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RESEARCH ARTICLE

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

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ABSTRACT

Despite the common assumption that the brain is malleable to surrounding conditions mainly 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 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.

KEY WORDS: Brain plasticity, Cognition, Reversal learning, Spatial learning

INTRODUCTION

Brain morphology shows remarkable variation both across and within species. Despite a massive research effort in recent 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 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,b; Iwaniuk and Nelson, 2003).

While plastic neural changes have been shown to be extremely pronounced during juvenile stages mainly as a result of 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 the 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, 2013).

The earliest evidence for plastic changes during adulthood stems 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 mass of the 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 march 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 regard to plastic adaptations in brain morphology. The use of social cues, for example, has been extensively studied in non-human 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. As cognition is expensive, in particular when associated with changes in costly neural tissue (Aiello and Wheeler, 1995; Isler and van Schaik, 2006, 2009; Kotrschal et al., 2016, 2013; Kozlovsky et al., 2014), it could be highly advantageous to avoid spending unnecessary energy on maintaining 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* W. Peters 1859) is a suitable model for studies of brain morphology plasticity. As is the case with

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many fishes, guppies experience continued growth and prolonged neurogenesis throughout their lifespan. The occurrence of adaptive plastic responses in neural architecture even in adult animals is therefore not unexpected (Ekström, 1994; Gonda et al., 2009b; Kihslinger 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 fill the gaps in our understanding of the factors driving plastic changes in brain morphology.

Here, we adopted 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 larger cerebella in these fish (Kihslinger 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 subregions (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 a high population size in Trondheim University for multiple generations, maintaining high genetic diversity (Kotrschal et al., 2013). Eighty female guppies were netted and individually housed in 4 l aquaria with a layer of light-coloured gravel ranging from 0.20 to 1 cm^3 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 six 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 end of the shelf was limited to one neighbour in the adjacent tank. To avoid any bias 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 6 days of the week. Only sexually mature female guppies were used in the study.

Prior to the start of any experimental procedures, females were randomly allocated to either of two experiments: reversal learning (n=40) or spatial learning (n=40). Within the reversal-learning test, we further divided the fish 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 a graphical representation of the different treatments). Animals in the learning environment control groups were treated identically to those in their respective treatment groups in terms of housing and handling, with the exception that they were not trained in each task. This was to test 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.

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).

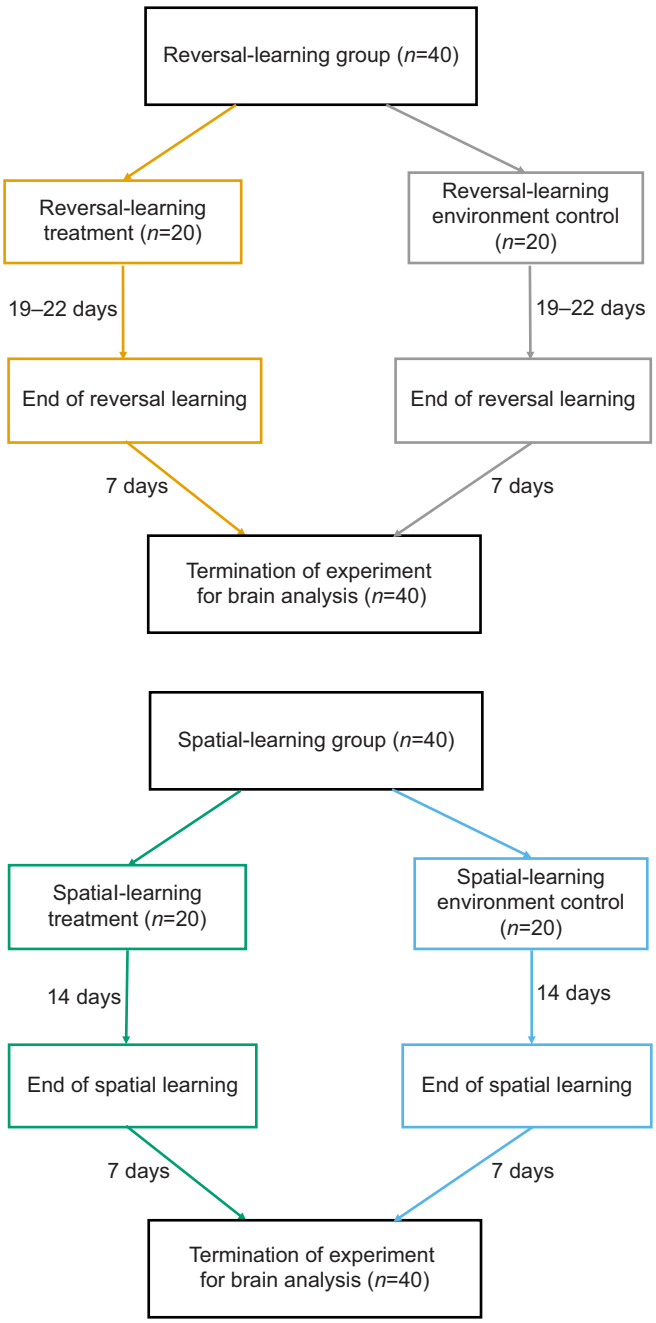

Fig. 1. Stepwise progression for the two learning groups. (A) Reversal-learning group. (B) Spatial-learning group.

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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 (25×15 cm) and an experimental compartment (15×15 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 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 phrase, 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 (A.B., S.D.B. and N.K., unpublished data). Fish in the reversal-learning environment control group were naive to the training but were fed similar amounts of frozen *Artemia* to fish in 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 it was still noted as a non-choice response. Fish were tested in blocks of three trials, up to a maximum of two blocks on each given day. This meant that individuals could receive a maximum of six trials per day. As individuals received the food reward at the end of the trial regardless of the first choice made, this ensured that the fish remained motived in the tests. We randomly allocated either one or two trial blocks to each test day, where a maximum of six trials per day was permitted for no more than 3 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 six consecutive blocks of 10 trials. This specific criterion was chosen in order to

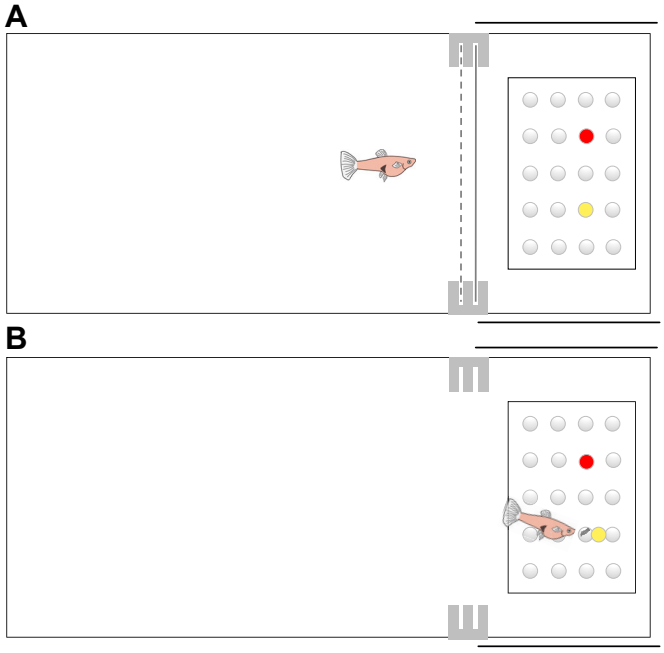

Fig. 2. Schematic diagram of the reversal-learning setup. (A) Experimental tank, showing the home compartment (left) and the experimental compartment (right), separated by transparent and opaque doors. (B) In this particular instance, the female has to dislodge the yellow disc in order to obtain the food reward hidden beneath it.

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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, because of time constraints, the total duration of the entire test for batches one and two took 22 and 19 days, respectively.

One week after the completion of the reversal-learning test, fish were killed in a water bath containing an overdose of benzocaine (0.4 g l^{-1}), and fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 5 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 dimensions of 100×50 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. 3). Between three and five frozen *Artemia* hatchlings were placed on a small plastic Petri dish (6 cm in diameter) located in the goal compartment (Fig. 3, area B). 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 was 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 h, when 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). Fish were killed 1 week following completion of the trials as per the reversal-learning treatment (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 (S.F.) to minimize inter-observer variability. Given that individuals could only be identified by their number, the experimenter remained blind to the treatment of each individual. The volume (V) of individual brain regions was quantified from their length (L), width (W) and height (H) via the ellipsoid model (Pollen et al., 2007; White and Brown, 2015b):

$$V = (L \times W \times H)\frac{\pi}{6}. \quad (1)$$

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

Data analysis

To first assess whether individuals assigned to the reversal-learning treatment had successfully learnt both the colour-association 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 $\text{model}_{\text{forward-learning}}$: $\text{response}_{(\text{correct/incorrect first choice})}\sim \text{trial}_{(1–30)}+(1+\text{trial}|\text{fish})$, family=binomial, lme4 syntax for R $\text{model}_{\text{reversal-learning}}$: $\text{response}_{(\text{correct/incorrect first choice})}\sim\text{trial}_{(1–66)}+(1+\text{trial}|\text{fish})$, 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+trial|fish)].

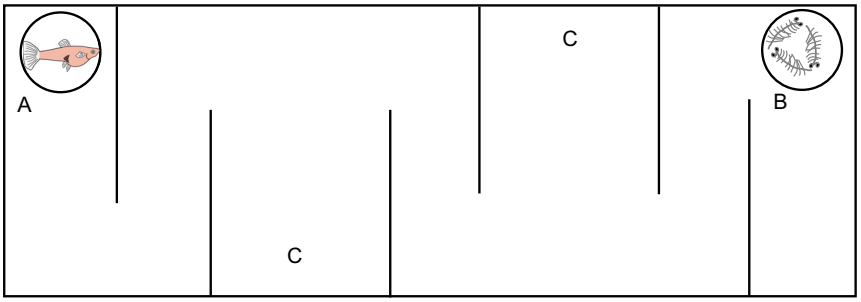

Fig. 3. Graphical representation of the spatial-learning setup. Individuals were allowed a 60 s acclimation period in a transparent Perspex ring following transfer (A). The food reward was placed in the goal compartment at the end of the maze (B). The two separate dead ends are indicated by C.

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In female guppies, body size tends to be indicative of their age. As sexually mature females of indeterminate age were randomly assigned to the different treatment groups, we wanted to test for any difference in body 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 four treatment groups, with total brain size or brain subregion size 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 analysed, 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 (https://CRAN.R-project.org/package=emmeans) (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 tests had an effect on brain morphology, brain measurements were specifically compared within each test (i.e. reversal-learning environment control versus reversal-learning treatment; spatial-learning environment control versus 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 those 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 versus spatial-learning environment control, and reversal-learning treatment versus spatial-learning treatment. All brain measurements and body size were log transformed prior to analysis. Analyses were performed using R statistical software v3.5.1 (RStudio 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-learning phase of the reversal-learning test (GLMM; trial: $z_{1,66}$=15.3, P<0.001). End performance levels (±s.d.) were comparable across individuals in both the forward-learning phase (89±1%) and the reversal-learning phase (82±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; see Table 1). *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; see Table 2). In contrast, we found a strong effect of the learning treatment environment on relative brain size (t_{73}=−3.30, P=0.0074; Table 2). Pairwise comparisons revealed that fish in the spatial-learning group (i.e. both treatment and control groups) had an overall larger relative brain size than those in the reversal-learning group (Table 2, Fig. 4A).

Table 1. Results of the overall GLM for relative brain size and relative size of the six major brain subregions across all four treatment groups

	Treatment	Covariate
Overall brain	$F_{3,73}$=7.33, ***P*<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*=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 subregions, respectively. Significant differences across treatments are 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.

Similar to relative brain size, no significant differences in the relative size of any of the major brain subregions was observed between the treatment groups and the control groups within each test (data not shown). 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>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 with 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

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

Treatment	Overall brain	Optic tectum
CRL–TRL	t_{73}=−0.885, P=0.758	t_{73}=0.645, P=1.00
CSL–TSL	t_{73}=−0.849, P=0.758	t_{73}=0.366, P=1.00
TRL–TSL	t_{73}=−3.17, ***P*=0.0088****	t_{73}=−2.32, P=0.117
CRL–CSL	t_{73}=−3.30, ***P*=0.0074****	t_{73}=−2.11, P=0.154
CRL–TSL	t_{73}=−4.12, ***P*<0.001*****	t_{73}=−1.69, P=0.289
TRL–CSL	t_{73}=−2.36, P=0.0628	t_{73}=−2.76, ***P*=0.0434***

CRL, reversal-learning environment control; TRL, reversal-learning treatment; CSL, spatial-learning environment control; TSL, spatial-learning treatment. Brain measurements 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 subregions, respectively. Significant differences are in bold. Values were calculated following Holm's adjustment for multiple comparisons. ***P<0.001, **P<0.01, *P<0.05.

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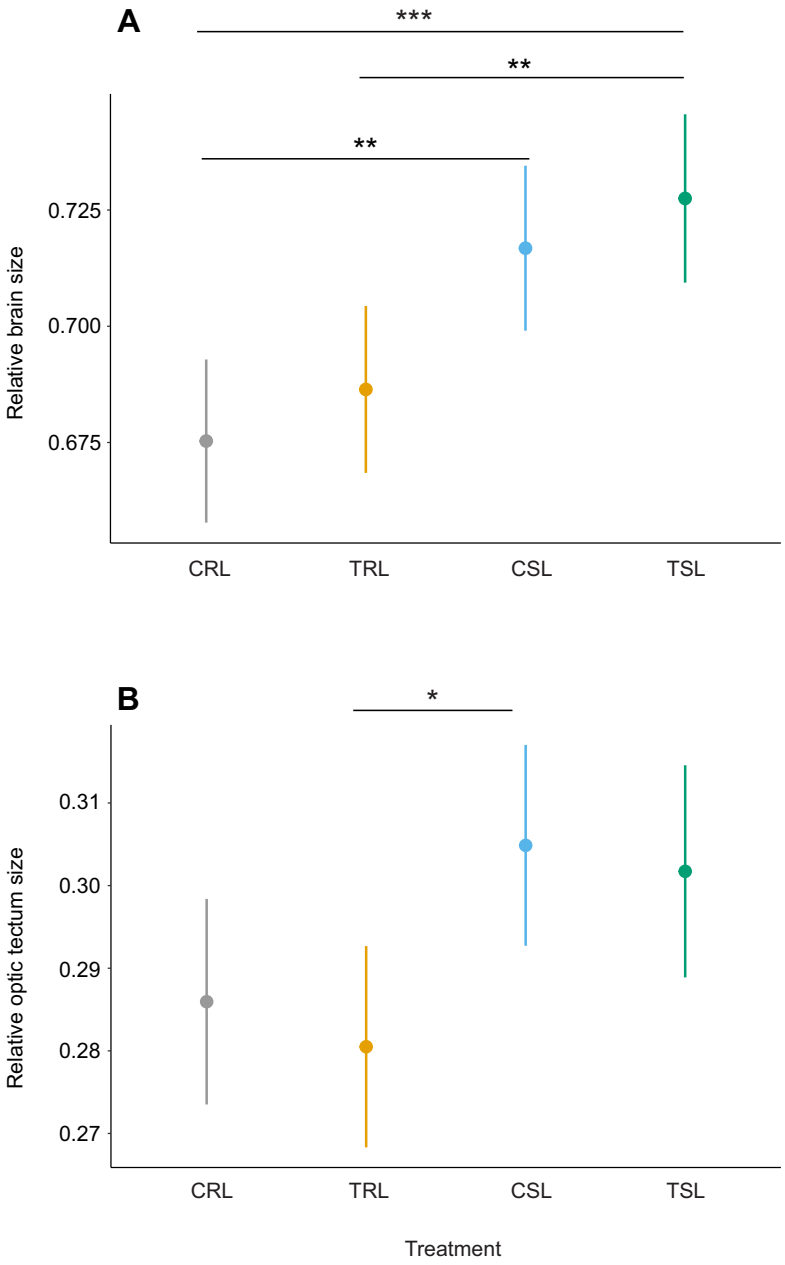

Fig. 4. Effects of treatment on brain morphology in female guppies. (A) Relative brain size. (B) Relative optic tectum size. Estimated marginal means including s.e.m. are shown on the *y*-axis, with the different treatment groups on the *x*-axis. Significant differences between treatments are marked with asterisks. *P*-values were calculated following Holm's adjustment for multiple comparisons: ***P<0.001, **P<0.01, *P<0.05. 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).

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 probably 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. Why then did we not detect any effects of learning on brain morphology in our experiments? 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 involved a longitudinal design, where training duration ranged from 5 days to 3 months. Studies involving animal models generally utilize a training regime with a duration that falls between 5 days in rodents and 14 days in macaques. Human studies, in contrast, tend to adopt training schedules spanning an extended period of time. For cases pertaining to the acquisition of a new skill, such as juggling, or a period of extensive learning, e.g. studying for a medical examination, 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 a period of 14 days. Given that the time allocated for each of the cognitive tasks falls within the range utilized by the aforementioned studies, we expected our test duration to be sufficiently long for plastic changes in brain morphology to occur.

Perhaps the 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 and 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 guppies' natural habitat, as 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 the 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 deviates further 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, our spatial learning test may involve a low level of complexity, given that the start and end positions remained 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 behaviour. Previous studies that have adopted this paradigm have shown that this ability to flexibly withhold a particular behaviour tends 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 relate to the nature of the 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 increase in the 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 taking place at the cellular level. Ideally, future studies should expand the sample size 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 the underlying neural modifications induced by learning. Employing such a variety 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 a 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 minimum duration of 46 days. In light of the fact that our guppies were placed in the spatial-learning setup for only 15 min 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 are 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 respond mainly to environmental cues (Gonda et al., 2011). For instance, it could be that the level of environmental complexity is always strongly linked to the level of cognitive demand in wild guppy populations. The main cognitive challenges for wild guppies include avoiding predation, finding food and finding a mate (Burns and Rodd, 2008). All three aspects have previously been 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). Additionally, variation in environmental factors is 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 with 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 of small plastic changes in brain morphology elicited by learning, and would be valuable to include 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.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: S.F., N.K.; Methodology: S.F., S.D.B., A.K., N.K.; Formal analysis: S.F., S.D.B.; Investigation: S.F., A.B.; Resources: N.K.; Writing - original draft: S.F.; Writing - review & editing: S.F., S.D.B., A.B., A.K., N.K.; Visualization: S.F.; Supervision: N.K.; Project administration: S.D.B.; Funding acquisition: N.K.

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Data availability
Data are available from the Dryad digital repository (Fong et al., 2019): dryad.c4q7h62

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