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RESEARCH

Brain anatomy of a clonal fish, the Amazon Molly *(Poecilia formosa)*: effects of early-life environment

Hayo G. Nadema[1] · Tyler J. Reich[2,4] · Eline J. E. Weenink[1] · Debbie Cunningham[3] · Elvis Wong[5] · Ingo Schlupp[2,4] · Alexander Kotrschal[1]

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Abstract
During ontogeny, environmental factors can shape trait development and resource allocation to aid survival, reflecting adaptive plasticity. This may include differential investment in brain regions in response to cognitive challenges. Especially the brain anatomy of fish seems to respond readily to biotic and abiotic factors. However, we currently lack sufficient data to determine the extent to which such changes are consistent across species. To extend the phylogenetic breadth in this endeavour, we studied how brain anatomy of Amazon mollies (*Poecilia formosa*) responds to the presence or absence of physical enrichment and/or visual neighbours. The Amazon molly is a clonal species and genetically highly uniform, so that differences in brain anatomy of individuals from the same clonal lineage are most likely due to the environmental conditions provided, rather than inherent genetic differences. We quantified brain anatomy by determining the volumes of six main brain regions (olfactory bulb; telencephalon; optic tectum; cerebellum; dorsal medulla; hypothalamus) and overall brain size. While brain anatomy was overall unaffected by the visual presence of a conspecific, we found that the cerebellum was larger in animals reared in an environmentally complex habitat. Our results corroborate the idea that use of certain cognitive domains fosters enlargement in the region governing those.

Keywords Amazon molly · Brain size · Ontogeny · Environmental effects · Environmental enrichment · Fish · Neighbour presence · Poecilia formosa

Introduction

The environment shapes many traits, often through phenotypic plasticity: extreme weather produces individuals adapted to famine or non-optimal temperatures (Reznick and Braun 1987; Dussault et al. 2004); high altitude produces more saturated blood and metabolic adaptations (Qui et al. 2012); predator presence shapes escape behaviour (Pollen et al. 2007). One form of plastic adaptation is the development of neural pathways in the brain (Taubert

Hayo G. Nadema and Tyler J. Reich contributed equally to this work.

Extended author information available on the last page of the article

et al. 2011). Neural pathways are dynamically created in response to the experiences of an individual and so underlie the processing of information and execution of actions. These experiences can come from acute events, such as getting sick after eating a foreign food object or seeing a conspecific being predated upon (du Toit et al. 1991; Kelley and Magurran 2003). Similarly, longer-term experiences, for instance via ecological variation, can shape neural pathways when individuals live in regions with higher predator density, different food sources, or different climates, which can affect escape behaviour, perception, and foraging behaviour (Huber et al. 1997; Ehlman et al. 2015; Reddon et al. 2018). Such phenotypic plasticity can be constrained by several factors, including the genetic background (Murren et al. 2015), leading to differences in scope for plasticity between genotypes within a population. Hence, to understand the role of plasticity in how animals respond to different ecological conditions it is vital to control for genetic differences. One approach is to use naturally clonal species (i.e., animals with no genetic differences) when investigating the impact of ecology on the developing brain. We did this by raising individuals of a clonal, livebearing fish, the Amazon molly (*Poecilia formosa*) under four different conditions and then examined their brain anatomy.

Teleost fishes in general, and livebearing fishes (Poeciliidae) in particular, provide widely used model species to investigate the effects of environmental conditions on brain region development (Ebbeson and Braithwaite, 2012, Maruska et al. 2019). They are a well-studied group, occurring in a wide range of habitats in nature (Evans et al. 2011). Among these, the genus *Poecilia* includes useful model species for research due to their short generation time, high sociality, and numerous studies characterizing many aspects of the ecology and evolution of these fishes (Houde 1997; Magurran 2005; Broglio et al. 2011). We already have solid data on different environmental factors affecting brain anatomy in the guppy, *Poecilia reticulata*, where diet and microhabitat use affect the size of the telencephalon (Huber et al. 1997); social grouping and interactions affect males and females in different ways (Kotrschal et al. 2012a, b, c, 2017), lotic environments produce more environmental diversity and result in an increased size of the optic tectum (Reyes et al. 2022); and first-generation of lab-rearing results in smaller brains than wild individuals (Burns et al. 2009).

Here, we specifically study the effects of physical and social enrichment on the brain anatomy of Amazon mollies, a close relative of the guppy with an important difference: this species is naturally asexual and clonal (Hubbs and Hubbs 1932). The Amazon molly is an all-female species of hybrid origin that reproduces via gynogenesis (Lamatsch and Stöck, 2010; Schlupp 2005). Amazon mollies clonally produce diploid eggs that are pseudofertilized by sperm from heterospecific males (Hubbs and Hubbs 1932). Amazon mollies have no functional meiosis (Dedukh et al. 2022; Rasch et al. 1982), and offspring are genetically identical with the mother, barring mutations and very rare paternal introgression (Nanda et al. 1995; Rasch et al. 1965). Especially, in clonal lineages that go back to a single mother, genetic differences are expected to be minimal (Makowicz et al. 2016), and certainly far lower as compared to species that reproduce sexually. Amazon mollies are capable of recognizing minimal differences between clonal lineages (Makowicz et al. 2016) and alter their behaviour based on this (Makowicz et al. 2018a, b), while related sexual species do not (Makowicz et al. 2018a, b). Thus, we posit that the Amazon molly, having a highly uniform genetic background, naturally provides a model for ontogenetic studies utilizing the very limited genetic variability of phenotypic plasticity between individuals to reduce genetic background noise (Laskowski et al. 2019).

Looking at the role each brain region has in the functioning of teleost fishes, it becomes possible to predict how environmental enrichment and neighbour presence may affect brain anatomy. For example, the olfactory bulb is primarily affected by olfactory environmental cues and can be less developed in microsmatic habitats, possibly due to energy constraints leading to trade-offs between brain regions during development (Pollen et al. 2007; Gonda et al. 2009). The function of the telencephalon has been related to habitat use, learning, and social interaction (van Staaden et al. 1995; Huber et al. 1997; Pollen et al. 2007). As a result, abiotic factors such as environmental enrichment may require more investment in the telencephalon as foraging and sheltering becomes more varied and conditional, whereas biotic factors such as social presence stimulate this region to develop more (Gonda et al. 2009). The optic tectum is relevant for perception and subsequently behavioural predator avoidance (Huber et al. 1997; Kotrschal et al. 2017; Corral-Lopez et al. 2023). Both skills would require more investment as the environment becomes more complex, but predator avoidance may decrease as social presence increases, because vigilance may be distributed across multiple individuals. While in female guppies, the optic tectum increased in animals kept with same sex?, compared to the opposite sex (Kotrschal et al. 2012a, b, c), it is unclear how the presence/absence of a neighbor may impact its plasticity. The cerebellum mainly governs motor functions, and is influenced by predator presence, as well as environmental complexity - as quick response and movement towards shelter can become more intricate when there are more possible obstacles around the habitat (Pollen et al. 2007; Kotrschal et al. 2017). In Steelhead salmon (*Oncorhynchus mykiss*), individuals reared in a more complex environment developed a larger cerebellum (Kihslinger and Nevitt 2006). The dorsal medulla shares functions with the cerebellum in motor skills but is also important in auditory processing and body balance (Kozloski and Crawford 2000). While relevant for predator detection, environmental complexity may not be as much of a driving force in this region as for the cerebellum and may occasionally reduce in size as developmental demands are drawn to other brain regions (White and Brown 2015). Finally, the hypothalamus plays a major role in homeostasis and appetite and shows little evidence of being affected by environmental complexity or sociality (Lin et al. 2000).

Here, we investigate the effects of visual neighbour presence and environmental enrichment on the development of the different brain regions in the Amazon molly. We measured the volumes of six main brain regions (olfactory bulb, telencephalon, optic tectum, cerebellum, dorsal medulla, hypothalamus) and the sum of those (brain size), and compared them across four different treatments differing in how much complexity the environment of the fishes provided. If the brains of Amazon mollies show similar phenotypic plasticity as those of other fishes described above, we expect to see reduced investment in the olfactory bulb in the presence of physical enrichment, but greater investment in the telencephalon, optic tectum, and cerebellum, and a larger overall brain size in Amazon mollies raised with physical enrichment than those raised without. For social enrichment, we abstain from such predictions as the literature is remarkably scarce on this topic.

Methods

Sample acquisition

The sample fish for this experiment were the offspring of 9 randomly selected female Amazon mollies, born between May 8, 2021, and August 3, 2021. They were selected at the International Stock Center for Livebearing Fishes, part of the Aquatic Research Facility of the Department of Biology at the University of Oklahoma. Females were eligible for selection if they had a known birthdate, had fed recently, and were showing no divergent behaviour. They were then kept individually in 9 L to 40 L tanks and checked daily for offspring. All 9 females produced offspring. We used individuals from a single clone (VI/17), identical to the one used in sequencing the Amazon molly genome (Warren et al., 2018). This ensured that any differences between the individual sample fish are most likely due to differences in treatment, rather than any genetic diversity caused by sexual reproduction and meiosis. Once offspring were detected, we returned the female to the stock tank and raised the offspring with *ad libitum* food until they were 30 days of age. These individuals were used in the experiment described below.

Experimental setup

After day 30, we isolated the sample fish ($N = 80$) and kept them individually in 5 L freshwater tanks until day 300. We performed weekly water changes and fed the offspring daily *ad libitum* with commercially available TetraMin flake food, occasionally supplemented with Hikari frozen bloodworms (mosquito larvae), *Daphnia*, and brine shrimp nauplii. We kept the tanks inside a temperature-controlled greenhouse under natural light conditions, placed across a dedicated rack. The tanks were set up to accommodate two different treatments: visual access to a neighbour, and the presence of environmental enrichment. Tanks with visual access to a neighbour were placed next to other tanks, allowing visual communication, while tanks without access were visually isolated using manila folders. Tanks with environmental enrichment contained a single layer of gravel on the bottom and a PVC half-pipe (8 cm long and 3 cm wide) to serve as a hiding place, and a sponge filter, while tanks without enrichment only contained a sponge filter. Tank placement was haphazard due to the requirements of the neighbour treatment: some tanks needed to be placed next to a visual neighbour. For a satellite study (Cunnigham et al., 2024), we removed the sample fish using a dip-net and took measurements of their size and mass every 30 days from day 90 onwards, up until day 300. Each tank was checked daily for sick or deceased individuals. Sick individuals were treated ($N = 1$), while deceased ones were discarded ($N = 12$). If the removal of an individual led to a fish in the neighbouring tank being isolated, we placed a replacement Amazon molly in the emptied tank to keep the treatment of the neighbour continuous.

Brain extraction & photography

On day 300, we humanely euthanised the sample fish using an overdose of buffered Tricaine S and subsequently conserved them in buffered formaldehyde. We measured the standard length (mm, from the tip to the snout to the end of the caudal peduncle) and body mass (g) of each sample fish before removing the brains and weighing them (mg). Brains that were

heavily damaged during extraction were discarded (N=6). These cases were evenly distributed across the treatments. After eliminating all damaged samples the final sample size was 62 ($N_{Unenriched, NoNeighbour}$ = 14, $N_{Enriched, NoNeighbour}$ = 16, $N_{Unenriched, Neighbour}$ = 13, and $N_{Enriched, Neighbour}$ = 19).

We took images of the sample brains using a microscopy camera with a resolution of 8.83 mm by 6.62 mm (1280 × 960 px). Samples were placed at a consistent depth in water to standardise light refraction across images and were placed so that paired lobes would not be affected by size shifts due to perspective (Kotrschal et al. 2012a, b, c, 2013a, b).

Volumetric measurements

For measuring brain region volumes, we defined length, width, and height parameters for the olfactory bulb, telencephalon, optic tectum, cerebellum, dorsal medulla, and the hypothalamus (Kotrschal et al. 2012a, b, c, 2013a, b) (Fig. 1). Length and height measurements were taken from lateral images, while width was taken from the dorsal and ventral images. The width of the olfactory bulb is the lateral projection from the midline, at the olfactory bulb-

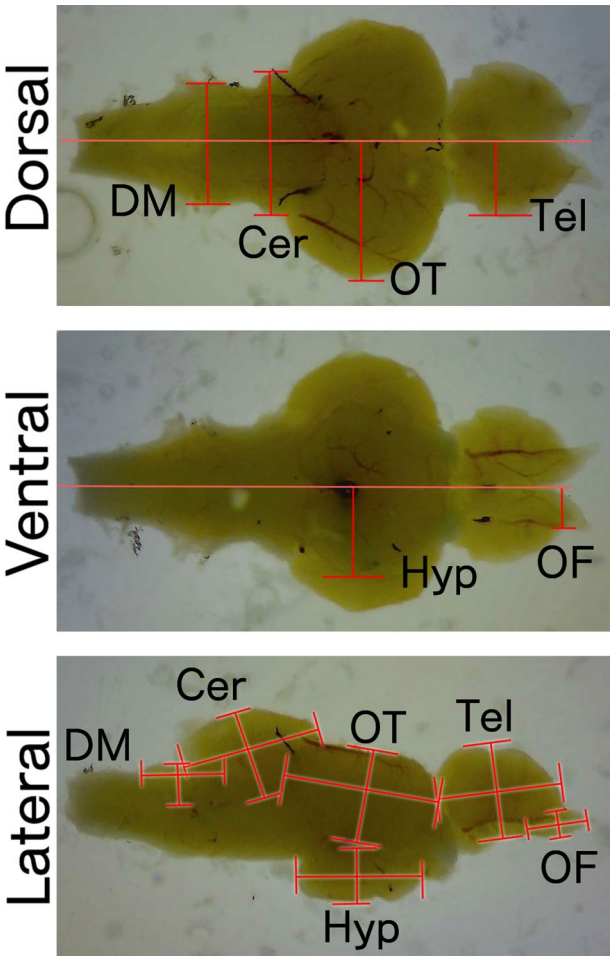

Fig. 1 Range parameters for measuring the volumes of Amazon molly (*P. formosa*) brains from dorsal (top), ventral (middle), and lateral (bottom) view: olfactory bulb (OF), telencephalon (Tel), optic tectum (OT), cerebellum (CER), dorsal medulla (DM), and hypothalamus (Hyp)

telencephalon cleft. The length is the largest distance between anterior and posterior apices of the olfactory bulb. The height is the largest distance between apices perpendicular to the length. The width of the telencephalon is the largest lateral projection from the midline. The length is the distance between the olfactory bulb-telencephalon cleft and the telencephalon-optic tectum cleft. The height is the distance between the dorsal surface and ventral surface perpendicular to the length, excluding the olfactory bulb and the optic chiasm. The width of the optic tectum is the largest lateral projection from the midline. The length is the distance between the anterior and posterior apices, denoted by a shift in light refraction in the images. The height is the distance between the dorsal and ventral apices, denoted by a shift in light refraction in the images. The width of the cerebellum is the largest distance between the lateral apices posterior to the optic tectum-cerebellum cleft, perpendicular to the midline. The length is the distance between the optic tectum-cerebellum cleft and the cerebellum-dorsal medulla cleft. The height is the distance between the dorsal surface of the cerebellum and the midline of the brain stem, perpendicular to the length. The width of the dorsal medulla is the largest distance between the lateral apices, perpendicular to the midline. The length is twice the distance between the cerebellum-dorsal medulla cleft and the dorsal medulla-brain stem cleft. The height is the distance between the dorsal apex and the midline of the brain stem. The width of the hypothalamus is the largest lateral projection from the midline. The length is the distance between the anterior and posterior apices. The height is the distance between the dorsal and ventral apices, denoted by a shift in light refraction in the images. Measurements were taken by the same person, blinded to the treatments. The volume of each brain region was calculated using the formula:

$$\mathrm{V} = \mathrm{LWH}(\pi/6)$$

Volumes for the olfactory bulb, telencephalon, optic tectum, and hypothalamus were multiplied by 2 to account for there being two lobes of each of these regions present. After measuring, we reviewed the data for outliers and errors using correlation scatterplots of the volumes of the brain regions and re-measured any data point that fell outside the general cloud. We also reviewed and re-measured any data point that was more than two standard deviations removed from the mean of its category. Details on brain volumes can be found in the Appendix (Table 2). To evaluate repeatability, we randomly selected 9 sample brains and remeasured them to calculate the intraclass correlation coefficient (ICC) based on a two-way model with agreement as type. The ICC for total brain volume was excellent (ICC = 0.974, $P < 0.001$). ICC scores of the different brain regions were moderate to excellent (ICC = 0.602–0.983, $p < 0.05$).

Statistical analysis

All analyses were performed in R (R Core Team 2024). We performed a linear regression to examine the relationship between brain weight and body size, environmental enrichment, and the presence of a neighbour. We used standard length as a measure of body size:

$$Brain\ weight \sim Body\ size * Treatment_{enrichment} * Treatment_{neighbour}$$

For the brain region volumes, we first calculated the volume of the rest of the brain volume (total brain volume minus the volume of the specific region) for each region. We ran linear regressions to determine the impact of enrichment and the presence of a neighbour on the brain volume of the different regions according to the following scheme:

$$V_{region} \sim V_{rest} * Treatment_{enrichment} * Treatment_{neighbour}$$

Subsequently, we conducted stepwise model simplifications by removing higher-order interactions and comparing nested models using likelihood ratio tests via analysis of variance (ANOVA). This continued until we obtained a minimal model that included the two treatments and the covariate, either without interactions or with only significant interaction terms. Final model parameteter estimates and p-values were obtained from the model summary.We examined model assumptions by inspecting diagnostic plots for linearity, normality, homoscedasity, and influential observations. In addition, we tested for heteroscedasticity on the full model using Breusch-Pagan tests from the *lmtest* package (Zeileis and Hothorn 2002). Across all regression models, Breusch-Pagan tests yielded non-significant results, suggesting that the assumption of homoscedascity was met (Table 3, Appendix).

To address potential allometric effects, we also fitted equivalent models to log-transformed numerical variables (brain weight, body size, volume of the brain region, and volume of the rest of the brain). Model selection and assumption checks followed the same procedure as for untransformed data. All data and statistical code can be found in the Appendix.

Results

We found that the mollies that experienced an enriched environment showed larger cerebella than mollies from tanks without enrichment (Fig. 2; Table 1). In addition, the increase in hypothalamus volume associated with each unit increase in the rest of the brain volume is smaller when visual neighbours are present (Table 1). Results of the allometric models revealed qualitatively similar results (Table 4, Appendix).

Discussion

We studied how rearing conditions affect brain anatomy in the Amazon molly. To the best of our knowledge, this is the first test of brain plasticity in any clonal fish species. We specifically used physical and social enrichment to trigger potential plastic changes in brain anatomy. While we did not find any brain anatomical effects in response to manipulations of the social environment, we did find an increase in the size of the cerebellum in the animals reared with physical enrichment as compared to such reared in barren tanks. We posit that the cerebellar increase is due to the changed locomotor demands of the molly in an environment with environmental enrichment. Such a three-dimensional structure provides more complex shelter and escape opportunities, which could mean that the animals in such environments utilise short bursts of movement during escape, in contrast to longer pursuits in environments lacking three-dimensional structures. This may therefore promote more prominent cerebellar development in structure-rich habitats (Kotrschal et al. 2012a, b, c).

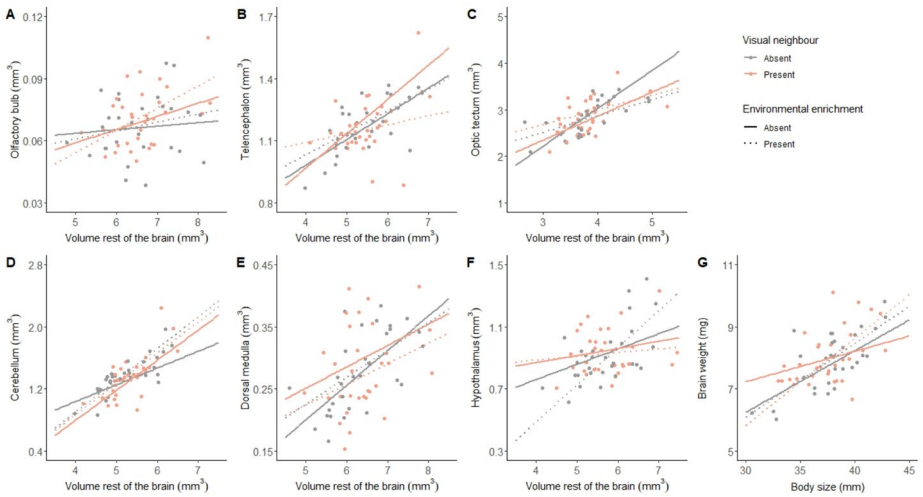

Fig. 2 Relationships between brain region volumes and the volume of the rest of the brain across different treatments for (**A**) olfactory bulb, (**B**) telencephalon, (**C**) optic tectum, (**D**) cerebellum, (**E**) dorsal medulla, and (**F**) hypothalamus. (**G**) shows the relationship between total brain weight and body size across treatments. Dots represent individual measurements. Colours indicate the presence or absence of a visual neighbour, whereas line type indicates absence or presence of environmental enrichment

Dedicated behavioural trials will be necessary to clarify if cerebellar volume differences indeed translate into differences in spatial behaviour.

The fact that we did not find any other effect of either physical or social enrichment on brain anatomy contrasts with several previous studies that generally find widespread plastic adjustments in fish brain in response to such treatments (Huber et al. 1997; Magurran 2005; Reyes et al. 2022). This may be due to three non-mutually exclusive reasons. First, fishes are the most speciose group of vertebrates and differ vastly in ecology (Helfman et al. 2009), hence also their holding conditions in the laboratory are very different from each other. Experiments comparing species in how their brains react to environmental factors will therefore need to use different manipulations, even when using the same language to describe the animal care conditions. For instance, 'physical enrichment' in a 4000 L Coho salmon (*Oncorhynchus kisutch*) aquaculture tank mimicking a temperate limnetic habitat (Kotrschal et al. 2012a) will inherently differ from physical enrichment in a 3 L aquarium mimicking tropical benthic habitat (Kotrschal et al. 2012b). Second, those same differences in ecology may predispose the brains of different species to different degrees of plasticity in response to environmental challenges. While pioneer species selected for fast adjustments to novel environments may show high levels of plasticity to facilitate niche exploration also on the cognitive level, specialist species adapted to very stable environments, such as deep-sea species, may not benefit from cognitive plasticity due to the high predictability of their environments, and hence show limited scope for brain plasticity. In the case of a clonal species, suchg as the Amazon molly used here, plasticity may be maladaptive as clonality is thought to evolve and persist especially in highly stable environmnets (Vrijenhoek 1979) where fine scale adjustments via plasticity may be not necessary. Indeed, in plants clonal species show much lower levels of plasticity than non-clonal species (Fazlioglu and Bonser 2016). In our animals, the fact that most of the brain regions did not respond to either of

Table 1 Estimate effects of environmental enrichment and visual neighbours on brain anatomy of Amazon mollies

Brain region	Coefficient	Estimate	S.E.	t-value	*P*-value
Brain weight	Body size	0.20	0.04	5.60	**<0.001**
	Neighbour	0.16	0.19	0.85	0.40
	Enrichment	0.06	0.19	0.33	0.74
Olfactory bulb	Rest of brain	0.01	0.00	2.27	**0.03**
	Neighbour	0.00	0.00	1.07	0.29
	Enrichment	0.00	0.00	0.43	0.67
Telencephalon	Rest of brain	0.11	0.02	4.94	**<0.001**
	Neighbour	−0.01	0.03	−0.28	0.78
	Enrichment	−0.02	0.03	−0.59	0.56
Optic tectum	Rest of brain	0.46	0.08	5.71	**0.001**
	Neighbour	0.05	0.07	0.68	0.50
	Enrichment	0.06	0.08	0.83	0.41
Cerebellum	Rest of brain	0.34	0.04	8.59	**0.001**
	Neighbour	−0.04	0.04	−1.04	0.30
	Enrichment	0.10	0.04	2.25	**0.03**
Dorsal medulla	Rest of brain	0.04	0.01	3.96	**<0.001**
	Neighbour	0.00	0.02	0.10	0.92
	Enrichment	−0.01	0.02	−0.65	0.52
Hypothalamus	Rest of brain	0.16	0.04	4.05	**<0.001**
	Neighbour	0.73	0.32	2.28	**0.03**
	Enrichment	−0.03	0.04	−0.73	0.47
	Rest*Neighbour	−0.13	0.06	−2.26	**0.03**

For brain size, we used body size as a covariate, for individual brain regions we used the rest of the brain (total brain volume minus the given brain region volume) as a covariate. Significant values ($P<0.05$) are highlighted in bold

the two factors supports such reasoning, while the fact that we did find an effect of environmental complexity in the cerebellum may oppose it. To test this idea, additional studies on brain plasticity in clonal fish are needed. Additionally, both meta-analysis (Arnqvist and Wooster 1995) and comparative studies will be necessary to determine the factors underlying potential differences in brain plasticity across species. Note also that our social enrichment consisted of visual contact between conspecifics only, while olfactory, tactile, or other interactions were excluded. Therefore, based on our results, we can conclude that visual social enrichment does not seem to be sufficient to cause any measureable brain anatomical changes in Amazon mollies. Full social contact may affect brain development; in the closely related guppy (*Poecilia reticulata*) social rearing impacts the development of inhibitory control (Lucon-Xiccato et al. 2022).

The telencephalon is generally associated with 'higher' cognitive tasks such as learning about habitat characteristics and social interactions (Triki et al. 2022). Manipulation of physical and social environment could have therefore lead to corresponding changes in telencephalon. The fact that we did not find such an effect may be due to the generally low cognitive demands in our holding conditions, with the fish receiving feed at regular intervals and no need to forage or travel through predator territory. Increasing the general need for cognitive capacities may well reveal telencephalon plasticity in the future.

Our study contributes to the growing number of studies that show how phenotypically plastic fish brains can respond to differences in the environment during ontogeny. Using a clonal fish with established strong genetic similarity allowed us to draw inferences from a relatively small sample size. Our data further suggest that rearing conditions are relevant to keeping fish. While we did not assess direct behavioural differences between animals of

Table 2 Mean volumetric measurements of each brain region, the calculated standard deviation and the calculated standard error ($N=62$). RSE for each region was below 3%

Brain region	Mean volume (mm^3)	Standard Deviation	Standard Error
Total Brain	6.6421	0.7613	0.0967
Olfactory Bulb	0.0689	0.0142	0.0018
Telencephalon	1.1709	0.1305	0.0166
Optic Tectum	2.8308	0.3661	0.0465
Cerebellum	1.3642	0.2573	0.0327
Dorsal Medulla	0.2803	0.0654	0.0083
Hypothalamus	0.9269	0.1644	0.0209

Table 3 Heteroscedasticity was assessed using the Breusch-Pagan test

Brain region	Original values			Log-transformed values		
	BP	df	*P*-value	BP	df	*P*-value
Brain weight	5.63	7	0.58	6.51	7	0.48
Olfactory bulb	7.41	7	0.39	8.98	7	0.25
Telencephalon	10.01	7	0.19	9.52	7	0.22
Optic tectum	6.53	7	0.48	5.40	7	0.61
Cerebellum	11.09	7	0.13	6.94	7	0.43
Dorsal medulla	3.19	7	0.87	3.20	7	0.87
Hypothalamus	9.16	7	0.24	5.39	7	0.61

Results indicated no significant heteroscedasticity in any of the models, suggesting that the assumption of constant variance was Met for both linear regression and allometric models across brain weight and the volume of the brain regions

different rearing conditions, it is clear that different rearing conditions can leave behind an imprint on the brain; impacts on the behavioural traits are likely. Future studies on the brain anatomy of Amazon mollies should consider measuring the brain regions at different stages of life to investigate if their brains are more plastic earlier in life and less so later in life.

In summary, we investigated the effects of physical and social environmental enrichment on the development of the brain of Amazon mollies and found little effects on gross brain anatomy apart from in the cerebellum. Comparisons with sexual species would be useful to test our claim that a clonal organism shows less variability than a sexual one. After all, even despite near identical rearing conditions, Amazon mollies of the same clonal lines show distinct differences in behaviour (Bierbach et al. 2017). Connecting such studies with studies on brain morphology is a promising avenue of research for the future of behavioural brain ecomorphology research.

Appendix

See Tables 2, 3 and 4.

Table 4 Estimate effects of environmental enrichment and visual neighbours on brain anatomy of Amazon mollies

Brain region	Coefficient	Estimate	S.E.	t-value	*P*-value
Brain weight	Body size	0.94	0.16	5.73	**<0.001**
	Neighbour	0.02	0.02	0.90	0.37
	Enrichment	0.01	0.02	0.34	0.73
Olfactory bulb	Rest of brain	0.45	0.23	1.94	0.06
	Neighbour	0.06	0.05	1.15	0.25
	Enrichment	0.03	0.05	0.58	0.56
Telencephalon	Rest of brain	0.49	0.10	4.74	**<0.001**
	Neighbour	−0.01	0.02	−0.33	0.74
	Enrichment	−0.01	0.03	−0.54	0.59
Optic tectum	Rest of brain	0.87	0.16	5.40	**<0.001**
	Neighbour	0.01	0.03	0.50	0.62
	Enrichment	0.60	0.28	2.10	**0.04**
	Rest*Enrichment	−0.43	0.21	−2.02	**0.048**
Cerebellum	Rest of brain	1.29	0.15	8.46	**<0.001**
	Neighbour	−0.04	0.03	−1.26	0.21
	Enrichment	0.07	0.03	2.29	**0.03**
Dorsal medulla	Rest of brain	1.01	0.25	4.05	**<0.001**
	Neighbour	0.00	0.05	0.00	1.00
	Enrichment	−0.04	0.06	−0.69	0.49
Hypothalamus	Rest of brain	0.94	0.23	4.09	**<0.001**
	Neighbour	1.25	0.58	2.17	**0.03**
	Enrichment	−0.04	0.04	−0.94	0.35
	Rest*Neighbour	−0.71	0.31	−2.14	**0.04**

For brain size, we used body size as a a covariate, for individual brain regions, we used the rest of the brain (total brain volume minus the given brain region) as a covariate. All numerical values were log-transformed. Significan values ($P<0.05$) were highlighted in bold

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10682-025-10361-4.

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Author contributions HGN performed brain measures and wrote a first draft, HGN and EJEW performed the statistics, TJR & DC & IS reared the fish under divergent conditions, EW performed the brain dissections, AK taught the brain dissections and measuring process, AK, IS & EJEW wrote the final draft, all authors contributed to the writing process.

Data availability No datasets were generated or analysed during the current study.

Declarations

Competing interests The authors declare no competing interests.

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Authors and Affiliations

Hayo G. Nadema[1] · Tyler J. Reich[2,4] · Eline J. E. Weenink[1] · Debbie Cunningham[3] · Elvis Wong[5] · Ingo Schlupp[2,4] · Alexander Kotrschal[1]

✉ Alexander Kotrschal
alexander.kotrschal@wur.nl

[1] Behavioural Ecology, Department of Animal Sciences, Wageningen University, De Elst 1, 6708 WD Wageningen, Netherlands

[2] School of Biological Sciences, University of Oklahoma, 730 Van Vleet Oval, Norman, OK 73072, USA

[3] Office of the Vice President, Oklahoma State University, Stillwater, OK 74078, USA

[4] International Stock Center for Livebearing Fishes, School of Biological Sciences, 730 Van Vleet Oval, Norman, OK 73072, USA

[5] Department of Fish Biology, Fisheries and Aquaculture, Leibniz Institute of Freshwater Ecology and Inland Fisheries, Müggelseedamm 310, 12587 Berlin, Germany