Experimental mosaic brain evolution improves main executive function abilities in the guppy

Authors: Zegni Triki*, Stephanie Fong, Mirjam Amcoff, Sebastian Vàsquez-Nilsson, and Niclas Kolm

Authors' affiliation: Department of Zoology, Stockholm University, Svante Arrheniusväg 18 B, Stockholm, Sweden

***Corresponding author:** Zegni Triki, email: zegni.triki@gmail.com

Abstract

Executive functions are a set of cognitive control processes required for optimizing goal-directed behaviour. Despite more than two centuries of research on executive functions, both in humans and nonhuman animal species, there is still a knowledge gap of what constitutes the mechanistic basis of evolutionary variation in executive function abilities. Here we show experimentally that changes in a forebrain structure (i.e., telencephalon) size, through mosaic brain evolution, underlie individual variation in executive functions capacities in a fish. For this, we used artificial selection lines of guppies (*Poecilia reticulata*) with substantial differences in relative telencephalon size. We tested fish from the large and small telencephalon lines in tasks for the three main core executive functions: cognitive flexibility, inhibitory control, and working memory, but also in a basic conditioning test that does not require executive functions. Individuals with larger telencephalons outperformed individuals with smaller telencephalons in all three executive function assays but not in the conditioning assay. Based on our discovery, we propose that the telencephalon is the executive brain in teleost fish. Also, selective enlargement of key brain structures, like the fish telencephalon, through mosaic brain evolution is a potent evolutionary pathway towards evolutionary enhancement of advanced cognitive abilities in vertebrates.

Keywords: telencephalon; reversal learning; detour task; object permanence; brain morphology; expensive tissue; trade-offs.

Introduction

There is a general agreement that there are three main executive functions: cognitive flexibility, self-control and working memory [1]. These three domains form the core top-down executive functions that regulate several cognitive subprocesses [1,2]. For instance, cognitive flexibility allows individuals to adapt and change their behaviour and strategy in response to the environment [3]. Self-control requires inhibitory control abilities to override motor impulses, resulting in adaptive goal-oriented behaviours when correctly performed [4,5]. Finally, working memory is holding and working with visual-spatial information (and verbal information in humans) that is no longer perceptually present [6,7]. These executive functions ultimately play a pivotal role in survival and reproduction and thus have important fitness consequences [8].

In mammals, neural structures in the neocortex, like the prefrontal lobes, are critical for regulating executive functions, and subsequently the neocortex has been referred to as the "executive brain" [8–10]. Therefore, the evolutionary expansion of the neocortex – suggested being the outcome of a mosaic change in brain structure (i.e., mosaic brain evolution) where independent evolutionary changes in brain region sizes can drive changes in specific cognitive abilities [11]– emerges as a promising candidate mechanism behind evolutionary changes in executive functions [10,12,13]. Nevertheless, most of this evidence is correlative and hence lacks experimental support.

Artificial selection experiments are a powerful experimental approach that can create a shortcut for evolutionary changes of a trait of interest within species [14–16]. Here, we take advantage of a recent artificial selection experiment on telencephalon size in the guppy [17] to test the hypothesis that mosaic evolution of the size of a neocortex "homologue" [18] leads to evolutionary changes in executive functions. In fish, the telencephalon is a part of the forebrain well known for its

role in various advanced perceptual and cognitive functions [19]. By comparing intact subjects to subjects with partly or wholly ablated telencephalon, researchers have determined the involvement of this region in regulating spatial learning, memory and decision-making [20–24]. Moreover, although functional differences certainly exist between fish and mammals, at least in some functions, the telencephalon has been suggested to be homologous to the mammalian neocortex [18]. However, a substantial knowledge gap remains concerning whether the telencephalon is the "executive brain" in fish as well, and what is the quantitative relationship between telencephalon size and executive functions. Recently, Triki et al., [25] showed that three generations of selection on telencephalon size, resulting in a 5% difference in relative telencephalon volume between up- and down-selected lines, was enough to cause significant differences in inhibitory control abilities, one of the core executive functions. Here, we built further on this finding and tested whether more neural tissue in the telencephalon would enhance performance in all three core executive functions in a fifth generation of the guppy telencephalon size selection lines.

Therefore, we tested up- and down-selected fish abilities in cognitive flexibility, inhibitory control and working memory in three different tasks. We used the reversal learning task to test for cognitive flexibility, a commonly used paradigm across species and taxa [26–30]. The task also allows assessing associative learning abilities (operant conditioning) during the initial learning phase, which served as a control test for non-executive cognitive ability. We first tested the fish in a two-colour discrimination task where choosing the correct colour led to a food reward. For those that learned the initial cue-reward association, we reversed the reward contingency where the previously unrewarded colour becomes the new rewarded cue. Thus, we tested fish's ability to adjust their behaviour after the reversal of the cue-reward contingency, a measure of behavioural and cognitive flexibility [3,27,31]. In the next task, we tested fish's inhibitory control

abilities (self-control), a commonly used detour paradigm across species and taxa known as the "cylinder task" [5,13,32]. In the cylinder task, individuals are often presented with a food reward placed inside a transparent cylinder open on either side. Animals lacking higher inhibitory control abilities move directly towards the visible food and hence get blocked by the barrier. Successful performance is when an animal can delay gratification by moving away from the goal and going around the see-through barrier without touching it to reach the food reward [13,25,30,32]. In the final task, we tested the fish's working memory in an object permanence task [33]. Object permanence tasks were initially designed to test the cognitive development of human infants [34] and later used to document object permanence abilities in primates [35], dogs and cats [36], marine mammals [37] and birds [38]. The task we performed tested whether fish can memorise the location of an object that has been visibly displaced behind an opaque screen and the knowledge that the object still exists when out of sight (see Supplementary Video S1).

An important aspect to consider when addressing the evolutionary link between brain morphology and cognitive ability is that neural tissue is energetically costly and constrained by the individual's total energy budget. Growing larger brains, for instance, is often a manifestation of an energy trade-off by selective investment in the brain at the expense of other expensive (energy-demanding) tissues like the gut [16,39]. For this reason, we tested whether increased telencephalon size was traded off against gut mass in the telencephalon size selection lines. Furthermore, we measured the volume of the five major brain regions (telencephalon, optic tectum, hypothalamus, cerebellum and dorsal medulla) in all tested fish. This was necessary to verify the individual telencephalon size from the fifth generation of selection lines but also served to test for potential size trade-offs among brain regions as a consequence of the selection experiment.

As predicted, we find that an enlarged telencephalon enables individuals to show overall higher performance across all three executive function domains (Table 1), but not in a non-executive associative learning assay. Together, these results suggest that the fish telencephalon is effectively "the executive brain" in teleost fish, and that evolutionary mosaic changes in brain structure can be energy-efficient drivers of cognitive evolution with regards to more advanced cognitive functions.

Results

For the cognitive tasks, we tested (N = 48) adult male guppies from the fifth generation of the up- and down-selected lines of telencephalon size. In the colour discrimination task (associative learning) (Supplementary Video S1), 79% of up-selected fish were successful in learning the association colour-food (cue-reward) within 42 test trials, vs 74% success in down-selected fish, which was not a statistically significant difference (Survival analysis: N = 37, Hazard Ratio (HR) = 1.107 [0.71, 1.73], p = 0.675, Fig. 2a) (see further statistical details in Supplementary Table S3). Interestingly, when we reversed the colour-reward contingency in the reversal learning task to test for the first aspect of executive function (cognitive flexibility), the up-selected fish exhibited higher performance by being faster and more successful at solving the task with 63% success vs 48% in the down-selected fish within 84 test trials (Survival analysis: N = 37, HR = 1.231 [1.01, 1.50], p = 0.039, Fig. 2e). In the second aspect of executive function (self-control), in the cylinder detour task, up-selected fish outperformed down-selected fish with 37% correct detours (detouring the transparent cylinder without touching it) on average vs 32% in the down-selected fish, where the up-selected fish showed improved performance across the 11 test trials in contrast to down-selected fish (Generalised LMM (GLMM): interaction term line x trials: N = 45, estimate = 0.74 [0.29, 1.19], X^2 = 10.191, p = 0.001). This effect was mainly driven by performance across time (trials), where the up-

selected had a positive slope of performance across trials (posthoc emtrend estimate = 0.110 [0.021, 0.20], p = 0.015), but a negative performance slope in down-selected fish (posthoc emtrend estimate = -0.123 [-0.24, -0.01], p = 0.03) (Fig. 3a). Finally, in the third aspect of executive function (working memory), the up-selected fish showed 60% success on average in memorising and following the correct path to the object location in the object permanence task vs 49% in the down-selected fish within 16 test trials (GLMM: N = 47, estimate = 0.49 [0.15, 0.84], X^2 = 7.809, p = 0.005, Fig. 3c). Additionally, we tested whether fish found the correct location of the object by chance (50% chance level of scoring correctly). We found that up-selected fish performed significantly above chance level (posthoc emmeans test: estimate = 0.424 [0.06, 0.79], p = 0.023), but this was not the case for down-selected fish (estimate = -0.069 [-0.44, 0.30], p = 0.712, Fig. 3c).

To verify that the tested fish really differed in telencephalon size, and to allow for linking individual variation in telencephalon size to cognitive performance, we measured telencephalon size (and the size of other main brain regions, see below) after the cognitive assays (N = 44) (see Methods). The tested fish from the selection lines did indeed differ in their relative telencephalon size (relative to the size of the rest of the brain), with up-selected fish having 9.6% larger telencephalon on average than down-selected fish (Linear Mixed Effects Model (LMM): 22 up- vs 22 down-selected fish, estimate = 0.09, 95% credible interval (95% CI) [0.06, 0.13], X^2 = 31.662, p < 0.001; marginal-R^2 = 0.74, conditional-R^2 = 0.81, Fig. 1). Building on this, we could analyse and reveal that the differences in performance between up- and down-selected fish were also linked to individual telencephalon size. First, we found a significant effect of relative telencephalon size on correct choices across test trials in the reversal learning task (GLMM: interaction term telencephalon size x rounds: N = 34, estimate = 0.13 [0.01, 0.26], X^2 = 4.315, p = 0.038). Posthoc analyses revealed that this was due to

individuals with larger than average telencephalon size (mean + 1SD) having a steeper learning slope (estimate = 0.42 [0.27, 0.56], $p < 0.001$) compared to individuals with average or smaller than average telencephalon size (mean: estimate = 0.28 [0.21, 0.36], $p < 0.001$; mean - 1SD: estimate = 0.15 [0.00, 0.30], $p = 0.05$, Fig. 2h). Second, in the detour task, there was a significant effect of telencephalon size on performance across test trials (GLMM: interaction term telencephalon size x trials: N = 42, estimate = 0.35 [0.01, 0.69], $X^2 = 3.911$, $p = 0.048$) that can be explained by individuals with larger than average telencephalon size having a positive and steeper learning slope (mean + 1SD: estimate = 0.38 [-0.04, 0.79], $p = 0.07$) compared to individuals with average or smaller than average telencephalon size (mean: estimate = 0.03 [-0.20, 0.25], $p = 0.80$; mean - 1SD: estimate = -0.32 [-0.73, 0.09], $p = 0.13$, Fig. 3b). Finally, we found an overall positive correlation relationship between performance in the object permanence task and telencephalon size (GLMM: N = 44, estimate =0.40 [0.10, 0.71], $X^2 = 6.517$, $p = 0.011$, Fig. 3d). Again, and similarly to the group level analyses, there was no statistically significant relationship between telencephalon size and performance in the colour discrimination task ($p > 0.05$, Fig. 2b and d) (for further details see Supplementary Table S3).

To further evaluate fish performance across the three tests of executive functions, we generated a composite score [13] in the form of a "performance rank score". To do so, we summed up the individual ranks in the three tasks: reversal learning (rank of the number of trials until success), detour (rank of the proportion of correct detours) and object permanence (rank of the proportion of successfully locating the object). Our analyses revealed that up-selected fish significantly outranked down-selected fish by 28% mean difference (GLMM: 24 up- vs 21 down-selected fish, estimate = 0.25 [0.03, 0.47], $X^2 = 5.08$, $p = 0.024$, marginal-$R^2 = 0.11$, conditional-$R^2 = 0.15$, Fig. 4a). We found a similar outcome with individual telencephalon size and performance

rank score in the form of a significant interaction term between telencephalon size and the size of the rest of the brain (GLMM: N = 42, estimate = -0.13 [-0.22, -0.03], X^2 = 7.231, p = 0.007, marginal-R^2 = 0.25, conditional-R^2 = 0.30, Fig. 4b). The posthoc test showed a significant positive correlation between telencephalon size and performance rank when rest of the brain was smaller than average (slope of telencephalon and performance estimate = 0.24 [0.04, 0.45], p = 0.02), but not when rest of the brain was average size (estimate = 0.11 [-0.08, 0.30], p = 0.26) or larger than average size (estimate = -0.02 [0.24, 0.20], p = 0.83, Fig. 4b).

For the analyses of the sizes of the other brain regions, we detected a significant difference in optic tectum size between the two selection lines, with up-selected fish having smaller optic tectum compared to down-selected fish (LMM: estimate = -0.04 [-0.09, -0.01], X^2 = 31.662, p = 0.045, marginal-R^2 = 0.53, conditional-R^2 = 0.53, Fig. 1). However, further analyses on whether the selection experiment affected total brain size or other brain regions (hypothalamus, cerebellum and dorsal medulla) as well as our quantification of an expensive tissue, gut weight, showed no statistically significant differences between up- and down-selected fish (all p-values > 0.05) (Supplementary Fig. S1, Tables S1, S2 and Fig. 1).

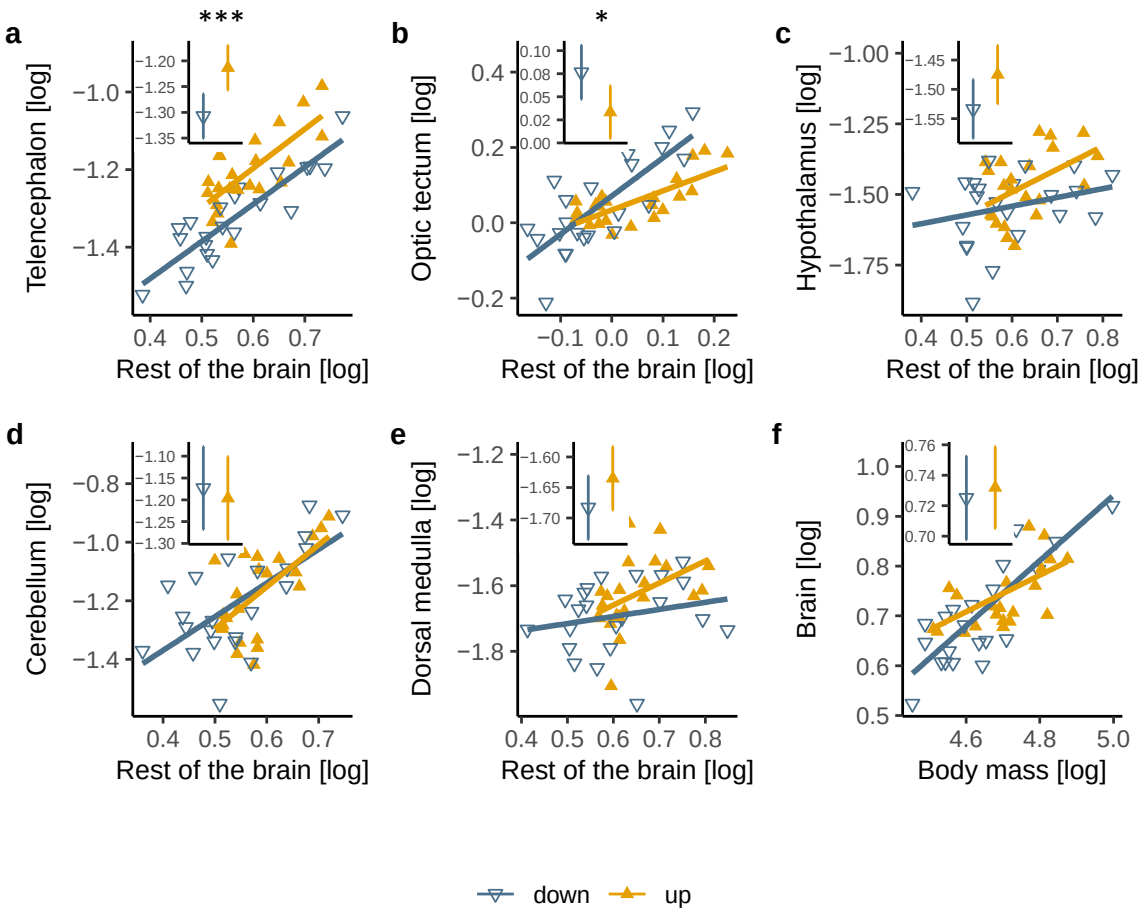

Figure 1. Individual brain morphology from the two selection lines. Regression lines and 95% CI of log-normal transformed volume (mm^3) of the brain region of interest (**a**) telencephalon, (**b**) optic tectum, (**c**) hypothalamus, (**d**) cerebellum, and (**e**) dorsal medulla on the log-normal transformed remainder of the brain without the volume of the corresponding region (mm^3), as a function of selection line. (**f**) Regression lines and 95% CI of log-normal transformed total brain volume (mm^3) on log-normal transformed body mass (mg) as a function of selection line. Inside each plot are depicted the estimated marginal means of up- and down-selected groups with 95% CI calculated from the statistical models. Twenty-two up-selected vs 22 down-selected fish: LMM, *$p < 0.05$, ***$p < 0.001$.

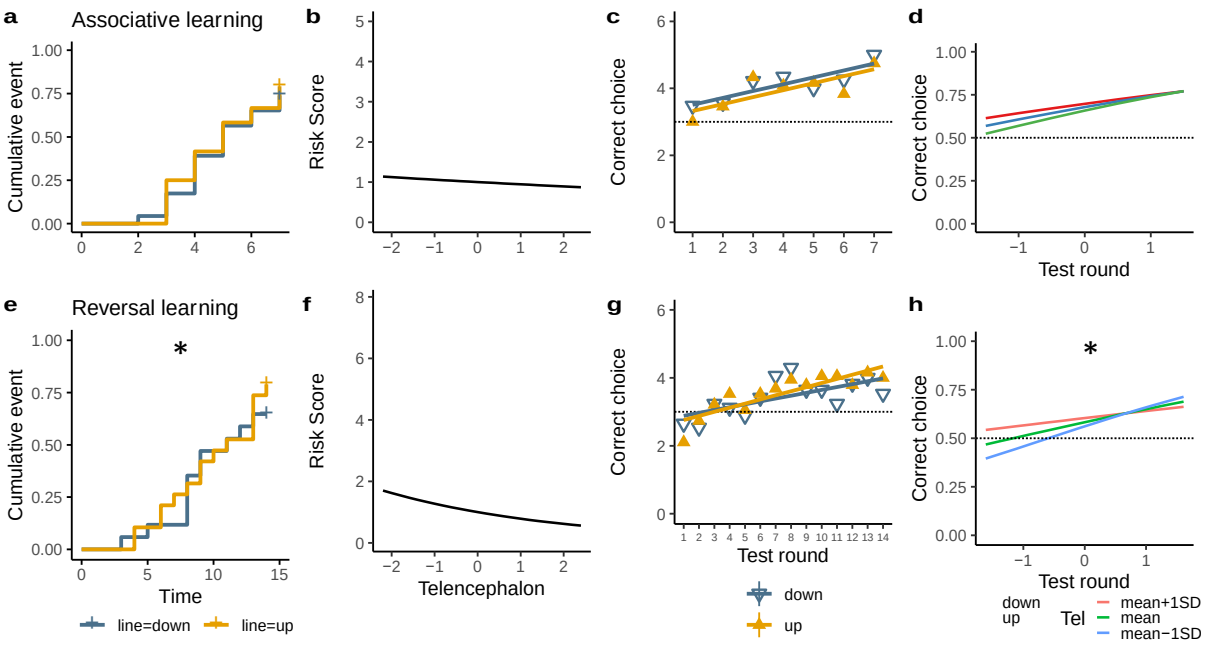

Figure 2. Performance in the associative (a, b, c and d) and reversal learning (e, f, g and h) tasks as a function of selection line and telencephalon size. Cumulative event curves of the incidence of success (i.e., learning significantly the rewarding colour) in the (**a**) associative (n = 24 up-selected vs n = 23 down-selected fish) and (**e**) reversal learning (n = 19 up-selected vs n = 17 down-selected fish) tasks as a function of selection line. Cox proportional hazards model predictions of the relationship between success score in the (**b**) associative task (N = 44) and (**f**) reversal learning task (N = 34) and individual telencephalon size. The y-axes in (**b**) and (**f**) indicate "Risk score", where higher scores read as higher success rate, and the grey area indicates the 95% CI. Average correct choices in every test round with regression slopes per selection line in the (**c**) associative and (**g**) reversal learning tasks. Dashed lines refer to the 50% success by chance. Interaction plots from the model's adjusted predictions of two continuous explanatory variables: individual telencephalon size (standardised log-transformed telencephalon size) indicated as mean, mean + 1SD, and mean - 1SD and test rounds (each round contains six test trials) in the (**d**) associative and (**h**) reversal learning task. Survival analysis; binomial GLMM; *$p < 0.05$.

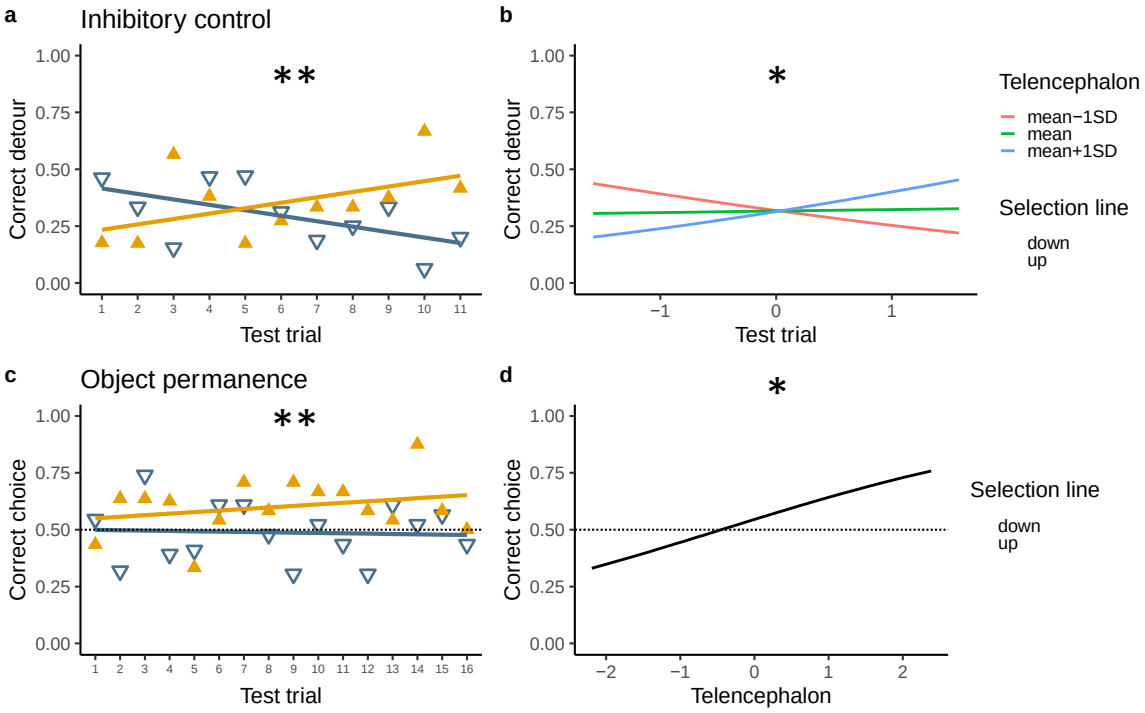

Figure 3. Performance in detour (a and b) and object permanence (c and d) tasks as a function of selection line and individual telencephalon size. (**a**) Average correct detours (i.e., detouring the transparent cylinder without touching it in the detour task) in every test trial and regression slopes as a function of selection lines (n = 24 up- selected vs n = 21 down-selected fish). (**b**) Interaction plot from the detour task model's adjusted predictions of two continuous predictor variables: individual relative telencephalon size (indicated as mean, mean + 1SD, and mean - 1SD) and test trial (N = 42). (**c**) Average correct choices (i.e., successful memorisation of object location in the object permanence task) in every test trial and regression slopes as a function of selection lines (n = 22 up-selected vs n = 22 down-selected fish). (**d**) Regression line and 95% CI estimated from adjusted predictions of the regression model testing correct choices in the object permanence task as a function of individual relative telencephalon size (standardised log-transformed telencephalon size) (N = 44). Dashed lines refer to the 50% success by chance in (**c**) and (**d**). GLMM, *$p < 0.05$, **$p < 0.01$.

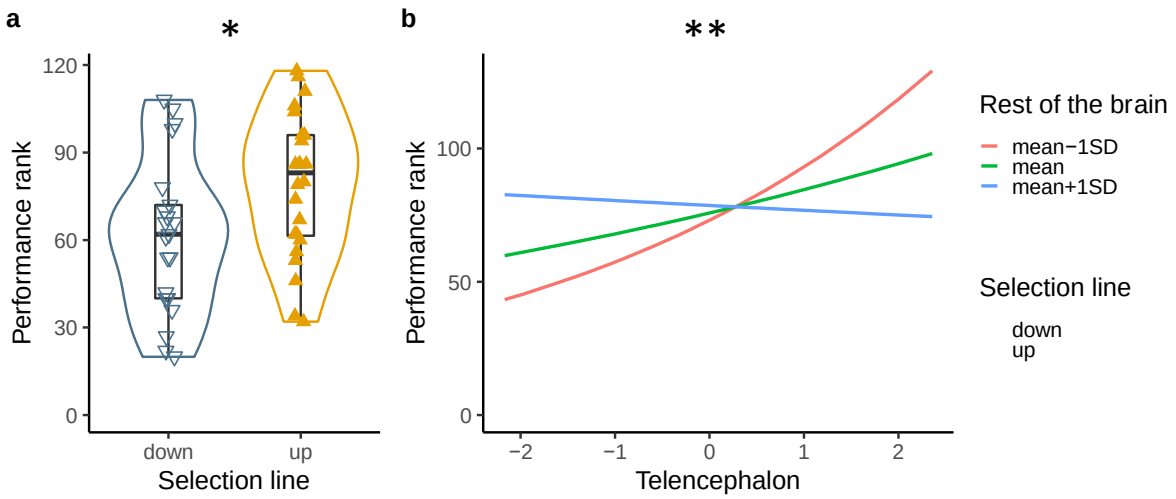

Figure 4. The individual sum of performance rank in the reversal learning, detour and object permanence tasks as a function of selection line and individual telencephalon size. (**a**) Violin and boxplots of individual rank performance as a function of selection lines (24 up- vs 21 down-selected fish). (**b**) Regression line and 95% CI estimated from the model's adjusted predictions of the relationship between individual rank performance and individual telencephalon size (standardised log-transformed telencephalon size) to the remainder of the brain (standardised and log-transformed, indicated as mean, mean + 1SD, and mean - 1SD) (N = 42). Negative binomial GLMM, *$p < 0.05$, **$p < 0.01$.

Table 1. Recapitulative table of the study findings.

Laboratory test	Cognitive ability	Telencephalon size selection lines	Individual relative telencephalon size
Colour discrimination task	Associative learning (Operant conditioning)	No differences ✕	No correlation relationship ✕
Reversal learning task	Cognitive flexibility	Up-selected lines outperformed down-selected lines ✓	Larger telencephalon facilitated learning across trials ✓
Detour task	Inhibitory control (self-control)	Up-selected lines outperformed down-selected lines across trials ✓	Larger telencephalon facilitated improved performance across trials ✓
Object permanence task	Memory of object location (working memory)	Up-selected lines outperformed down-selected lines ✓	Larger telencephalon facilitated better performance ✓

Discussion

Our results, based on artificial selection, support our hypothesis that mosaic brain evolution of telencephalon size can be a driver of executive functions. This is a finding with several important implications. First, mosaic brain evolution can be a rapid driver of cognitive evolution. In only five generations, artificial selection has resulted in ~10% divergence in relative telencephalon size, with demonstrated functional implications. Given the high costs of neuronal tissue [16,40,41], mosaic brain evolution has been suggested to be a highly energy-efficient driver of cognitive evolution whereby changes in specific brain regions match specific selective demands from the environment [17,42–46]. In the present study, we tested whether an "expensive tissue" like gut size has potentially been traded off against telencephalon size, but we found no evidence for differences in gut size as a function of the selection experiment. This result adds to the recent study by Fong et al. [17] that failed to detect any link between selection on telencephalon size and offspring reproduction, another highly costly biological aspect that previously has been found to be negatively associated with brain investment [16,41]. This is a very different picture than the one obtained during artificial selection for relative brain size in the

guppy [16]. In the guppy brain size selection lines (with matching differences in neuron number [47]), multiple energetically costly traits were reduced in the large-brained lines compared to the small-brained lines, for instance, offspring production and gut size [16] as well as immune function [48]. Based on the evidence collected so far, and in support of theory [43], mosaic evolutionary expansion of separate brain regions appears to be a much more energy-efficient driver of cognitive evolution than the expansion of the entire brain.

Second, our results suggest that relative telencephalon size is important for executive functions in the guppy and in extension that the fish telencephalon may be a homologous structure to the neocortex in mammals, at least in some functions (see also [18,19]). This finding supports recent work that has identified multiple similarities in the sensory pathways to the telencephalon between mammals, birds and sometimes even teleost fish (e.g. [49], also reviewed by Karten [50]). The emerging consensus is that more similarities than previously thought exist in telencephalon function in terms of "neocortex-like functions" across these taxa. Given the effects of telencephalon size on individual cognitive performance, our results are also in line with correlational studies documenting a positive association between executive functions and the size of the prefrontal cortex in mammals [12,51], and the pallium in birds [52]. Our experimental approach, together with this correlational evidence, suggests that more neural tissue in these structures improves cognitive capacities across vertebrates. Interestingly, we detect no differences in our non-executive function assay, the colour discrimination learning. This might be because this task does not acquire complex processing but rather basic association formation through operant conditioning [53]. This is consistent with findings from fish with the entire telencephalon being removed successfully performing simple associative learning tasks [54], while they failed in more complex tasks like reversal learning [55]. One possible mechanistic explanation is that a larger telencephalon contains more neurons and therefore possesses higher

information processing and storing capacities. It has previously been shown in the guppy that an increase in overall brain size leads to an increase in overall neuron number [47]. We suggest that a similar pattern occurs in the telencephalon size selection lines, with a higher overall number of neurons in the up-selected fish. But other possibilities exist, including differentiation in telencephalon sub-region sizes, or in connectivity.

Third, while executive functions in fish have been demonstrated many times before, suggesting substantial cognitive abilities also exist in this taxa [19,56–58], we are not aware of any previous demonstration in fish for our third assay of executive function: object permanence. Success in object permanence tasks, as performed in our study, are highly cognitively challenging and require at least substantial working memory and potentially the ability to create a mental picture of an object out of sight (Call [35] and references therein). Our results thus suggest substantial cognitive abilities in teleost fish, at least in individuals with relatively larger telencephalons. An important finding is that the large-telencephalon size selection lines displayed performance significantly higher than the null expectation (Fig 3c). This suggests that object permanence is not always a species-specific trait, but that substantial within-species variation can exist. It will be highly interesting to assay different populations in an attempt to capture the environmental variables that are linked with the expression of this trait.

Fourth, we found that up-selected fish with a larger telencephalon had smaller optic tectum, while down-selected fish with a smaller telencephalon had larger optic tectum. However, we did not find any significant changes between the up- and down-selected fish in the size of the other brain regions investigated (hypothalamus, cerebellum and dorsal medulla). The optic tectum is a brain region known as the visual processor in fish [59], and it is often comparatively larger in species that rely heavily on visual information [60,61]. That the up-selected fish performed

better in executive functions despite a smaller optic tectum, show that differences in visual perception abilities between the lines are unlikely to explain the cognitive differences. Moreover, although this negative association between telencephalon size and optic tectum size was not evident in earlier generations (see [17]), the pattern potentially reveals negative genetic correlations between the telencephalon and optic tectum. This result may indicate an energy trade-off between investment into the telencephalon and the optic tectum. But it may also mean that these two brain regions share a common developmental basis during brain regionalization. Negative associations between telencephalon size and the size of the optic tectum have been discussed before. For example, Striedter and Charvet [62] showed, based on comparing parakeet and quail, that parakeet has substantially larger telencephalon but smaller optic tectum and vice versa for quail. The authors suggest that a combination of delayed timing of telencephalic development and the amount of tissue allocated to the optic tectum could have generated such a pattern. More work is still needed to fully understand the stability across generations and the mechanistic background to the negative association between telencephalon and optic tectum sizes in these telencephalon size selection lines.

Conclusion

Our study provides experimental support for mosaic brain evolution with the selective enhancement of the telencephalon size yielding cognitive advantages in the three core executive functions: cognitive flexibility, self-control and working memory. Furthermore, costs appear to be much lower for such mosaic evolutionary changes in a brain region than for changes in overall brain size since the only cost we have revealed so far is a potential trade-off between investment in the telencephalon and the optic tectum. Finally, we add to the list of fish capabilities (with evidence for intraspecific variation) that they can also solve relatively

"complex" cognitive tasks [57,58] through our demonstration of object permanence [63], facilitated here by a larger telencephalon.

Materials and methods

Study animals

We conducted the study between April and June 2021 in the fish laboratory facilities at Stockholm University Zoology department in Sweden. We tested 48 male guppies generated from three replicated laboratory lines of Trinidadian guppies (*Poecilia reticulata*) artificially selected for having large (up-selected) or small (down-selected) relative telencephalon size. These lines were created by Fong et al. [17] in our fish lab facilities by setting up 225 breeding pairs (F0) of laboratory-based wild-type guppies and allowing them to produce at least two clutches. Then, the descendants were ranked based on the relative (telencephalon volume against the volume of the rest of the brain) size of their parents' telencephalon either as higher-rank (top 20 %) or lower-rank (bottom 20 %) individuals. Afterwards, they set up new pairs from the high- and low-ranking individuals and used them to produce the next generation (F1), which resulted in up- and down-selected lines with significantly divergent telencephalon size [17]. Next, Fong et al. repeated this process for successive generations until generation F4. In this study, we generated the fifth generation (F5) from these lines following similar methods.

Fish from the telencephalon size selection lines were kept in housing tanks separated by selection line, sex and replicate. For males, each housing tank had the capacity of 40 adult males. We then collected 48 male guppies (24 up-selected and 24 down-selected) from these housing tanks and transferred them to individual experimental aquaria (length x width x height; 40 x 15 x 15 cm) with continuously aerated water and enriched with 2 cm gravel and artificial plant. Every experimental aquarium had two guillotine doors, one see-through and one opaque.

The two doors divided the aquarium into housing and test compartments. The experimental room had an ambient temperature of ~26 °C with a light schedule of 12 hours light and 12 hours dark. In the housing aquaria, guppies received food *ad libitum* in the form of fish flakes and newly hatched brine shrimp six days per week. Once in the experimental aquaria, guppies received daily food in the form of defrosted adult brine shrimps delivered with a 1 mL transparent plastic pipette. This helped to acclimate fish to feed from the plastic pipettes, which we later used to deliver food as a positive reinforcement in the cognitive tests, where fish acquired food solely from test trials. We used only males in the present study to maximise the sample size for the tested traits instead of having two sexes with a smaller sample size for each (to fit our logistic capacities). To control for potential subconscious observer bias during data collection, the real identity of all tested fish, such as selection line treatment, was concealed by running numbers (#1, #2, etc.). We started the cognitive tests after an acclimation period of five days, and we did not perform any tests during the weekends. Furthermore, there was always at least one day break between every two cognitive tests. Unfortunately, during the acclimation period, one fish from the down-selection lines (fish ID #23) was found dead on the floor after jumping out of the experimental tank during the night. It is noteworthy to mention that repeated fish testing is unlikely to affect brain plasticity since no such short-term effects have been found in the guppy (Fong et al. 2019).

Cognitive tests

1- Colour discrimination test

The colour discrimination test consisted of a simple two-choice test to estimate fish learning abilities through operant conditioning [65] in associating a food reward with a distinct colour cue (i.e., yellow vs red). We placed a white plastic tablet with 20 small wells (10 mm ⌀ and 5 mm depth) in every experimental aquarium at the bottom of the test compartment. Only two wells

(always the same) were used repeatedly for the colour cues throughout the test. We then placed two small plastic discs (14 mm ⌀) with a small silicone knob allowing them to fit on top of these two wells, one red and one yellow. On the first day, we exposed fish to three acclimation trials to minimise excessive training, during which we placed a defrosted adult brine shrimp on top of the red disc and allowed the fish to interact with both discs and consume the food reward. On the next day, test trials started with the experimenter pulling up the opaque sliding door followed by the see-through door, which gave the fish a few seconds to see the set-up before having access to the test compartment. The experimenter then delivered a small defrosted adult brine shrimp with a plastic pipette as a food reward directly once the fish chose the correct disc, in this case the red disc even when the fish chose the red disc after inspecting the yellow disc first. We scored a choice as "correct" if a fish chose the rewarded colour (red) at its first attempt, and we scored a choice as "failure" if a fish chose the wrong colour (yellow) at its first attempt. The red disc was always the rewarding disc in this test, so we did not need to have colour as another variable in the statistical analyses. The fish (N = 47) received 42 test trials over seven days, with six trials per day (i.e., six trials = one test round) per fish. To control for side bias, we randomly presented the rewarding cue 50% of the time on the left and 50% on the right side in every test round, with no more than three presentations on the same side in succession. We set two alternative learning criteria to evaluate individual fish performance. A fish had to score either six correct choices out of six consecutive trials (i.e., during one round of six trials = 100% success) or five correct choices out of six trials in two consecutive rounds (i.e., > 80% success in each round). Additionally, these criteria meant that the probability of learning by chance was $p < 0.05$ (with a binomial test).

2- Reversal learning test

Thirty-seven fish out of the 47 successfully learned the colour discrimination test within 42 trials and passed the learning criterion, and were then admitted to the reversal learning test to estimate their cognitive flexibility abilities. After successfully learning to associate the red disc with a food reward in the discrimination learning test, the fish had to unlearn that association and learn to associate the yellow disc with a food reward in the reversal learning test (see example in Supplementary Video S1). In this test, if a fish went first to the red disc and then to the yellow disc, it did not receive a food reward. We delivered a food reward only if the fish chose the yellow disc on their first approach to any disc. In total, we ran 84 test trials of reversal learning for each individual over 14 days, with one round of tests per day (one round = six trials). Here, we also randomised the presentation of the rewarding cue on the left or the right side, as described above. We evaluated individual performance using the same criteria as in the colour discrimination learning test.

3- Detour test

The detour test evaluated self-inhibitory abilities using a transparent cylinder, a widely used test paradigm across vertebrates [5,32,66]. On the morning of Day1, we fed the fish twice with small defrosted adult brine shrimps placed on top of a green spot in the test compartment so they could familiarise themselves with the association between the colour green and food. Then, for about one hour, we exposed the fish with a transparent Plexiglas cylinder (5 cm in length and 4 cm ⌀) open on either side, but with no food reward. This served as an acclimation opportunity for the fish to explore a transparent barrier without the goal of reaching the food. In the afternoon, we started the actual tests where we placed a food reward inside the transparent cylinder on top of a green spot drawn inside the cylinder. The trial started when the experimenter pulled up the opaque and transparent barriers simultaneously and allowed the fish

to enter the test compartment. To reach the food reward, fish had to detour the physical barrier (i.e., the cylinder walls) and swim inside the cylinder to retrieve the food placed on the green spot. We recorded whether the fish detoured without touching the cylinder ("success") (see example in Supplementary Video S1) or if they touched the cylinder before retrieving the food ("failure"). In total, we ran 11 test trials per fish over three days, with one trial on Day1 and five trials on both Day2 and Day3. Two fish from the down-selected lines did not participate in the detour task yielding an overall sample size of 45 fish for this test.

4- Object permanence test

The object permanence test evaluates the ability to memorise the location of an object and the knowledge that the object still exists when out of sight [65]. Research on humans indicates that object permanence abilities develop in young children (aged between 18 and 24 months) in six stages that gradually increase in difficulty [34]. These stages range from visual tracking of moving objects (Stage 1 and 2), retrieving partially hidden objects (Stage 3 and 4), retrieving objects after visual displacement until fully hidden (Stage 5) to retrieving objects that have been invisibly displaced (Stage 6) [34,38,63]. Here, we tested 47 fish (adult guppies) in Stage 5 of the object permanence stage with visual displacement of an object until out of sight behind an opaque screen.

The object in this test consisted of a 1 mL plastic pipette cut to 9.5 cm in length, filled with water and sealed with glue so it did not float in water. We decorated the pipette with red and yellow adhesive tapes to increase its salience. This should have been particularly effective since all the tested fish had repeated exposures to these two colour cues during the previous colour discrimination and reversal learning tests. Before starting the actual object permanence tests, we exposed the fish to one acclimation exposure with the object (the coloured plastic pipette),

so they could explore it freely and receive a food reward upon being physically close to the object. All fish successfully approached the object and consumed a food reward on the first day, so we started the test trials the next day.

During the test, we placed a small Plexiglas apparatus in the test compartment. The apparatus consisted of an opaque Plexiglas screen (length x height, 6 x 8 cm) mounted on a transparent Plexiglas platform (6.5 x 6.5 cm), so it stood inside the experimental aquaria, and had another Plexiglas piece glued on its back (length x height, 3.5 x 8 cm) that prevented fish from swimming behind the opaque screen to the other side. This apparatus allowed the experimenter to visually displace the object in front of the test subject until it fully disappeared behind the screen. A test trial started by the experimenter first pulling up the opaque door (separating the home and test compartments), so the fish could see the test compartment without access. The experimenter then introduced the object in the middle of the test compartment and ensured the fish was facing (seeing) the object before displacing it. Only then the experimenter slowly moved the object either on the left or right side until it became completely hidden behind the screen. Next, fish were allowed to enter the test compartment, and the experimenter recorded whether they followed the object's path successfully or not. Locating the object by successfully following the correct path on the first attempt led fish to receive a food reward (see example in Supplementary Video S1), while failing to locate the object led to the termination of the trial without a food reward. It is worth noting that fish could not access the object if they swam the wrong path. This avoided that the subject could find the object simply by learning to search behind the screen. In total, we tested each fish in 16 trials over three days, with five trials on Day1 and Day2 and six trials on Day3. Importantly, we controlled for potential side biases and side-learning by randomising the visible displacement of the object on either side across the test trials, where we displaced the object 50% of the time on the left and 50% of the time on

the right side in random sequences with no more than three displacements on the same side in succession.

Gut size measurements

At the end of all four cognitive tests, fish were left in their test aquaria for two more days but with no food, so their guts evacuated the remaining food. We euthanised the 47 adult male guppies with an overdose of benzocaine bath (0.4 g/1). Using a digital scale, we measured their body weight to the nearest 0.01 milligrams (N = 47, mean ± SD: 107.9 ± 13.32 mg). Then, under a stereo zoom microscope Leica MZFLIII®, we dissected their guts and weighed them to the nearest 0.001 mg (range 3.98 ± 0.85 mg).

Brain morphology measurements

After dissecting the guts, with a transection cut behind the gills, we removed the heads and placed them in a 4% paraformaldehyde phosphate-buffered saline (PBS) fixation solution for five days. Upon fixation, we washed the samples twice in PBS for 10 min each before storage at 4°C pending dissection. First, we dissected the whole brain out of the skull and photographed it from the dorsal, ventral, right lateral and left lateral view under a stereo zoom microscope Leica MZFLIII® with a digital camera Leica DFC 490. Second, we estimated the length (*L*), width (*W*) and height (*H*) of the telencephalon, optic tectum, cerebellum, dorsal medulla, hypothalamus and olfactory bulb with the open-access software ImageJ [67]. Finally, we fitted the *L*, *W*, and *H* measurements in an ellipsoid function to calculate the volume (*V*) of every brain region (in mm^3) ($V = (L \times W \times H)\ \pi\ /6$) [and 44,based on 68]. Three brain samples (two up-selected and one down-selected) were damaged during the dissection process and hence provided no data. This yielded an overall sample size of 44 fish with brain morphology data.

Data analysis

We used the open-access software R version 4.2.1 [69] to run all statistical analyses and generate the figures. We fitted selection line as the explanatory variable in the analyses that focused on testing group level effects by comparing up-selected to down-selected lines. In the individual level analyses, where we tested for the effect of individual telencephalon size on performance, we fitted telencephalon size (volume in mm^3) as a continuous explanatory variable and the rest of the brain as a control covariate (mm^3) (both log-transformed and then standardised with the *scale* function [70]).

We used survival analyses with the Cox proportional hazards models to evaluate learning performance in the colour discrimination and reversal learning tests (*coxph* function from R package *survival*). For this, we replaced "death" in the classic survival analyses with "success" in the learning tests [53]. These types of *Coxph* models simultaneously test both the rate of success and failure and the time to succeed. We used the functions *ggeffect* and *ggpredict*, from R package *ggeffects*, to plot *Coxph* model predictions. Furthermore, we used a set of Linear Mixed Effect Models (LMMs) (from R package *lme4*) to test for size differences of the telencephalon, brain and gut between the up- and down-selected fish, where we fitted selection line replicate as a random factor. Also, we used a set of Generalised Linear Mixed Effect Models (GLMMs) (from R package *lme4*) with binomial error distribution to test performance (success vs failure) across test trials in the colour discrimination, reversal learning, detour, and object permanence tests. In these models, either test rounds (learning tests) or trial numbers (detour and object permanence) were standardised and added as continuous explanatory variables to the corresponding statistical model. In addition, fish identity was fitted as a random factor to account for individual repeated testing across trials. Additionally, in the GLMMs testing for group level effects, replicate was added to the models as a random factor. By

summing up individual performance rank across the three tests of executive functions: the reversal learning, detour and object permanence (see above), we fitted two GLMMs with a negative binomial distribution (due to overdispersion issues with the Poisson distribution) to test for the effects of selection line and individual relative telencephalon size.

Finally, for the post hoc analyses, we used functions from the Estimated Marginal Means R package (*emmeans*). This package allows post hoc analyses in models involving interaction terms between categorical factors and continuous predictors. We also used the function *sim_slopes* from the R package (*interactions*), which allowed us to generate statistics from an interaction term between two continuous explanatory variables. The function reduces variance in one of the two continuous variables into three values per default (mean, mean + 1SD, and mean - 1SD) and generates predictions based on the statistical model for these three values [71]. We checked that all models met their corresponding assumptions, such as normality of residuals and homogeneity of variance, dispersion in the mixed models, and the proportional hazards assumptions for *Coxph* models. For further details, please refer to our step-by-step code provided along with the data via the shared link in the Data and Code accessibility statement.

Ethics

This work was approved by the ethics research committee of the Stockholm Animal Research Ethical Permit Board [permit numbers: Dnr 17362-2019, 17402-2020].

Data availability

Data will be made available upon publication.

Author contribution

ZT and NK conceived and designed the study. SF established the telencephalon selection lines. SF and MA prepared generation F5 of the telencephalon size selection lines. ZT, MA and SVN collected the data. ZT analysed the data and generated the figures. ZT wrote the first draft. ZT and NK finalised the manuscript with input from all authors. All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

Competing interests

All authors declare that they have no conflict of interest.

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