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Research Report

Experimental expansion of relative telencephalon size improves the main executive function abilities in guppy

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Abstract

Executive functions are a set of cognitive control processes required for optimizing goal-directed behavior. Despite more than two centuries of research on executive functions, mostly in humans and nonhuman primates, there is still a knowledge gap in what constitutes the mechanistic basis of evolutionary variation in executive function abilities. Here, we show experimentally that size changes in a forebrain structure (i.e. telencephalon) underlie individual variation in executive function capacities in a fish. For this, we used male guppies (*Poecilia reticulata*) issued from artificial selection lines with substantial differences in telencephalon size relative to the rest of the brain. We tested fish from the up- and down-selected lines not only in three tasks for the 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 relatively larger telencephalons outperformed individuals with smaller telencephalons in all three executive function assays but not in the conditioning assay. Based on our findings, we propose that the telencephalon is the executive brain in teleost fish. Together, it suggests that selective enlargement of key brain structures with distinct functions, like the fish telencephalon, is a potent evolutionary pathway toward evolutionary enhancement of advanced cognitive abilities in vertebrates.

Keywords: telencephalon, reversal learning, detour task, object permanence, brain morphology

Significance Statement

Executive functions are advanced cognitive abilities that make it possible to "take the time to think before acting when meeting unanticipated challenges." They are hence essential in the day-to-day life of humans and other vertebrates. While substantial literature exists, mostly in humans and nonhuman primates, on the ontogenetic development of executive functions, virtually nothing is known about how they evolve at the species level. Based on a combination of multiple generations of artificial selection experiments on brain morphology in the guppy and cognitive assays, we show that telencephalon enlargement improves individual abilities of the main executive functions. These findings have far-reaching implications in advancing our understanding of the underlying mechanisms of executive function capacities.

Introduction

Executive functions are processes of general purpose that serve to control goal-oriented behaviors. 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 modulate several cognitive subprocesses, and hence, they regulate cognition dynamics (1, 2). For instance, cognitive flexibility is the capacity to switch attention and adjust behavior with changing demands. This allows individuals to adapt and change their behavior and strategy in response to the environment (3). Self-control requires inhibitory control abilities or response inhibition capabilities by pausing and overriding motor impulses in response to a specific stimulus, resulting in adaptive goal-oriented behaviors when correctly performed (4, 5). Finally, working memory is holding temporary information and working with visual–spatial information (and verbal information in humans) that is no longer perceptually present, thus guiding decision-making and behavior (6, 7). These executive functions ultimately play a pivotal role in survival and reproduction and therefore have important fitness consequences (8, 9).

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, 11). Therefore, the evolutionary expansion of the neocortex—suggested to be 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 (12)—emerges as a promising candidate mechanism behind evolutionary changes in executive functions

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(11, 13, 14). However, 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 in a given trait (15–17). Here, we take advantage of a recent artificial selection experiment on relative telencephalon size in the guppy (18) to test whether size changes in this region, which include homolog structures to the mammalian neocortex (19), lead to evolutionary changes in executive functions. Across four generations, Fong et al. (18) selected for relative telencephalon size (relative to the rest of the brain) by consistently pairing fish with relatively larger telencephalons together and pairing fish with relatively smaller telencephalons together. All treatments had three independent replicates. This resulted in up-selected lines of guppies having relatively larger (about 10%) telencephalons and down-selected guppies having relatively smaller telencephalons. Their selection experiment changed the relative telencephalon size with no further apparent changes in the other regions. Importantly, the up- and down-selected fish differed from wild Trinidadian populations by having relatively larger and smaller telencephalons, respectively (18).

In fish, the telencephalon is a part of the forebrain well known for its role in various advanced perceptual and cognitive functions (20). By comparing intact subjects to subjects with partly or wholly ablated telencephalons, researchers have determined the involvement of this region in regulating spatial learning, memory, and decision-making (21–25). Moreover, although functional differences certainly exist between fish and mammals, at least in some functions, telencephalon structures like the pallium have been suggested to be homologous to the mammalian neocortex (19). However, a substantial knowledge gap remains concerning whether the telencephalon is the "executive brain" in fish and the quantitative relationship between telencephalon size and executive functions. Here, we tested whether more neural tissue in the telencephalon would enhance performance in all three core executive functions in a new generation (the fifth generation) of the guppy telencephalon size selection lines.

We tested up- and down-selected fish for their 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). We first tested the fish in a two-color discrimination task where choosing the correct color led to a food reward. For those that learned the initial cue–reward association, we reversed the reward contingency where the previously unrewarded color becomes the new rewarded cue. Thus, we tested fish's ability to adjust their behavior after reversing the cue–reward contingency, a measure of behavioral and cognitive flexibility (3, 9, 27). The task also allows for the assessment of associative learning abilities (operant conditioning) during the initial learning phase, which serves as a control test for nonexecutive cognitive ability. This is mainly due to forming associations (associative learning) not requiring complex cognitive processes. The task is also often excluded when looking into complex cognitive abilities, such as general intelligence (31, 32). 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, 14, 33). 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 toward 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 (14, 30, 33, 34). In the final task, we tested the fish's working memory in an object permanence task (8, 35–37). Object permanence tasks were initially designed to test the cognitive development of human infants (38) and later used to test the cognitive development of human infants (38) and later used to document object permanence abilities in primates (39), dogs and cats (40), marine mammals (41), and birds (42). The task tested whether fish can memorize the location of an object visibly displaced behind an opaque screen and the knowledge that the object still exists when out of sight (see 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 (17, 43). For this reason, we first tested whether an overall increase in brain volume accompanied the increased telencephalon size. Then, we tested whether changes in this brain region or the total brain as a function of the selection experiment might have yielded energetic trade-offs against gut mass. 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 not only 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 nonexecutive 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 regard to more advanced cognitive functions.

Table 1. Recapitulative table of the study findings.

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

Results

In four different cognitive tasks, we tested ($N = 48$) adult male guppies from the fifth generation of the up- and down-selected lines of telencephalon size. Upon finishing the cognitive tests, we measured the telencephalon's volume and the other four main brain regions (optic tectum, hypothalamus, cerebellum, and dorsal medulla; see Materials and Methods section). This verified that the tested fish differed in their telencephalon size as a function of the selection experiment and allowed for linking such individual variation in telencephalon size to cognitive performance. We found that the tested fish 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), $\chi^2 = 31.662$, $P < 0.001$; marginal-$R^2 = 0.74$, conditional-$R^2 = 0.81$; Fig. 1; see also Fig. S1]. There were no significant differences in the total brain size due to the selection experiment [LMM: 22 up- vs. 22 down-selected fish, estimate = 0.01 (−0.03, 0.05), $\chi^2 = 0.12$, $P = 0.732$, Fig. 3D].

For the cognitive tasks, in the color discrimination task (associative learning) (Video S1), 79% of up-selected fish were successful in learning (reaching a learning criterion of scoring significantly above chance level; see Materials and Methods section) the color–food (cue–reward) association within 42 test trials, versus 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 Table S1). Interestingly, when we reversed the color–reward contingency in the reversal learning task to test for cognitive flexibility, the up-selected fish exhibited significantly higher performance by being faster and more successful at solving the task with 63% success versus 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]. Given that we continued with testing the fish also after they reached the fixed learning criterion (see Materials and Methods section), we were able to test the amount of correct choices scored per test round (each round had six test trials). Statistical analyses of this data showed no significant differences between up- and down-selected fish in color discrimination or reversal tasks ($P > 0.05$; Fig. 1C and G; see statistics in Table S1).

In the cylinder detour task, up-selected fish outperformed down-selected fish with 37% correct detours (detouring without touching the transparent cylinder) on average versus 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 [generalized LMM (GLMM): interaction effect of selection line and test trial: $N = 45$, estimate = 0.74 (0.29, 1.19), $\chi^2 = 10.191$, $P = 0.001$; Fig. 3A]. This effect was mainly driven by performance across time (test trials), where the up-selected had improved performance across test trials [post hoc *emtrend* estimate = 0.110 (0.021, 0.20), $P = 0.015$] but down-selected fish had a deteriorating performance across trials [post hoc *emtrend* estimate = −0.123 (−0.24, −0.01), $P = 0.03$] (Fig. 3A). Finally, in the object permanence task, the up-selected fish showed 60% success on average in memorizing and following the correct path of the displaced object versus 49% in the down-selected fish within 16 test trials [GLMM: $N = 47$, estimate = 0.49 (0.15, 0.84), $\chi^2 = 7.809$, $P = 0.005$; 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 [post hoc emmeans test: estimate = 0.424

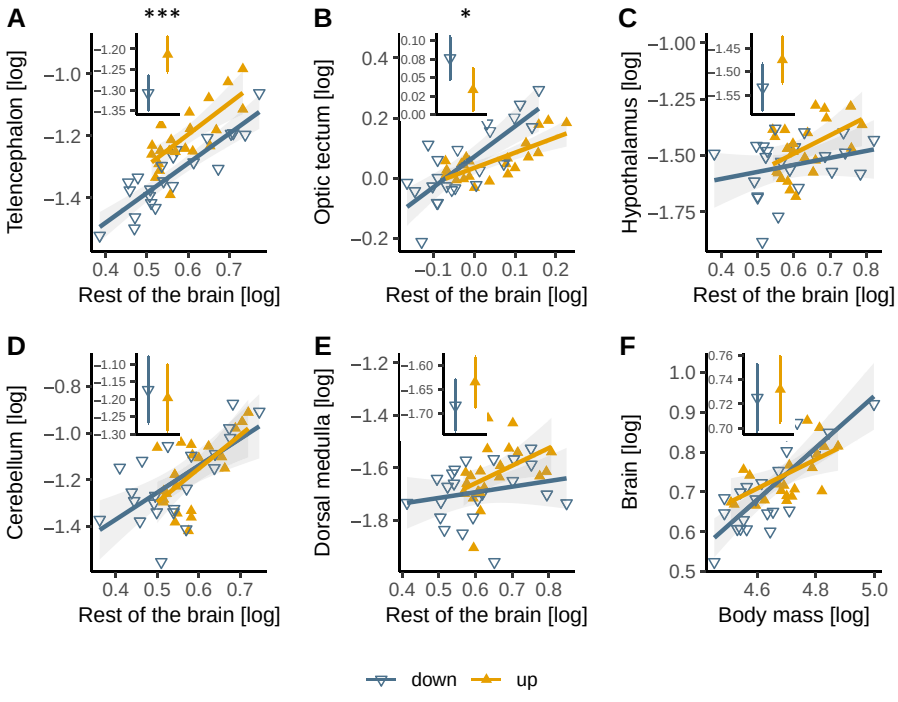

Fig. 1. Individual brain morphology from the two selection lines. Regression lines and 95% CI of log-normal transformed volume (mm^3) of the brain region of interest A) telencephalon, B) optic tectum, C) hypothalamus, D) cerebellum, and E) dorsal medulla on the log-normal transformed remainder of the 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 the depicted estimated marginal means of up- and down-selected groups with 95% CI calculated from the statistical models. Twenty-two up-selected versus 22 down-selected fish: LMM, $^*P < 0.05$, $^{***}P < 0.001$.

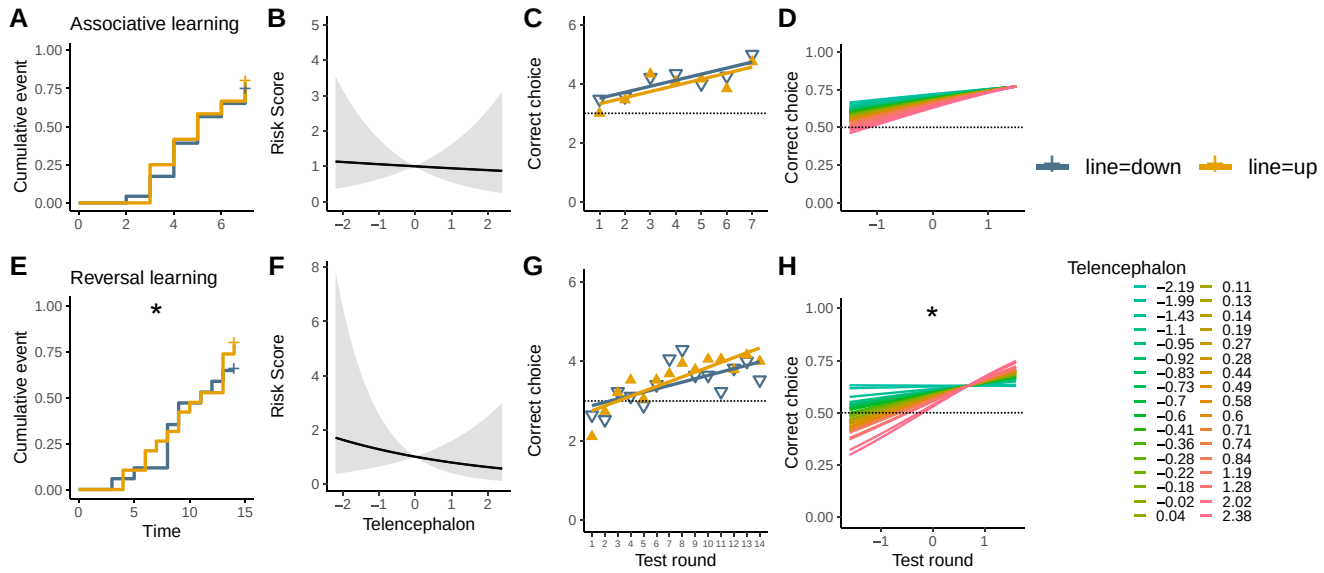

Fig. 2. Performance in the associative A, B, C, and D) and reversal learning E, F, G, and H) tasks as a function of selection line and relative telencephalon size. Cumulative event curves of the incidence of success (i.e. learning significantly the rewarding color) in the A) associative ($n = 24$ up-selected 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 relative telencephalon size. The *y*-axes in B) and F) indicate the "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 (each round contains six test trials) with regression slopes per selection line in the C) associative and G) reversal learning tasks. Dashed lines refer to the 50% success by chance. Individual learning curve as the proportion of correct choices (*y*-axis) per test round (standardized values on x-axis) for each relative telencephalon size value, shown in the color legend (standardized and log-transformed), in the D) associative and H) reversal learning task. Survival analysis; binomial GLMM; $^{*}P < 0.05$.

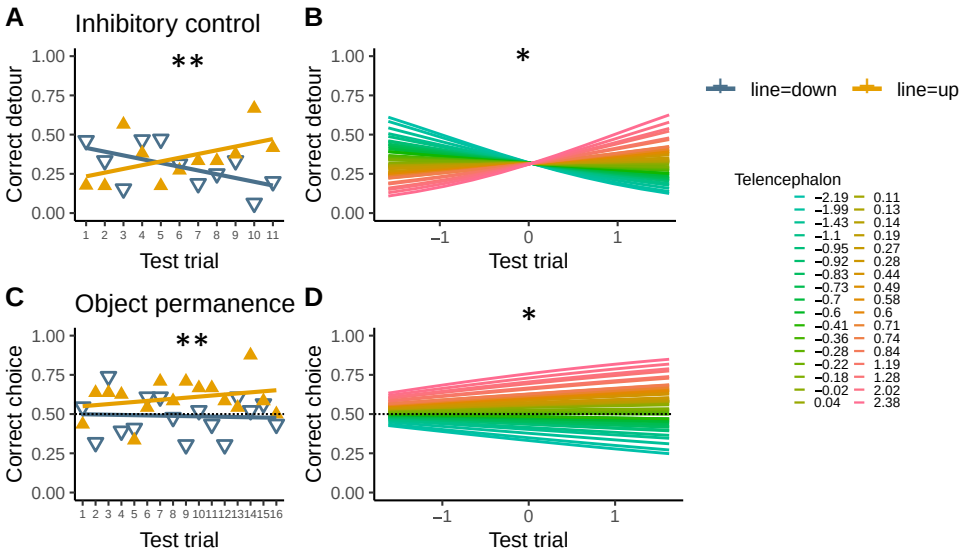

Fig. 3. Performance in detour a and B) and object permanence C and D) tasks as a function of selection line and relative telencephalon size. A) Average correct detours (i.e. detouring the transparent cylinder without touching it in the detour task) in every test trial and regression slope as a function of selection lines ($n = 24$ up-selected vs. $n = 21$ down-selected fish). B) Individual performance curve as the proportion of correct detours (*y*-axis) per test trial (standardized values on x-axis) for each relative telencephalon size value shown in the color legend (standardized and log-transformed) ($N = 42$). C) Average correct choices (i.e. successful memorization of object location in the object permanence task) in every test trial and regression slope as a function of selection lines ($n = 22$ up-selected vs. $n = 22$ down-selected fish). d) Individual performance curve as the proportion of correct localization of the object (*y*-axis) per test trial (standardized values on x-axis) for each relative telencephalon size value shown in the color legend (standardized and log-transformed) ($N = 44$). Dashed lines refer to the 50% success by chance in C) and D). GLMM, $^{*}P < 0.05$, $^{**}P < 0.01$.

(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].

Furthermore, by analyzing the individual data on brain morphology, we tested whether differences in performance between up- and down-selected fish were also linked to individual telencephalon size. Similarly to the group-level analyses, there was no statistically significant relationship between telencephalon size and performance in the color discrimination task ($P > 0.05$; Fig. 2B and D) (for further details, see Table S1). However, in the reversal learning task, although the relative telencephalon

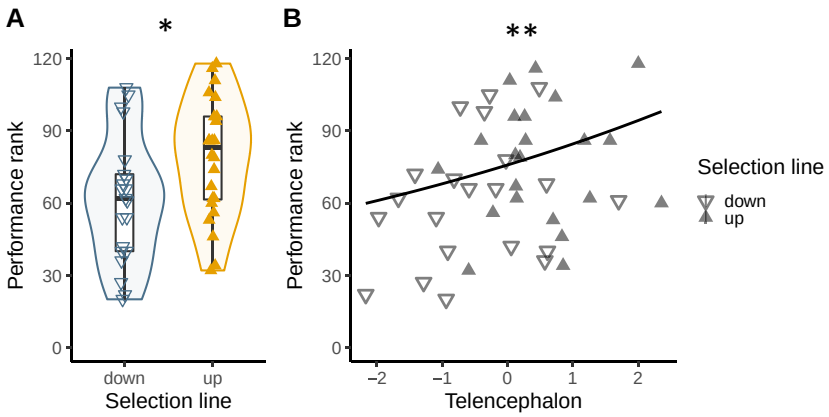

Fig. 4. The individual sum of performance rank in the reversal learning, detour, and object permanence tasks as a function of selection line and relative telencephalon size. **A)** Violin and boxplots of individual rank performance as a function of selection lines (24 up- vs. 21 down-selected fish). **B)** Regression line and 95% CI estimated from the model's adjusted predictions of the relationship between individual rank performance and relative telencephalon size (standardized and log-transformed) ($N = 42$). Negative binomial GLMM, $^{*}P < 0.05$, $^{**}P < 0.01$.

size did not explain fish performance when scored as success and failure [survival analysis: $N = 37$, HR = 0.785 (0.39, 1.57), $P = 0.63$; Fig. 2F], it did show a significant positive effect on correct choices across time (test rounds) [GLMM: interaction effect of telencephalon size and test rounds: $N = 34$, estimate = 0.13 (0.01, 0.26), $\chi^2 = 4.315$, $P = 0.038$; Fig. 2H].

In the detour task, there was also a significant effect of relative telencephalon size on performance across time (test trials) [GLMM: interaction effect of telencephalon size and test trials: $N = 42$, estimate = 0.35 (0.01, 0.69), $\chi^2 = 3.911$, $P = 0.048$; Fig. 3B]. In the final test of object permanence, we found a significant positive effect of relative telencephalon size on performance, i.e. following the correct path to the hidden object [GLMM: $N = 44$, estimate = 0.40 (0.10, 0.71), $\chi^2 = 6.517$, $P = 0.011$; Fig. 3D].

To further evaluate fish performance across the three tests of executive functions, we generated a composite score (14) 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 a 28% mean difference [GLMM: 24 up- vs. 21 down-selected fish, estimate = 0.26 (0.04, 0.48), $\chi^2 = 5.47$, $P = 0.019$, marginal-$R^2 = 0.11$, conditional-$R^2 = 0.15$; Fig. 4A]. With individual brain morphology data, the analyses showed a positive effect of relative telencephalon size on performance rank [GLMM: interaction effect of telencephalon size and rest of the brain: $N = 42$, estimate = −0.12 (−0.22, −0.03), $\chi^2 = 7.231$, $P = 0.009$, marginal-$R^2 = 0.23$, conditional-$R^2 = 0.28$; Fig. 4B].

Additionally, we explored potential size changes in the other brain regions. The analyses showed a significant difference only in the optic tectum size, with up-selected fish having relatively smaller optic tectum (relative to the rest of the brain, i.e. total brain minus optic tectum) compared with down-selected fish [LMM: estimate = −0.04 (−0.09, −0.01), $\chi^2 = 31.662$, $P = 0.045$; Fig. 1B], with no significant changes in the other regions, hypothalamus, cerebellum, and dorsal medulla ($P > 0.05$; Fig. 1C–E). Finally, our quantification of an expensive tissue, the gut mass, showed no statistically significant differences between up- and down-selected fish, nor being traded off against either total brain mass or relative telencephalon size (all P's > 0.05) (Figs. S2 and S3 and Tables S2 and S3).

Discussion

Based on artificial selection, the results show how telencephalon expansion through mosaic brain evolution can improve executive functions. In both the reversal learning and detour tasks, a relatively larger telencephalon appeared to correlate positively with performance in these two tasks (see also Triki et al. (34, 44)). This suggests that relative telencephalon size facilitated flexible learning. For the detour task, increased experience and familiarization with the transparent cylinder often yield improved detours across test trials (5). In our study, this means that fish with a relatively larger telencephalon exhibited better inhibitory control once familiarized with the cylinder. Moreover, relative telencephalon size also improved object permanence performance suggesting that fish with relatively larger telencephalon size possess enhanced working memory capacities aiding them in locating an object displaced out of sight.

Our findings have several important implications. First, artificial selection has resulted in ~10% divergence in relative telencephalon size in only five generations, with demonstrated functional implications. Given the high costs of neuronal tissue (17, 45, 46), 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 (18, 47–51). In the present study, we tested whether an "expensive tissue," like the gut, has potentially been traded off against the brain and/or telencephalon size due to the selection experiment, but we found no evidence for such trade-offs. This adds to the recent findings by Fong et al. (18) that did not detect a link between selection on relative telencephalon size and offspring reproduction, another highly costly biological aspect often associated negatively with brain investment (17, 46). This is different from another artificial selection experiment selecting for total brain size (relative to body size) in the guppy (17), where multiple energetically costly traits were reduced in the large-brained lines compared with the small-brained lines, like offspring production and gut size (17) as well as immune function (52). This absence of energy trade-offs in the selective expansion of separate brain regions suggests that mosaic brain evolution 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 contain functionally homologous structures to the neocortex in mammals (see also Mueller et al. and Ebbesson and Braithwaite (19, 20)). 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. Trinh et al. (53), also reviewed by Karten (54)). The emerging consensus is that more similarities than previously thought do 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 (13, 55) and the pallium in birds (56). 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 nonexecutive function assay, the color discrimination learning. This might be because this task does not require complex processing but rather basic association formation through operant conditioning (44). This is consistent with the findings from fish whose entire telencephalon being ablated can still perform successfully in simple associative learning tasks (57), while they failed in more complex tasks like reversal learning (58). One possible mechanistic explanation is that a larger telencephalon contains more neurons and therefore possesses higher information processing and storage capacities. It has previously been shown in the guppy that an increase in overall brain size leads to a rise in overall neuron number (59). We suggest a similar pattern occurs in the telencephalon size selection lines, with a higher number of neurons in the up-selected fish. But other possibilities exist, including differentiation in telencephalon subregion sizes or connectivity. Further studies tackling detailed morphological changes within the telencephalon due to the selection experiment and parallel studying of executive functions in males and females are needed to fully understand the cognitive function of a relatively larger telencephalon in these selection lines.

Third, while executive functions in fish have been demonstrated many times before, suggesting that substantial cognitive abilities also exist in this taxa (20, 60–62), we are not aware of any previous demonstration in fish for our third assay of executive function: object permanence. As performed in our study, success in object permanence tasks might pose cognitive challenges and require at least substantial working memory and the ability to create a mental picture of an object out of sight (Call (39) and references therein). Our results thus suggest that relatively complex cognitive processes exist in teleost fish, at least in individuals with relatively larger telencephalons. An important finding is that the up-selected lines displayed performance significantly higher than the null expectation (Fig. 3C). This suggests that object permanence is not always a species-specific trait, but 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 relatively larger telencephalon had smaller optic tectum, while down-selected fish with a smaller telencephalon had larger optic tectum. However, we found no 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 (63), and it is often comparatively larger in species that rely heavily on visual information (64, 65). The up-selected fish performing better in executive functions despite a relatively smaller optic tectum shows that differences in visual perception abilities between the lines are unlikely to explain the cognitive differences. If anything, one would expect fish with relatively larger optic tectum to perform better if visual artifacts in the cognitive test paradigms biased the information used in generating the observed behavioral output. Moreover, although this negative association between telencephalon size and optic tectum size was not evident in earlier generations (see Fong et al. (18)), 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 (66) 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 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.

Finally, using a composite score for all three executive functions served as a general measurement of cognitive performance across all three tasks (14). The concept of general intelligence is that some species (G) or individuals (g) outperform others across an array of cognitive tasks (9). The composite score generated previously by MacLean et al. (14) for self-control tests across primates correlates positively with G for these species (9). Still, tests for executive functions are often not included in calculating these intelligence scores, and yet they are known to correlate positively with such scores (9). Moreover, both MacLean et al.'s (14) composite score and Deaner et al.'s (67) G score correlated positively to absolute brain size in primates. Finding that both group-level and individual-level analyses of relative telencephalon size correlated positively with our composite cognitive score indicates, to some extent, that a "general intelligence" pattern might be facilitated by enlarged key brain regions like the telencephalon also in teleost fish (but see Aellen et al. (32)).

Conclusion

Our study provides experimental support that mosaic brain evolution with selective enhancement of telencephalon size yields 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 those for changes in overall brain size since the only cost we have revealed 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 (61, 62) through our demonstration of object permanence (68), facilitated here by a relatively 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 telencephalons. These lines were created by Fong et al. (18) 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' telencephalons either as higher-rank (top 20%) or lower-rank (bottom 20%) individuals. Afterward, 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 (18). 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 for 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 × width × height: 40 × 15 × 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-h light and 12-h dark. In the housing aquaria, guppies received food ad libitum in the form of fish flakes and newly hatched brine shrimp 6 days/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 maximize 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 5 days and did not perform any tests on weekends. Furthermore, there was always at least 1-day break between every two cognitive tests. Unfortunately, during the acclimation period, one fish from the down-selected 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 (see Fong et al. (69)). In all tests, the between-trial interval for every fish was about 50 min.

Cognitive tests

Color discrimination test

The color discrimination test consisted of a simple two-choice test to estimate fish learning abilities through operant conditioning (70) in associating a food reward with a distinct color 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 color 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 the fish to three acclimation trials to minimize 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 setup 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 (by touching the disc and/or swimming very close to the disc as shown in Videos S1), 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 color (red) at its first attempt, and we scored a choice as "failure" if a fish chose the wrong color (yellow) on its first attempt. The red disc was always the rewarding disc in this test. We chose red—guppies often have a color preference toward red (28)—as the first rewarding color to facilitate the associative learning phase and avoid adding color as an extra variable in our statistics (e.g. Triki and Bshary (30)). 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). Regardless of passing or failing, all fish ($N = 47$) received 42 test trials over seven days, with six trials per day (i.e. six trials = one test round) per fish. This number of trials allowed at least 70% of the tested fish to pass the test.

Reversal learning test

Thirty-seven fish out of the 47 successfully learned the color 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 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. Here, we also randomized 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 color discrimination learning test. 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). This number of trials allowed at least 70% of the tested fish to pass the test.

Detour test

The detour test evaluated self-inhibitory abilities using a transparent cylinder, a widely used test paradigm across vertebrates (5, 33, 71). On the morning of day 1, 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 familiarize themselves with the association between the color green and food. Then, for about 1 h, we exposed the fish to a transparent Plexiglas cylinder (5 cm long 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 began 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 Video S1) or if they touched the cylinder before retrieving the food (failure). In total, we ran 11 test trials per fish over 3 days, with one trial on day 1 and five trials on both days 2 and 3 (with one more trial than the usual 10 trials for detour tests (34)). 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.

Object permanence test

The object permanence test evaluates the ability to memorize the location of an object and the knowledge that the object still exists when out of sight (70). 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 (38). These stages range from visual tracking of moving objects (stages 1 and 2), retrieving partially hidden objects (stages 3 and 4), and retrieving objects after visual displacement until fully hidden (stage 5) to retrieving objects that have been invisibly displaced (stage 6) (38, 42, 68). 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 color cues during the previous color discrimination and reversal learning tests. Before starting the actual object permanence tests, we exposed the fish to one acclimation exposure with the object (the colored 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 × height: 6 × 8 cm) mounted on a transparent Plexiglas platform (6.5 × 6.5 cm), so it stood inside the experimental aquaria and had another Plexiglas piece glued on its back (length × height: 3.5 × 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 was started by the experimenter by 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 on the right side until it became completely hidden behind the screen. Next, within the first 10 s of having the object out of sight, the 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 the fish to receive a food reward (see example in 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 (number of trials decided based on previous studies using between 15 and 20 test trials (32, 41)) over 3 days, with five trials on days 1 and 2 and six trials on day 3. Importantly, we controlled for potential side biases and side learning by randomizing 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 2 more days but without food, so their guts evacuated the remaining food. We euthanized the 47 adult male guppies with an overdose of benzocaine bath (0.4 g/L). Using a digital scale, we measured their body weight to the nearest 0.01 mg ($N = 47$, 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 from 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 (72). 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 White and Brown (49), based on Pollen et al. (73)). 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 (74) to run all statistical analyses and generate the figures. We fitted the selection line as the explanatory variable in the analyses that tested 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 standardized with the *scale* function (75)].

We used survival analyses with the Cox proportional hazards models to evaluate learning performance in the color 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 (44). 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 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 GLMMs (from R package *lme4*) with binomial error distribution to test performance (success vs. failure) across test trials in the color discrimination, reversal learning, detour, and object permanence tests. In these models, either test rounds (learning tests) or trial numbers (detour and object permanence) were standardized 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 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 availability 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).

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Supplementary material

Supplementary material is available at *PNAS Nexus* online.

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Author contribution

Z.T. and N.K. conceived and designed the study. S.F. established the telencephalon selection lines. S.F. and M.A. prepared generation F5 of the telencephalon size selection lines. Z.T., M.A., and S.V.-N. collected the data. Z.T. analyzed the data and generated the figures. Z.T. wrote the first draft. Z.T. and N.K. finalized 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. A preprint version of this article is available on EcoEvoRxiv (76).

Data availability

Source data from this study are archived at the Figshare data repository by Triki et al. (77) (https://doi.org/10.6084/m9.figshare.19738435). Analyses reported in this article can be reproduced using the code archived at the Figshare data repository by Triki et al. (77) (https://doi.org/10.6084/m9.figshare.19738435).

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