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology* **141**, 327-335.
- Lenth, R.** (2018). emmeans: Estimated Marginal Means, aka Least-Squares Means. *R package version 1.2.4*.
- Lerch, J. P., Yiu, A. P., Martinez-Canabal, A., Pekar, T., Bohbot, V. D., Frankland, P. W., Henkelman, R. M., Josselyn, S. A. and Sled, J. G.** (2011). Maze training in mice induces MRI-detectable brain shape changes specific to the type of learning. *NeuroImage* **54**, 2086-2095.
- Lucon-Xiccato, T., Manabe, K. and Bisazza, A.** (2019). Guppies learn faster to discriminate between red and yellow than between two shapes. *Ethology* **125**, 82-91.
- Maguire, E. A., Gadian, D. G., Johnsrude, I. S., Good, C. D., Ashburner, J., Frackowiak, R. S. and Frith, C. D.** (2000). Navigation-related structural change in the hippocampi of taxi drivers. *Proceedings of the National Academy of Sciences* **97**, 4398.
- Nilsen, A., Hagen, Ø., Johnsen, C. A., Prytz, H., Zhou, B., Nielsen, K. V. and Bjørnevik, M.** (2019). The importance of exercise: Increased water velocity improves growth of Atlantic salmon in closed cages. *Aquaculture* **501**, 537-546.
- Patel, S. N., Clayton, N. S. and Krebs, J. R.** (1997). Spatial learning induces neurogenesis in the avian brain. *Behavioural Brain Research* **89**, 115-128.
- Peper, J. S., Brouwer, R. M., Boomsma, D. I., Kahn, R. S. and Hulshoff Pol, H. E.** (2007). Genetic influences on human brain structure: a review of brain imaging studies in twins. *Human Brain Mapping* **28**, 464-473.
- Pollen, A. A., Dobberfuhl, A. P., Scace, J., Igulu, M. M., Renn, S. C. P., Shumway, C. A. and Hofmann, H. A.** (2007). Environmental complexity and social organization sculpt the brain in Lake Tanganyikan cichlid fish. *Brain, Behavior and Evolution* **70**, 21-39.
- Quallo, M. M., Price, C. J., Ueno, K., Asamizuya, T., Cheng, K., Lemon, R. N. and Iriki, A.** (2009). Gray and white matter changes associated with tool-use learning in macaque monkeys. *Proceedings of the National Academy of Sciences*.
- Reader, S. M. and Laland, K. N.** (2002). Social intelligence, innovation, and enhanced brain size in primates. *Proceedings of the National Academy of Sciences* **99**, 4436.
- Rosenzweig, M. R., Krech, D., Bennett, E. L. and Diamond, M. C.** (1962). Effects of environmental complexity and training on brain chemistry and anatomy: A replication and extension. *Journal of Comparative and Physiological Psychology* **55**, 429-437.
- Sallet, J., Mars, R. B., Noonan, M. P., Andersson, J. L., O'Reilly, J. X., Jbabdi, S., Croxson, P. L., Jenkinson, M., Miller, K. L. and Rushworth, M. F. S.** (2011). Social network size affects neural circuits in macaques. *Science* **334**, 697.
- Salvanes, A. G. V., Moberg, O., Ebbesson, L. O. E., Nilsen, T. O., Jensen, K. H. and Braithwaite, V. A.** (2013). Environmental enrichment promotes neural plasticity and cognitive ability in fish. *Proceedings of the Royal Society B: Biological Sciences* **280**.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B. et al. (2012). Fiji: an open-source platform for biological-image analysis. *Nature Methods* **9**, 676.

Scholz, J., Klein, M. C., Behrens, T. E. and Johansen-Berg, H. (2009). Training induces changes in white-matter architecture. *Nature Neuroscience* **12**, 1370.

Shors, T. J., Anderson, M. L., Curlik, D. M. and Nokia, M. S. (2012). Use it or lose it: how neurogenesis keeps the brain fit for learning. *Behavioural Brain Research* **227**, 450-458.

Sørensen, C., Johansen, I. B. and Øverli, Ø. (2013). Neural plasticity and stress coping in teleost fishes. *General and Comparative Endocrinology* **181**, 25-34.

Swaab, D. F. (1991). Brain aging and Alzheimer's disease, "wear and tear" versus "use it or lose it". *Neurobiol Aging* **12**, 317-324.

Team, R. (2015). RStudio: Integrated Development for R. *RStudio, Inc., Boston, MA*.

Templeton, C. N. S., W. M. (2004). Multiple selection pressures influence Trinidadian guppy (*Poecilia reticulata*) antipredator behavior. *Behavioral Ecology* **15**, 673-678.

van der Bijl, W., Thyselius, M., Kotrschal, A. and Kolm, N. (2015). Brain size affects the behavioural response to predators in female guppies (*Poecilia reticulata*). *Proceedings of the Royal Society B: Biological Sciences* **282**.

van Schaik, C. P., Isler, K. and Burkart, J. M. (2012). Explaining brain size variation: from social to cultural brain. *Trends in Cognitive Sciences* **16**, 277-284.

White, G. E. and Brown, C. (2015a). Microhabitat Use Affects Brain Size and Structure in Intertidal Gobies. *Brain, Behavior and Evolution* **85**, 107-116.

White, G. E. and Brown, C. (2015b). Variation in Brain Morphology of Intertidal Gobies: A Comparison of Methodologies Used to Quantitatively Assess Brain Volumes in Fish. *Brain, Behavior and Evolution* **85**, 245-256.

Zupanc, G. K. H. (2006). Neurogenesis and neuronal regeneration in the adult fish brain. *Journal of Comparative Physiology A* **192**, 649.

Tables

Table 1. Results of the GLM for relative brain size and relative size of the six major sub regions across all treatment groups.

	Treatment	Covariate
Overall brain	$F_{3,73} = 7.33, p < \mathbf{0.001}^{***}$	$F_{1,73} = 17.6, p < 0.001^{***}$
Telencephalon	$F_{3,73} = 0.926, p = 0.433$	$F_{1,73} = 70.5, p < 0.001^{***}$
Optic tectum	$F_{3,73} = 3.18, p = \mathbf{0.0291}^*$	$F_{1,73} = 79.5, p < 0.001^{***}$
Cerebellum	$F_{3,73} = 0.231, p = 0.874$	$F_{1,73} = 50.6, p < 0.001^{***}$
Dorsal medulla	$F_{3,73} = 2.45, p = 0.0700.$	$F_{1,73} = 19.6, p < 0.001^{***}$
Hypothalamus	$F_{3,73} = 1.34, p = 0.269$	$F_{1,73} = 47.9, p < 0.001^{***}$
Olfactory bulb ^a	$F_{3,70} = 1.08, p = 0.364$	$F_{1,70} = 43.5, p < 0.001^{***}$

a: values show the interaction with batch.

All brain correlates presented are relative measures, i.e. with standard length and rest of the brain as covariates for overall brain size and the separate brain sub regions, respectively.

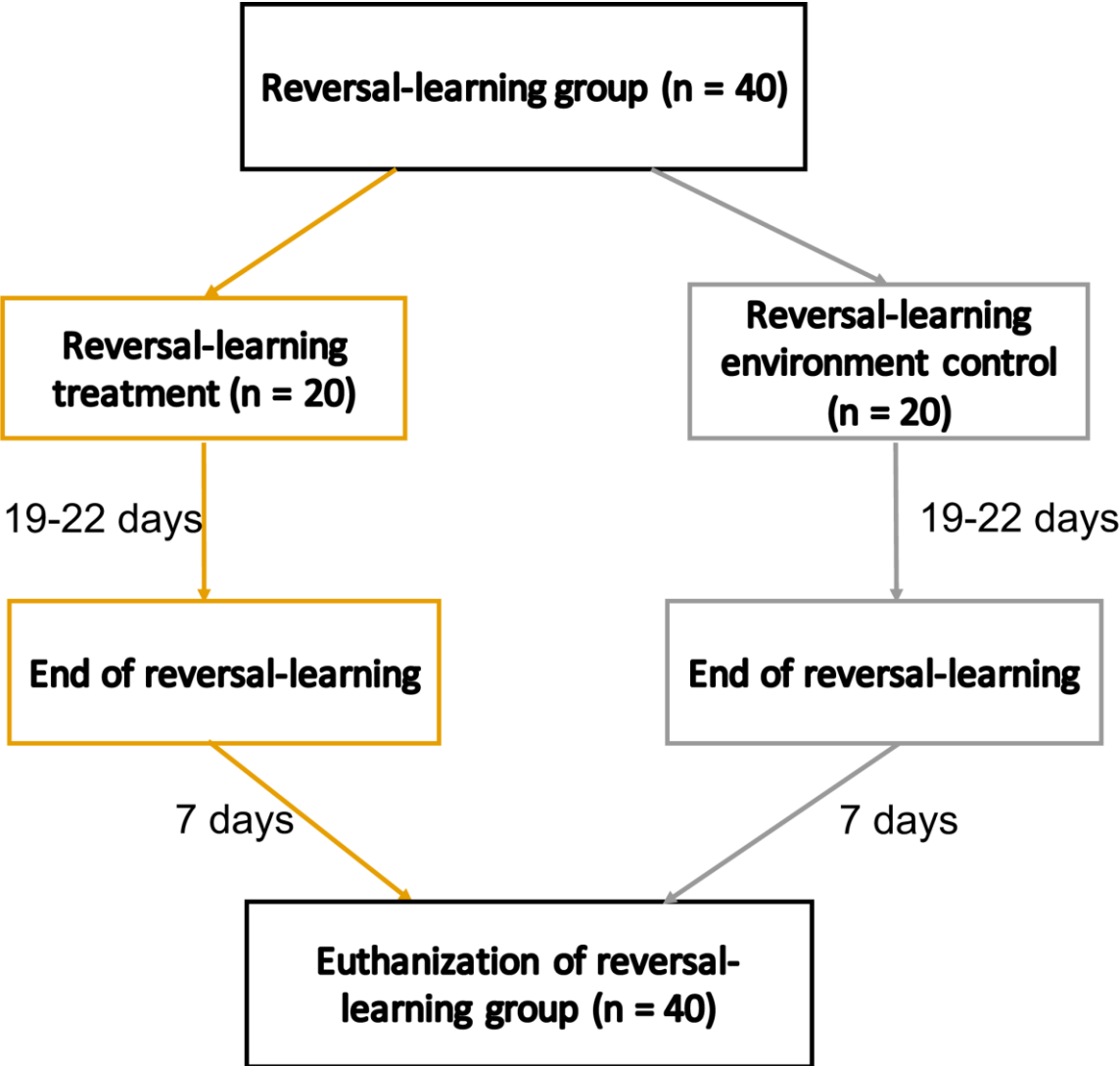
Significant differences are highlighted in bold. Interactions between batches and treatment groups were excluded from the model where the interaction was not found to be significant ($p > 0.05$). *** $P < 0.001$, * $p < 0.05$.

Table 2. Post-hoc pairwise comparison of relative brain size and optic tectum size across the different treatment groups.

Treatment	Overall brain	Optic tectum
Reversal-learning environment control – Reversal-learning treatment	$t_{73} = -0.885$, $p = 0.758$	$t_{73} = 0.645$, $p = 1.00$
Spatial-learning environment control – Spatial-learning treatment	$t_{73} = -0.849$, $p = 0.758$	$t_{73} = 0.366$, $p = 1.00$
Reversal-learning treatment – Spatial-learning treatment	$t_{73} = -3.17$, $p = 0.0088^{**}$	$t_{73} = -2.32$, $p = 0.117$
Reversal-learning environment control – Spatial-learning environment control	$t_{73} = -3.30$, $p = 0.0074^{**}$	$t_{73} = -2.11$, $p = 0.154$
Reversal-learning environment control – Spatial-learning treatment	$t_{73} = -4.12$, $p < 0.001^{***}$	$t_{73} = -1.69$, $p = 0.289$
Reversal-learning treatment – Spatial-learning environment control	$t_{73} = -2.36$, $p = 0.0628$.	$t_{73} = -2.76$, $p = 0.0434^*$

Brain measures are presented in relative terms, i.e. with standard length and rest of the brain as covariates for overall brain size and the separate brain sub regions, respectively. Significant differences are highlighted in bold. Values are calculated following Holm's adjustment for multiple comparisons. *** $P < 0.001$, ** $p < 0.01$, * $p < 0.05$.

Figures



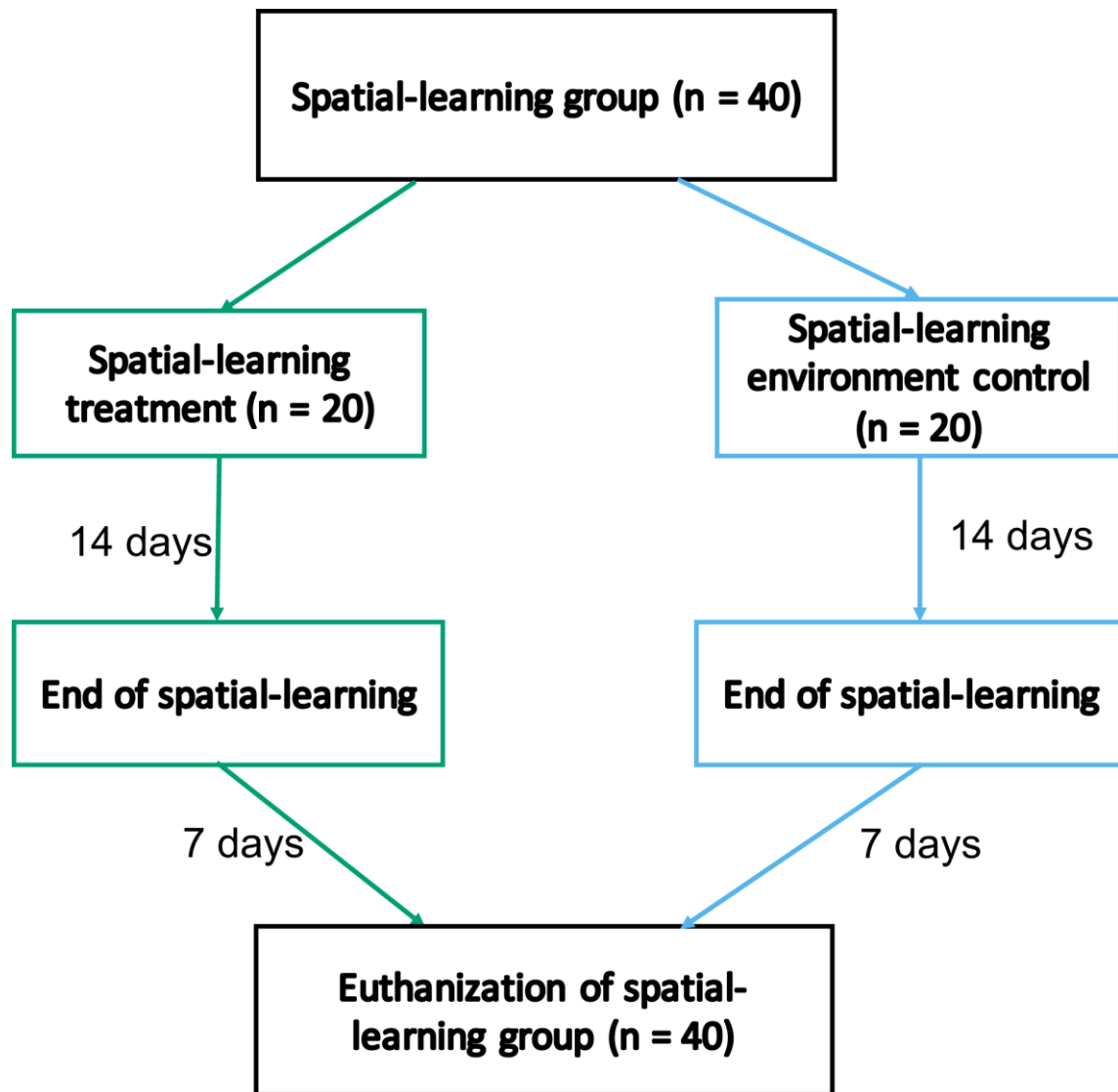


Figure 1. Stepwise progression for the reversal-learning group (A) and spatial-learning group (B).

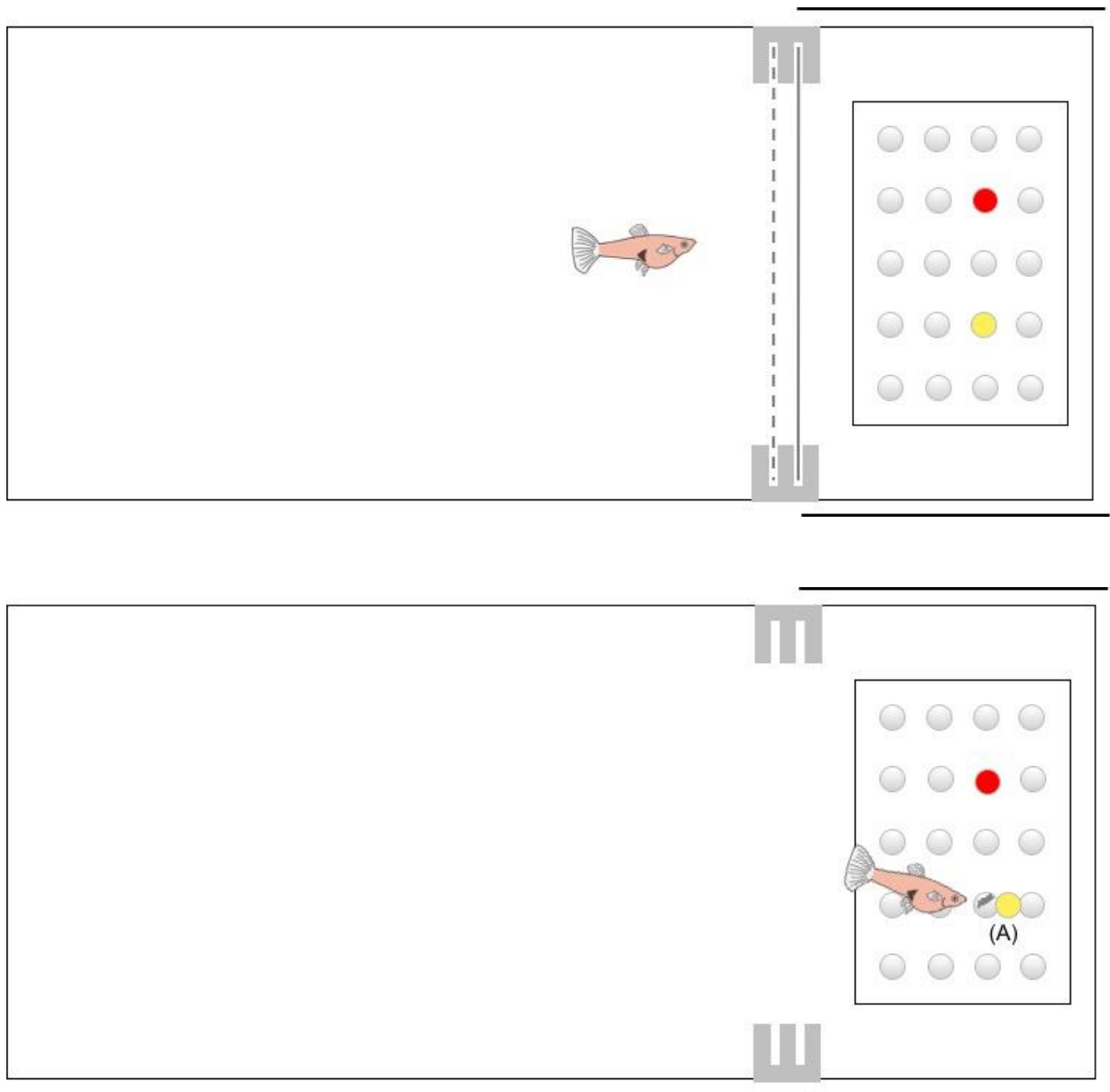


Figure 2. **Schematic diagram of the reversal-learning setup.** A) In this particular instance, the female has to dislodge the yellow disc in order to obtain the food reward hidden beneath it.

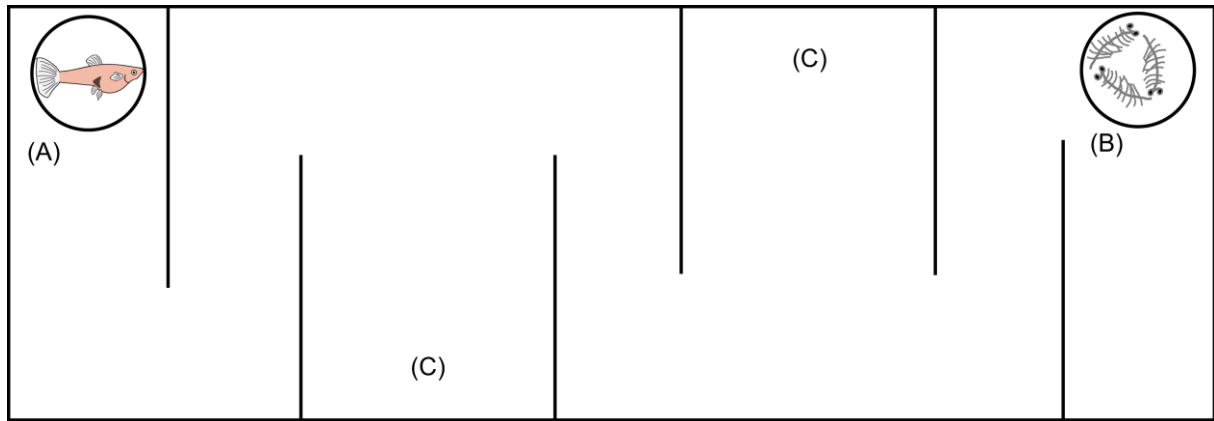
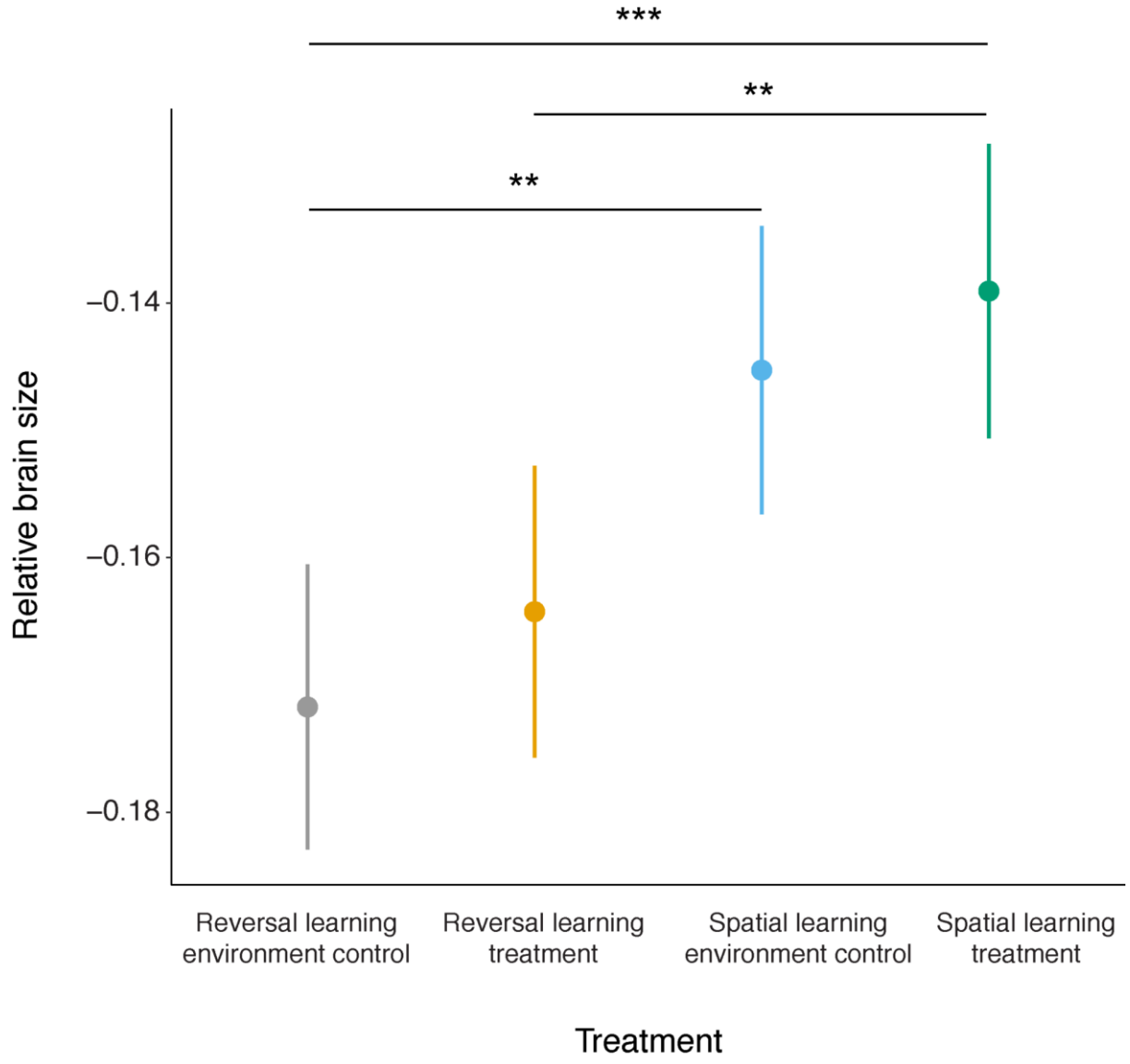


Figure 3. **A graphical representation of the spatial-learning setup.** Individuals were allowed a 60 s acclimation period in a transparent Perspex ring following transfer (A). The food reward was placed in the goal compartment at the end of the maze (B). The two separate dead ends are indicated by (C).



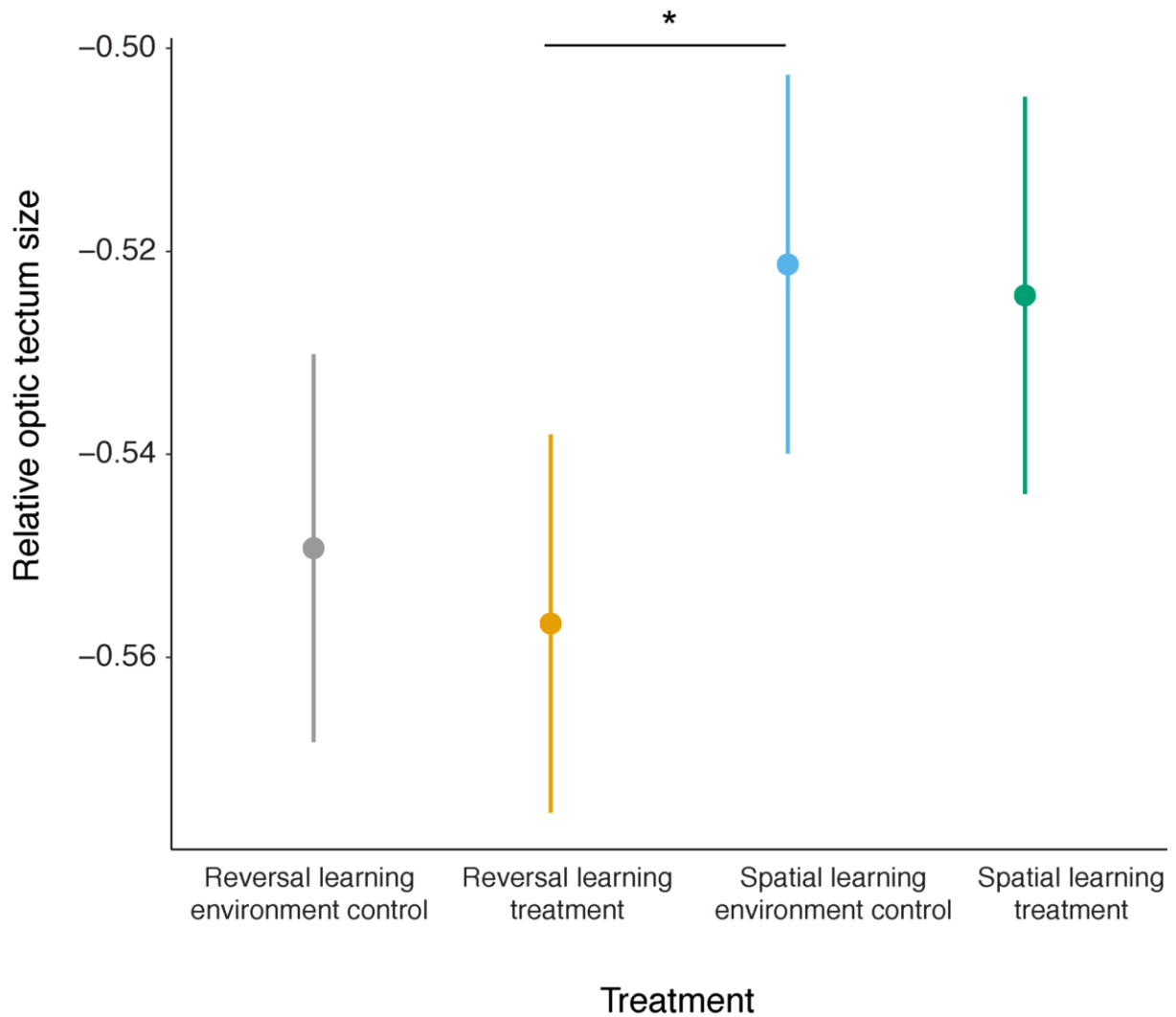


Figure 4. **Effects of treatment on (a) relative brain size and (b) optic tectum size in female guppies.** Estimated marginal means including standard errors are shown on the y-axis, with the different treatment groups on the x-axis. Significant differences between treatments are marked with asterisks. P-values are calculated following Holm's adjustment for multiple comparisons. Abbreviations: CRL, reversal-learning environment control (n = 20); TRL, reversal-learning treatment (n = 20); CSL, spatial-learning environment control (n = 20); TSL, spatial-learning treatment (n = 19). ***P < 0.001, **p < 0.01, *p < 0.05.